THE ROLE OF IONIC K\(^+\) IN THE
REGULATION OF APOPTOSIS

Thesis submitted for the degree
of Doctor of Philosophy at the
University of Leicester

by

Gwilym J. Thompson,
M.A. (Cambridge), M.Sc. (Edinburgh)
Department of Pathology
University of Leicester

April 2001
THE ROLE OF IONIC K⁺ IN THE REGULATION OF
APOPTOSIS

Gwilym J. Thompson, Medical Research Council Toxicology Unit, Hodgkin Building, Lancaster Road, Leicester LE1 9HN, U.K.

Abstract

Cell shrinkage is a major characteristic of apoptotic cell death and is associated with a decrease in intracellular K⁺ concentration. Intracellular K⁺ ions have an important role in setting and modulating the plasma membrane potential and can profoundly affect the activity of a number of cellular enzymes.

Following initial investigations into the role of K⁺ in cell survival and apoptosis in primary cultures of cerebellar granule neurons, flux of cellular K⁺ following induction of apoptosis by death receptor ligation or chemical stress was assessed in Jurkat T cells loaded with the K⁺ ion surrogate ⁸⁶Rb⁺. A time-dependent efflux of intracellular K⁺ was demonstrated that accompanies cell shrinkage, reduction of mitochondrial transmembrane potential (Ψₘ) and phosphatidylserine (PS) externalisation. An apparent increase in mitochondrial K⁺ concentration following treatment of cells with anti-CD95 antibody was also demonstrated. Induction of CD95- or chemical-mediated apoptosis results in depolarisation of the plasma membrane that accompanies PS externalisation and reduction of Ψₘ. Both depolarisation of the plasma membrane and efflux of intracellular K⁺ are dependent upon caspase activation in CD95- but not chemical-mediated apoptosis. Consistent with the hypothesis that efflux of intracellular K⁺ is required for the progression of apoptosis, formation of the caspase-activating ~700 kDa Apaf-1-containing apoptosome complex is inhibited in vitro by 50 mM K⁺.

To further clarify the role of K⁺ ions in apoptosis, cytoprotection by elevated extracellular [K⁺] (K⁺) was investigated. Supraphysiological concentrations of extracellular K⁺ were required to alter intracellular conditions by decreasing or reversing the driving force of K⁺ ions across the plasma membrane. Elevated [K⁺] inhibits CD95-mediated apoptosis by preventing activation of caspase-8 at the death inducing signalling complex, prior to mitochondrial perturbation. Chemical-mediated apoptosis is inhibited upstream of mitochondrial perturbation and caspase activation. The findings of this study are not consistent with the hypothesis that elevated [K⁺] inhibits apoptosis by preventing efflux of intracellular K⁺ alone and instead suggest that apoptosis is inhibited at multiple loci. The data imply that K⁺ plays an important role in the regulation and progression of apoptosis by influencing transmembrane voltage or ion-sensitive enzymatic processes.
Dedication

I would like to dedicate this thesis to my parents,
John and Felicity Thompson.
Acknowledgements

I would like to extend my thanks to my two supervisors, Prof. Gerald M. Cohen and Dr. Edward C. Conley, for their support, ideas and guidance.

I would like to thank the following for useful discussions and their technical assistance: Mr. Roger Snowden, Dr. Paul Richards, Dr. David Ray, Mr. Andrew Hubbard, Mr. Kulvindar Sikand, and Ms. Claudia Langlais.

I would also like to thank all those who advised, assisted and supported me during my time in Leicester, particularly Darren Roberts, Nicholas Harper, Jason Almond, Wendy Merrison, Marion MacFarlane, Shawn Bratton, Xiao-Ming Sun, David Brown and everybody else in lab 416.

I would like to thank the Medical Research Council for funding my studies at the University of Leicester.

Finally I would like to thank Lizzy, for her support, advice and for helping to keep everything in perspective.
TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION.

1.1 The physiological role of apoptosis ............................................................ 1
1.2 Genetic control of programmed cell death in Caenorhabditis elegans .......... 2
1.3 Morphological changes in apoptosis ............................................................ 5
1.4 Biochemical changes in apoptosis ............................................................... 7
  1.4.1 The caspase family ................................................................................. 7
  1.4.2 Death receptor-mediated apoptosis ....................................................... 10
  1.4.3 Apoptosis induced by cell stress or DNA damage ............................... 14
1.5 The involvement of mitochondria in apoptosis ......................................... 16
1.6 The Bcl-2 family ....................................................................................... 18
1.7 Nuclear changes in apoptosis ..................................................................... 20
1.8 Phagocytosis ............................................................................................. 21
1.9 Comparison between mechanisms of apoptosis and necrosis ................. 22
1.10 Ionic changes during apoptosis ................................................................. 23
  1.10.1 The intracellular ionic environment .................................................... 23
  1.10.2 Ionic fluxes during apoptosis ............................................................... 25
1.11 Ion channels in apoptosis ........................................................................ 27
  1.11.1 Potassium ion channels ....................................................................... 27
  1.11.2 Potential candidate ion channels in apoptosis ................................. 30
1.12 Aims and objectives of the thesis .............................................................. 31

CHAPTER 2. MATERIALS AND METHODS.

2.1 Materials .................................................................................................... 33
2.2 Animals ..................................................................................................... 33
2.3 Antibodies .................................................................................................. 34
2.4 Preparation and culturing of cerebellar granule neurons ........................ 35
2.5 Assessment of cell death in CGN cultures .............................................. 35
2.6 Tissue culture of cell lines ......................................................................... 36
2.6.1 Leukaemic cell lines ................................................................. 36
2.6.2 Media of defined ionic composition ........................................ 37
2.6.3 Induction of apoptosis ......................................................... 38
2.6.4 Treatment with pharmacological agents ............................ 39
2.7 Flow cytometry ........................................................................... 39
2.7.1 Principles of flow cytometry ................................................. 39
2.7.2 Fluorophores used in flow cytometry .................................... 42
2.8 \(^{86}\text{Rb}^+\) efflux assay ............................................................. 51
2.9 Bradford method for quantification of protein content .......... 53
2.10 Western blotting ...................................................................... 53
2.11 Investigation of subcellular fractions by Western blot ....... 55
2.11.1 Cytochrome c release ............................................................ 55
2.11.2 Membrane recruitment of FADD and caspase-8 .......... 56
2.12 Gel filtration chromatography ............................................... 56
2.12.1 Preparation of cell lysates .................................................... 56
2.12.2 Fluorimetric assay of caspase activity ................................. 57
2.12.3 Chromatographic methods ................................................. 57
2.12.4 Assay of apoptosomal effector caspase activating activity .... 58
2.13 Molecular biology ..................................................................... 58
2.13.1 cDNA and plasmids ............................................................ 58
2.13.2 Bacterial strains and growth media .................................... 59
2.13.3 Preparation of plasmid DNA ............................................... 59
2.13.4 Precipitation of oligodeoxynucleotide primers ............... 60
2.13.5 Quantification of nucleic acid concentration by spectrophotometry 60
2.13.6 DNA agarose gels ............................................................... 61
2.13.7 Enzymic manipulation of DNA .......................................... 61
2.13.8 Mutagenesis of \(K_{IR6.1}\) and \(K_{IR6.2}\) .................................... 62
2.13.9 Transformation ................................................................. 63
2.13.10 Preparation of cDNA stocks (Maxipreps) ....................... 64
2.13.11 \textit{In vitro} transcription and translation ............................ 65
2.13.12 Caspase cleavage of \(^{35}\text{S}\)-labelled peptides ................. 65
2.13.13 Autoradiography .............................................................. 65

VI
CHAPTER 3. INFLUENCE OF EXTRACELLULAR [K⁺] ON THE INDUCTION OF APOPTOSIS IN MATURE CEREBELLAR GRANULE NEURON CULTURES.

3.1 Introduction ................................................................. 68
3.2 Results ................................................................................. 71
3.2.1 Assessment and quantification of cell death in CGN cultures
by fluorescence microscopy .................................................... 71
3.2.2 Induction of apoptosis in CGN cultures by reduction
of [K⁺]₆₉ and withdrawal of serum ........................................... 73
3.2.3 Inorganic Hg²⁺ induces both apoptosis and necrosis in CGN cultures ...... 76
3.2.4 Pharmacologically-active concentrations of potassium channel
openers do not induce apoptosis in CGN cultures ....................... 78
3.3 Discussion ........................................................................... 80
3.4 Summary ........................................................................... 82

CHAPTER 4. INDUCTION OF APOPTOSIS IN JURKAT T CELLS IS ASSOCIATED WITH PLASMA MEMBRANE DEPOLARISATION AND EFFLUX OF INTRACELLULAR K⁺.

4.1 Introduction ........................................................................ 83
4.2 Results ................................................................................. 84
4.2.1 The plasma membrane of apoptotic cells is depolarised
relative to normal cells .......................................................... 84
4.2.2 Plasma membrane depolarisation is upstream of caspase
activation in etoposide- but not anti-CD95 antibody-induced apoptosis. 91

VII
4.2.3 Apoptotic cells have a decreased $[K^+]_i$ relative to resting cells............... 94
4.2.4 Decreased $[K^+]_i$ in anti-CD95 antibody-induced apoptosis is
dependent upon caspase activation ......................................................... 98
4.2.5 $K^+$ efflux accompanies the increase in PS externalisation, cell
shrinkage and reduction of $\Psi_m$ during the induction of apoptosis......... 101
4.2.6 $K^+$ efflux is not dependent upon effector caspase
activity in etoposide-induced apoptosis................................................... 102

4.3 Discussion ............................................................................................. 104
4.4 Summary ............................................................................................... 108

CHAPTER 5. LOCALISATION OF $K^+$ FLUX FOLLOWING INDUCTION OF APOPTOSIS.

5.1 Introduction ........................................................................................... 109
5.2 Results ........................................................................................................ 114
5.2.1 Effect of $K^+$ ion channel block on apoptosis........................................ 115
5.2.2 Sensitivity of $K_{IR6,x}$ ion channels to caspase digestion ......................... 119
5.2.3 Possible changes in mitochondrial matrix $K^+$
concentration during apoptosis............................................................... 122
5.2.4 Expression of mitochondrially-targeted constitutively
open $K_{IR6,x}$ channels............................................................................. 125
5.3 Discussion ............................................................................................... 129
5.4 Summary ............................................................................................... 132

CHAPTER 6. MECHANISM OF INHIBITION OF APOPTOSIS BY ELEVATED
EXTRACELLULAR $[K^+]$. 

6.1 Introduction ........................................................................................... 134
6.2 Results ...................................................................................................... 135
6.2.1 Elevated $[K^+]_{ext}$ inhibits apoptosis prior to caspase activation.............. 135
6.2.2 Treatment of cells in elevated $[K^+]_{ext}$ with apoptotic stimuli
does not cause a change in cell volume..................................................... 136
6.2.3 Elevated $[K^+]_{ext}$ does not inhibit binding of anti-CD95 antibody............ 139

VIII
6.2.4 Elevated $[K^+]_{ext}$ inhibits apoptosis upstream of cytochrome c release and reduction of mitochondrial transmembrane potential ($\Psi_m$) .......................... 141

6.2.5 Elevated $[K^+]_{ext}$ does not prevent anti-CD95 antibody-induced recruitment of FADD and caspase-8 to the DISC .............................................. 142

6.2.6 Properties of inhibition of CD95-mediated apoptosis by elevated $[K^+]_{ext}$ ............................... 145

6.2.7 Elevated $[K^+]_{ext}$ is required from initiation of signal transduction by anti-CD95 antibody to inhibit apoptosis .............................................. 147

6.2.8 Inhibition of CD95-mediated apoptosis is independent of decreased extracellular $[Na^+]$ ........................................................................... 150

6.2.9 Cell death in elevated $[K^+]_{ext}$ is independent of capase-8 activation over 24 h ......................................................................................... 152

6.2.10 Elevated $[K^+]_{ext}$ inhibits apoptosis induced by a range of stimuli .............................................. 154

6.2.11 Elevated $[K^+]_{ext}$ inhibits apoptosis in THP.1 cells ........................................................................ 154

6.2.12 Addition of $K^+$ or $Na^+$ to cytosolic lysates inhibits capase activation and formation of the ~700 kDa apoptosome complex .......................... 159

6.3 Discussion ........................................................................................................... 164

6.4 Summary ............................................................................................................ 169

CHAPTER 7. MECHANISMS OF N-ACETYLLEUCINE CHLOROMETHYLKETONE-INDUCED APOPTOSIS.

7.1 Introduction ........................................................................................................ 171

7.2 Results .................................................................................................................. 174

7.2.1 Concentration-dependent inhibition of ACPH activity and induction of apoptosis by ALCK ................................................................. 174

7.2.2 Dissociation between the inhibition of ACPH and induction of apoptosis using dichlorvos in Jurkat cells ......................................................... 177

7.2.3 Response of SKW6.4 and U937 cell lines to ALCK and dichlorvos: effects on cell viability and ACPH activity .............................................. 178

7.2.4 Dissociation between the inhibition of ACPH and induction of apoptosis in Jurkat, U937 and SKW6.4 cells using mipafox and chlorpyrifos methyl oxon .......................................................... 179
### Chapter 7.2.5 Investigation into the specificity of the reaction of ALCK with serine hydrolases

- 7.2.6 Potentiation of apoptosis by ALCK

### Chapter 7.3 Discussion

### Chapter 7.4 Summary

---

#### Chapter 8. General Discussion.

**8.1 Flux of intracellular ions during apoptosis**

- 8.1.1 Efflux of intracellular K⁺
- 8.1.2 Mitochondrial K⁺ flux
- 8.1.3 Depolarisation of the plasma membrane
- 8.1.4 Caspase inhibition of CD95-mediated apoptosis
- 8.1.5 Caspase inhibition of etoposide-induced apoptosis

**8.2 Mediation of intracellular K⁺ efflux during apoptosis**

**8.3 Cytoprotection by extracellular K⁺**

- 8.3.1 Cerebellar Granule Neurons
- 8.3.2 Inhibition of CD95-mediated apoptosis by elevated [K⁺]_{ext}
- 8.3.3 Inhibition of etoposide-induced apoptosis by elevated [K⁺]_{ext}

**8.4 Role of intracellular K⁺ efflux in apoptosis**

**8.5 Intracellular apoptotic signalling mechanisms**

**8.6 Future Directions**

---

#### Chapter 9. References

#### Appendix
LIST OF FIGURES

Figure 1.1 Genes involved in the regulation of programmed cell death in C. elegans and their human analogues................. 3
Figure 1.2 Morphological changes associated with apoptosis................................. 6
Figure 1.3 Phylogenetic relationship of the caspase family.................................. 8
Figure 1.4 Signal transduction pathways of death receptor-mediated apoptosis, as exemplified by the CD95 pathway........... 12
Figure 1.5 Chemical stress- or DNA damage-mediated apoptosis........................ 15
Figure 1.6 Ionic environment of the cell................................................................. 24
Figure 1.7 Transmembrane structure of voltage-gated K+ (Kv) channels........... 28
Figure 1.8 Transmembrane structure of inward rectifying K+ (Kir) channels.... 29
Figure 2.1 Optics of the FACScan flow cytometer (simplified)............................ 40
Figure 2.2 Assessment of cell size and density and cell shrinkage by flow cytometry.......................................................... 43
Figure 2.3 Quantification of apoptotic cells by Annexin V-FITC and PI............. 45
Figure 2.4 Assessment of apoptosis by reduction of mitochondrial transmembrane potential (Ψm) using DiOC6(3) and PI.................................................................................. 47
Figure 2.5 DiBAC4(3) fluorescence increases in response to plasma membrane depolarisation.............................................................. 48
Figure 2.6 TMRE is a measure of mitochondrial transmembrane potential........ 50
Figure 2.7 Assessment of reduction of Ψm by TMRE staining............................ 51
Figure 2.8 Calibration of Compugamma γ-counter for peak emissions of 86Rb+........................................................................ 52
Figure 3.1 Structure of potassium channel openers............................................. 70
Figure 3.2 Staining and photomicroscopy of mature CGN cultures (7 DIV)....... 72
Figure 3.3 Reduction of [K+]ext and serum withdrawal induces apoptosis in CGN cultures ............................................................... 75
Figure 3.4 Induction of apoptosis and necrosis in CGN cultures by Hg²⁺ is concentration-dependent........................................ 76
Figure 3.5 Induction and assessment of apoptosis in mature CGN cultures (7 DIV)......................................................................... 77
Figure 3.6  Treatment of CGN cultures with KCOs does not induce apoptosis at pharmacologically active concentrations

Figure 4.1  Assessment of apoptosis and plasma membrane depolarisation by DiBAC<sub>4</sub>(3) and PI

Figure 4.2  Plasma membrane depolarisation correlates with PS externalisation in apoptotic cells

Figure 4.3  Assessment of apoptosis by DiBAC<sub>4</sub>(3)/PI is comparable with Annexin V/PI and DiOC<sub>6</sub>(3)/PI

Figure 4.4  Plasma membrane depolarisation accompanies PS externalisation and cell shrinkage

Figure 4.5  z-VAD.fmk inhibits anti-CD95 antibody-induced apoptosis upstream of cell shrinkage, PS externalisation and plasma membrane depolarisation

Figure 4.6  z-VAD.fmk inhibits etoposide-induced PS externalisation but not cell shrinkage or plasma membrane depolarisation

Figure 4.7  Reduction of $\Psi_m$ correlates with PS externalisation

Figure 4.8  Cells with reduction of $\Psi_m$ have decreased $[K^+]$

Figure 4.9  Cells with PS externalisation have decreased $[K^+]$

Figure 4.10  z-VAD.fmk inhibits anti-CD95 antibody-induced apoptosis upstream of cell shrinkage, PS externalisation and $K^+$ efflux

Figure 4.11  z-VAD.fmk inhibits etoposide-induced apoptosis upstream of cell shrinkage, PS externalisation and $K^+$ efflux

Figure 4.12  Efflux of intracellular $K^+$ from apoptotic cells is concomitant with PS externalisation, reduction of $\Psi_m$ and cell shrinkage

Figure 5.1  Hypothetical release of mitochondrial proteins by rupture of the outer mitochondrial membrane

Figure 5.2  Chemical structures of the pharmacological agents used and amino acid sequence of recombinant Margatoxin (rMgTX)

Figure 5.3  Etoposide-induced apoptosis is inhibited by co-treatment with glyburide or ouabain
Inhibition or potentiation of apoptosis by pharmacological agents does not correlate with cell shrinkage.

Representation of $\text{K}_{\text{IR}2.3}$, $\text{K}_{\text{IR}6.1}$ and $\text{K}_{\text{IR}6.2}$ expressed in reticulate lysate.

Candidate ion channels are not substrates of dilute recombinant caspase-3 and -8.

The mitochondrial [$K^+$] of anti-CD95 antibody-treated cells is increased relative to the cytoplasm.

Design of truncation mutations of $\text{K}_{\text{IR}6.x}$ ion channels.

Representation of $\text{K}_{\text{IR}6.x}$ truncation mutations.

Western blot of CHO cell pellets 36 h after transfection with eGFP or $\text{K}_{\text{IR}6.1}{\Delta C61}$, probed for GFP.

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

Elevated [$K^+$]$_{\text{ext}}$ inhibits apoptotic cell volume changes induced by anti-CD95 antibody and etoposide.

Elevated [$K^+$]$_{\text{ext}}$ does not prevent binding of anti-CD95 antibody to Jurkat cells.

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

Inhibition of anti-CD95 antibody-induced apoptosis by elevated [$K^+$]$_{\text{ext}}$ is downstream of FADD and procaspase-8 recruitment to the plasma membrane.

Treatment of cells with anti-CD95 antibody and etoposide in varying [$K^+$]$_{\text{ext}}$.

Elevated [$K^+$]$_{\text{ext}}$ is required from the outset of anti-CD95 antibody treatment to inhibit apoptosis.

Cytoprotection by elevated [$K^+$]$_{\text{ext}}$ is independent of [$Na^+$]$_{\text{ext}}$ for anti-CD95 antibody- but not etoposide-induced apoptosis.
Figure 6.9  Cell death in elevated \([K^+]_{ext}\) is independent of caspase-8 activation over 24 h, though cytoprotection by 5K/130 NMDG medium against etoposide-induced apoptosis is lost............. 153

Figure 6.10 Elevated \([K^+]_{ext}\) inhibits apoptosis induced by TRAIL, STS and MG132 upstream of caspase activation................................................................. 155

Figure 6.11 Elevated \([K^+]_{ext}\) inhibits etoposide- and MG132- but not STS-induced apoptosis upstream of caspase activation in THP.1 cells.... 158

Figure 6.12 Pre-treatment of THP.1 lysates with KCl or NaCl inhibits caspase activation................................................................. 160

Figure 6.13 KCl inhibits formation of the ~700 kDa apoptosome in Jurkat T cell lysates................................................................. 161

Figure 6.14 Both NaCl and KCl inhibit ~700 kDa apoptosome complex formation in THP.1 lysates................................................................. 163

Figure 7.1 Structure of organophosphate ACPH inhibitors................................................................. 173

Figure 7.2 ALCK inhibits ACPH and induces apoptosis in Jurkat cells................................................................. 175

Figure 7.3 ALCK induces PARP cleavage and caspase-3 processing in Jurkat, SKW6.4 and U937 cells................................................................. 176

Figure 7.4 Response of Jurkat cells to dichlorvos................................................................. 177

Figure 7.5 Inhibition of \([H^3]-DFP\) labelling by ACLK................................................................. 181

Figure 7.6 Potentiation of apoptosis by ALCK but not dichlorvos................................................................. 183

Figure 8.1 Inhibition of CD95-mediated apoptosis by z-VAD.fmk................................................................. 188

Figure 8.2 Inhibition of etoposide-induced apoptosis by z-VAD.fmk................................................................. 190

Figure 8.3 Elevated \([K^+]_{ext}\) inhibits CD95-mediated apoptosis by preventing caspase-8 activation at the DISC................................................................. 194

Figure 8.4 Elevated \([K^+]_{ext}\) inhibits etoposide-induced apoptosis upstream of cytochrome c release and reduction of \(\Psi_m\)................................................................. 197

XIV
LIST OF TABLES

Table 1.1  Comparison between the key features of apoptosis and necrosis ............... 22
Table 2.1  Antibodies used for Western blotting .......................................................... 34
Table 2.2  Comparison of components of RPMI 1640 complete medium, media of defined ionic composition and dialysis buffer .................. 38
Table 2.3  Spectral properties of fluorophores used and cell parameters measured... 42
Table 2.4  Constituents of SDS-PAGE gels ................................................................. 54
Table 5.1  Predicted and observed sizes of expressed peptides and caspase cleavage products ................................................................. 122
Table 7.1  Effect of ACPH inhibitors and etoposide on culture viability and ACPH activity ................................................................. 179
### ABBREVIATIONS

In addition to standard abbreviations for metric measurements (e.g., ml) and chemical symbols (e.g., HCl), the abbreviations and acronyms below are used throughout this thesis.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>AANA</td>
<td>N-acetyl-alanyl-p-nitroanilidealanyl-p-nitroanilide</td>
</tr>
<tr>
<td>ACPH</td>
<td>acylpeptide hydrolase</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ALCK</td>
<td>N-acetyl-leucyl-chloromethylketone</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxymethyl ester</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated deoxyribonuclease</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DIABLO</td>
<td>direct IAP binding protein with low PI</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglyco-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
</tbody>
</table>
eV  electron volts
FADD  Fas-associated death domain
FCS  foetal calf serum
FITC  fluorescein isothiocyanate
g  relative centrifugal force
G  guanine
GFP  green fluorescent protein
gm  gram
h  hour
HEPES  N-(2-hydroxyethyl)piperidine-N'-(2-ethanesulphonic acid)
ICAD  inhibitor of caspase-activated deoxyribonuclease
ICE  interleukin 1-β converting enzyme
IMS  industrial methylated spirits
KCO  potassium channel opener
[K+]ext  extracellular potassium concentration
[K+]i  intracellular potassium concentration
m-CCCP  carbonyl cyanide m-chlorophenylhydrazone
MEM  minimal essential medium
min  minutes
MG132  carbobenzoxy-Leu-Leu-Leu-CHO
[Na+]ext  extracellular sodium concentration
[Na+]i  intracellular sodium concentration
NMDG  N-methyl-D-glucamine
OP  organophosphate
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PE  phycoerythrin
PI  propidium iodide
PI-3-K  phosphatidylinositol 3-kinase
PI-3-K  phosphatidylinositol 3-kinase
PIPES  piperazine-N,N'-bis[2-ethane-sulphonic acid]
PLAD  pre-ligand assembly domain
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PTPC</td>
<td>permeability transition pore complex</td>
</tr>
<tr>
<td>rMgTX</td>
<td>recombinant Margatoxin</td>
</tr>
<tr>
<td>S</td>
<td>Siemen</td>
</tr>
<tr>
<td>Smac</td>
<td>second mitochondrial activator of caspases</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>STS</td>
<td>staurosporine</td>
</tr>
<tr>
<td>T</td>
<td>thymidin</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>half-life</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TEMED</td>
<td>$N,N',N''$-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>v/v</td>
<td>volume for volume</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>w/w</td>
<td>weight for weight</td>
</tr>
<tr>
<td>z-DEVD.AFC</td>
<td>benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin</td>
</tr>
<tr>
<td>z-VAD.fmk</td>
<td>benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone</td>
</tr>
</tbody>
</table>
Chapter One. Introduction

1.1 The physiological role of apoptosis

Apoptosis (programmed cell death) is the mechanism by which extraneous, damaged or dysfunctional cells are disposed of efficiently and without inflammation in metazoans. The term apoptosis was coined by Kerr et al. (1972) and is derived from a Greek word (ἀπότομος) describing the shedding of petals from flowers or leaves from trees. Almost all cells carry within themselves the machinery to undergo apoptosis if the elimination of the cell is required by the organism. In this way apoptosis has been rather colourfully compared to a spy carrying a cyanide capsule with which to commit suicide if captured (Schendel et al., 1998). Apoptosis is a physiological process that:

i) controls homeostatic regulation of cell numbers, balancing against the rate of cell division and removing cells that are generated in excess, for example neuronal apoptosis in the developing central nervous system in vertebrates.

ii) removes cells that have no function (for example evolutionary vestiges) and sculpts tissues during development, for example removal of interdigital webbing in mammals during development.

iii) carries out the disposal of damaged or dysfunctional cells that are potentially harmful to the organism and cells that have developed improperly or have served their purpose.

iii) defends against pathogens. Apoptosis is fundamental to the development of the immune system as it regulates lymphocyte maturation, receptor repertoire selection and homeostasis, is responsible for the removal of potentially self-reactive lymphocytes and is also responsible for the termination of immune responses (reviewed in Krammer, 2000).

Apoptosis in Disease

Disregulation of apoptosis can lead to a number of pathological conditions (reviewed in Thompson, 1995). Failure of cells to undergo apoptosis is associated with the pathogenesis of many cancers, viral infections and autoimmune diseases. Cells in a wide range of human tumours have a decreased ability to undergo apoptosis in response to some physiological stimuli and so accumulation of neoplastic cells occurs. Metastatic tumour cells have the ability
to survive away from their parent tissues and so can form cancerous growths by dividing and growing in other tissues. The inability of the immune system to eliminate self-reactive lymphocytes by apoptosis can lead to autoimmunity. Cells infected by viruses often undergo apoptosis to limit viral infection. To prevent host cell apoptosis and counter the immune response, thus prolonging viral infection, several viruses have evolved inhibitors of apoptosis.

In contrast with the diseases detailed above caused by insufficient apoptosis, several pathological states are associated with excessive apoptosis. The human immunodeficiency virus (HIV) coat protein induces apoptosis in CD4$^+$ T cells, thus contributing to immunodepletion observed in acquired immunodeficiency syndrome (AIDS). Several neurodegenerative diseases are associated with apoptosis in select populations of neurons, including Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis (ALS) and various forms of cerebellar degeneration. A number of haematologic diseases are caused by excessive apoptosis outbalancing production of blood cells. Cell death in myocardial infarction and stroke also has an apoptotic element. When tissues are reperfused after occlusion of blood vessels, the rapid and uncontrolled increase in intracellular calcium and free radicals can cause apoptosis in cells surrounding the necrotic umbra. Specific apoptosis-inducing agents are therefore required for the therapeutic treatment of neoplasia and inhibitors of apoptosis are required to limit tissue damage resulting from neurodegenerative disorders, stroke or infarction. Further understanding of apoptosis should open new approaches to rational treatment strategies.

1.2 Genetic control of programmed cell death in *Caenorhabditis elegans*

Much of the early progress made in research into the regulation of apoptosis can be attributed to investigation of the genetic control of cell death during development of the nematode *Caenorhabditis elegans* (reviewed in Ellis et al., 1991; Metzstein et al., 1998). During normal development of the organism, 131 out of 1090 somatic cells generated die by apoptosis in an essentially invariant pattern, with linearly equivalent cells undergoing programmed cell death from animal to animal. While over a dozen cell death genes (called ced for cell death abnormal) have been identified in *C. elegans*, three genes are essential for normal regulation of apoptosis: ced-3 and ced-4 encode proteins that promote apoptosis (Ellis and Horvitz, 1986), while ced-9 expression is inhibitory (Hengartner and Horvitz, 1994).
CED-3 is a cysteine protease that cleaves specific substrates after self-activation. CED-4 binds to and promotes proteolytic activation of CED-3. Normally CED-9 forms an inactive complex with CED-4 that binds to and prevents the activation of CED-3 (Spector et al., 1997), but induction of apoptosis causes dissociation of CED-9 from this complex, allowing CED-3 to be activated and the cell to undergo apoptosis. The EGL-1 (egg-laying defective) protein negatively regulates CED-9 by direct binding and inhibition of its anti-apoptotic function (Conradt and Horvitz, 1998), causing CED-4 to be released from the CED-9/CED-4 complex and to translocate to the nuclear membrane. The relationship between the principal genes that control apoptosis in *C. elegans* is illustrated in Figure 1.1.

---

**Figure 1.1:** Genes involved in the regulation of programmed cell death in *C. elegans* and their human analogues. It should be noted that the human genes homologous to each *C. elegans* gene represent entire gene families in most cases, except for ced-4, for which only one human homologue has been identified at present.
The mechanism of programmed cell death appears to be conserved, at least in part, in vertebrates. The first evidence for this was the discovery that the mammalian anti-apoptotic protein Bcl-2 can substitute for CED-9 in *C. elegans* (Vaux et al., 1992) and is a direct homologue of CED-9 (Hengartner and Horvitz, 1994). Further mammalian homologues of *C. elegans* genes associated with the control of apoptosis have since been identified (Figure 1.1). CED-3 corresponds to the caspase family (see section 1.4.1) and CED-4 shares limited homology with Apaf-1 (Zou et al., 1997; see section 1.4.3), though there may be as yet undiscovered mammalian homologues of CED-4. Experiments with Apaf-1<sup>−/−</sup> knockouts imply the existence of other CED-4 mammalian homologues, as staurosporine (STS) -induced apoptosis in Apaf-1<sup>−/−</sup> embryonic fibroblasts occurs at almost normal levels (Cecconi et al., 1998; Yoshida et al., 1998). EGL-1 is equivalent to pro-apoptotic BH3-only members of the Bcl-2 family (see section 1.6).

Multiple homologues of each *C. elegans* gene exist in vertebrates perhaps because there is a wider range of physiological situations in which induction of apoptosis is required and where a more complete control is required. The different stages of apoptosis can be divided into initiation, commitment and execution, though this division is to some extent arbitrary. Figure 1.1 outlines only one potential vertebrate pathway of initiation of apoptosis; initiation can occur by multiple pathways, each 'private' to the various apoptotic stimuli. For example, intracellular apoptotic signalling pathways can be activated upon detection of DNA damage or cell stress and are sometimes referred to as intrinsic signalling pathways. In contrast, initiation of apoptosis can occur by extracellular receptors, for example killing of infected cells by cytotoxic T cells by ligation of death receptors in the plasma membrane. The effector stage of apoptosis is far less diverse and is made up of characteristic changes common to many forms of apoptosis. The degradation stage is common to almost all apoptotic processes and constitutes key changes in the morphology, metabolism and biochemical state of the cell and its disposal.
1.3 MORPHOLOGICAL CHANGES IN APOPTOSIS.

Initiation of apoptosis in vertebrate cells in vivo is associated with a characteristic sequence of morphological changes. These changes can be described as occurring in three stages (reviewed in Arends and Wyllie, 1991; Figure 1.2). First there is a reduction in nuclear size (pyknosis), with condensation of the chromatin into crescentic caps at the periphery of the nucleus, and disintegration of the nucleolus. Cells undergoing apoptosis in vivo detach themselves from their neighbours, and in vitro from the culture substratum. The cell exterior smoothens and specialised surface structures such as microvilli are lost. Cell volume decreases, cytoplasmic organelles cluster and become compacted and the smooth endoplasmic reticulum (ER) dilates.

In the second phase, there is blebbing of the cell surface and nuclear surface. Both the nucleus and cytoplasm may split into several fragments, with the cell becoming a cluster of membrane-bound apoptotic bodies. These apoptotic bodies may be shed from the epithelial surfaces or phagocytosed by neighbouring cells or macrophages. In addition, ER-derived vacuoles are often observed, which produce a characteristic 'bubbling' of the cytoplasm. In the third phase, the remaining nuclear and cytoplasmic structures degenerate, membranes disappear and organelles become unrecognisable. In vivo this often occurs within the phagosome once the cells have been phagocytosed. In cultured cell lines, the absence of cells capable of phagocytosis leads to lysis of late-stage apoptotic cells, referred to as secondary necrosis.
Figure 1.2 Morphological changes associated with apoptosis. Visual changes in cell morphology of cells undergoing apoptotic degradation can be divided into three stages, as described in the text. Figure adapted from Kerr et al. (1972).
1.4 Biochemical changes in apoptosis.

Apoptosis is an active cellular process requiring the consumption of ATP. A multitude of biochemical processes occur within the cell following induction of apoptosis (reviewed in Hale et al., 1996; Nicholson and Thornberry, 1999; Yeh et al., 1999; Hengartner, 2000), some of which result in the morphological changes described in section 1.3. Many of these morphological changes are mediated by proteolytic processing of cellular substrates by the caspase family of proteases during the execution stage of apoptosis.

1.4.1 The caspase family

The caspase family are the mammalian equivalents of the CED-3 protease in C. elegans. Fourteen members have been found to date (Figure 1.3), of which twelve mammalian homologues are known (reviewed in Cohen, 1997; Earnshaw et al., 1999; Nicholson, 1999). The nomenclature corresponds to cysteinyl aspartate-specific proteases (Alnemri et al., 1996). Caspases universally contain an active site that has a four-peptide substrate recognition motif with an almost absolute specificity for an aspartate in the P1 position of the substrate cleavage site. The preferred tetrapeptide cleavage motif differs between members of the caspase family and accounts for their diverse physiological roles (Thornberry et al, 1997). Caspases are synthesised as inactive zymogens with three domains: an N-terminal prodomain, a large subunit containing the active site cysteine and a C-terminal small subunit. The crystal structures of caspase-1 and -3 have been resolved (reviewed in Nicholson, 1999), showing that two large/small subunit heterodimers associate to form a tetramer with two independent active sites. Importantly, specific aspartic acid cleavage sites exist between the prodomain and each of the subunits, potentially allowing for caspase autoactivation. Upon activation, the linker between large and small subunits is degraded and the prodomain usually cleaved.
### Figure 1.3 Phylogenetic relationship of the caspase family

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE subfamily</td>
<td></td>
</tr>
<tr>
<td>caspase-13 (ERICE)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-5 (ICErel-III, TY)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-4 (TY, ICH-2, ICE-rel-II)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-11</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-12</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-1 (ICE)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-7 (Mch3, ICE-LAP3, CMH-1)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-3 (CPP32, Yama, apopain)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-6 (Mch2)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-8 (MACH, FLICE, Mch5)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-10 (Mch4)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-2 (ICH-1)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-9 (ICE-LAP6, Mch6)</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>caspase-14</td>
<td>Effector protease in inflammation and stroke</td>
</tr>
<tr>
<td>CED-3 subfamily</td>
<td></td>
</tr>
</tbody>
</table>

About two thirds of caspases have a role in apoptosis (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999), and caspases can thus be functionally divided into two groups (Figure 1.3). Interleukin 1-β converting enzyme (ICE) -related caspases (caspases -1, -4, -5, -11, -12, and -13) function predominantly in cytokine maturation and inflammatory response. The remaining caspases function in a manner similar to CED-3, mediating the execution of apoptosis. While CED-3 likely acts as both an initiator and an effector protease, these roles have diverged in vertebrates, as the CED-3 subfamily of caspases can be further divided
between initiator (such as caspases -2, -8, -9 and -10) and effector caspases (such as caspases -3, -6 and -7). The role of the initiator caspases is to activate the effector caspases, which mediate much of the proteolytic cleavage of cellular substrates during the execution stage of apoptosis. Initiator caspases have long pro-domains allowing for interaction with adaptor protein complexes, which recruit initiator caspases and bring them into close proximity. Several caspase zymogens (particularly caspases-8 and -9) have limited proteolytic activity and their close proximity in recruitment complexes has been proposed to lead to autoactivation (Salvesen and Dixit, 1999). Initiator caspases in extrinsic death signalling pathways, such as those transduced by the tumour necrosis factor (TNF) family of death receptors, are activated at a death inducing signalling complex (DISC; see section 1.4.2). Activation of caspases by intrinsic signalling pathways occurs at the apoptosome complex (see section 1.4.3). Recruitment of caspase zymogens to activating complexes is by means of recruitment domains within the caspase sequence. Caspases -8 and -10 contain death effector domains (DEDs) that allow recruitment to the DISC by homologous interaction, though only recruitment of caspase-8 has been shown in vivo. The caspase activation and recruitment domain (CARD) of caspases -1, -2, -4 and -9 potentially allows recruitment to the apoptosome, though only caspase-9 recruitment has been demonstrated.

Due to the aspartic acid cleavage site between caspase subunits, caspases can be activated both by themselves and other caspases. This leads to the possibility of amplificatory activation cascades. As detailed above, the initiator caspase that is activated upon apoptotic insult differs depending upon the apoptotic stimulus, and these can trigger downstream caspase cascades in a hierarchical relationship. Caspase-8 is the apical caspase in death receptor pathways (Medema et al., 1997; Muzio et al., 1997); caspase-9 is the apical caspase in intrinsic death pathways (Sun et al., 1999). Effector caspases have short pro-domains, and are activated by initiator caspases. Effector caspases are activated in most, if not all, caspase cascades.

The activation and activity of caspases can be inhibited by IAPs (inhibitor of apoptosis proteins; reviewed in Deveraux and Reed, 1998). Seven mammalian IAPs have been identified so far. Three of them, XIAP, c-IAP-1 and c-IAP-2 have been shown to preferentially bind to activated caspases -3, -7 and -9. XIAP binds to and inhibits the caspase-9/Apaf-1 apoptosome
complex (see section 1.4.4), thus preventing its caspase-activating function (Srinivasula et al., 2001). Inhibition by IAPs thus prevents the initiation of caspase cascades, thereby setting a threshold for the quantity of activated caspase required before cascades are initiated and preventing accidental induction of apoptosis.

The role of caspases in the execution of apoptosis. Over one hundred caspase substrates have been identified that are cleaved during apoptosis (reviewed in Stroh and Schulze-Osthoff, 1998; Earnshaw et al., 1999) and this cleavage is responsible for many of the morphological changes observed in apoptotic cells. Pro-life signals that would otherwise interfere with the apoptotic response are over-ridden by the cleavage of their mediators, for example caspase cleavage of Akt (Widmann et al., 1998), a downstream effector of many survival factors. Caspases cleave proteins involved in regulation of the cell cycle and proliferation and disable normal repair processes to prevent simultaneous counter-productive events. Since apoptosis is an active process requiring ATP, cellular ATP levels might be depleted if repair enzymes were not incapacitated, thus jeopardising completion of the apoptotic program. In order to disable DNA replication and to prevent repair of genomic DNA, two key DNA-repairing enzymes are disabled, these being PARP and DNA-PK. Caspase cleavage of nuclear lamins disrupts the nuclear structure, leading to nuclear shrinking and budding (Orth et al., 1996; Takahashi et al., 1996). Loss of cell shape is a result of caspase-mediated reorganisation of the cytoskeleton and digestion of cytoskeletal proteins such as fodrin and gelsolin (Kothkota et al., 1997). Caspases also cut off contacts with other cells, detaching the cell from its neighbours or culture substratum, induce the cell to display markers on the cell surface for engulfment by phagocytes and mediate the disintegration of the cell into apoptotic bodies.

1.4.2 Death receptor-mediated apoptosis

Apoptosis is induced extrinsically by the ligation of death receptors in the plasma membrane (reviewed in Ashkenazi and Dixit, 1998). Death receptors belong to the TNF superfamily, and are characterised by similar cysteine-rich extracellular domains (Smith et al., 1994). Members include CD95 (Fas/APO-1), TNFR1 (p55/CD120a), TRAIL-R2 (Apo-2/DR5/TRICK 2/KILLER). Triggering of death receptors with their cognate ligands or agonistic antibodies results in receptor oligomerisation (most likely trimerisation) and
activation (Boldin et al., 1995). There is recent evidence, however, that receptors of the TNF family already exist as trimers in quiescent cells (Siegel et al., 2000), by association of receptor pre-ligand assembly domains (PLAD; Chan et al., 2000). The PLAD is physically distinct from the ligand-binding domain of the receptor and is essential for efficient ligand binding and receptor function. Receptor oligomerisation promotes recruitment of adaptor proteins, leading to the activation of initiator caspases and caspase cascades.

The CD95 death receptor pathway. Perhaps the best understood death receptor pathway is the CD95 pathway (Figure 1.4). CD95 plays an important role in apoptosis in several physiological situations (reviewed in Nagata, 1997): i) removal of activated mature T cells after an immune response; ii) induction of apoptosis in viral-infected or cancerous cells by cytotoxic T cells and natural killer cells; iii) deletion of inflammatory cells at immune-privileged sites such as the eye. CD95-mediated apoptosis is initiated by ligation of the CD95 receptor, leading to formation of the DISC. Formation of the DISC is an almost instantaneous process that can be detected within seconds of receptor cross-linking (Kischkel et al., 1995; Scaffidi et al., 1997). DISC formation is initiated by binding of CD95 ligand or anti-CD95 antibody to the CD95 receptor, which results in receptor oligomerisation, leading to clustering of the receptors' death domain (DD). Fas-associated death domain (FADD; also called MORT1; Boldin et al., 1995; Chinnaiyan et al., 1995), which contains both a C-terminal DD sequence and an N-terminal DED sequence, is recruited to the DD of the aggregated receptors (Kischkel et al., 1995). Association of FADD molecules with the receptor complex brings into close proximity the FADD DEDs. The two N-terminal DEDs (Boldin et al., 1996; Muzio et al., 1996) of procaspase-8 associate with the DED of FADD molecules (Medema et al., 1997), thus recruiting procaspase-8 to the DISC. As procaspase-8 has limited enzymic activity, close proximity of procaspase-8 molecules results in their autoactivation (Muzio et al., 1998). Formation of the DISC therefore initiates a caspase cascade with caspase-8 as the apical caspase (Medema et al., 1997; Muzio et al., 1997).
Figure 1.4: Signal transduction pathways of death receptor-mediated apoptosis, as exemplified by the CD95 pathway. Apoptotic signalling and activation of effector caspases can be independent of mitochondria (type I cells) or mediated by a mitochondrial amplification step (type II cells). The precise stoichiometry of the apoptosome is currently unknown, and is represented as an octamer for aesthetic reasons only.
Type I and type II cells. Distal to caspase-8 activation at the DISC there are two possible mechanisms by which the effector caspases can be activated (Scaffidi et al., 1998). The mechanism employed is dependent upon whether sufficient DISC is formed and caspase-8 generated to activate effector caspases directly. Cells where effector caspases are activated directly are referred to as type I cells (Figure 1.4). In contrast, type II cells generate little DISC and active caspase-8, and consequently the apoptotic signal requires an amplificatory step via the mitochondria. Caspase-8 cleaves the Bcl-2 family member Bid to form a pro-apoptotic truncated form of Bid (tBid), which induces the release of cytochrome c from mitochondria (Li et al., 1998; Yin et al., 1998). Cytochrome c oligomerises with Apaf-1 and caspase-9 in the presence of dATP to form the caspase-activating apoptosome complex (Li et al., 1997; see section 1.4.3), which activates a post-mitochondrial caspase cascade.

Experiments examining the over-expression of Bcl-2 support the distinction between type I and type II cells. Since the anti-apoptotic action of Bcl-2 is primarily on the mitochondria, it follows that in type II cell lines which require amplification of the apoptotic signal via the mitochondria, apoptosis is prevented by Bcl-2 over-expression (Scaffidi et al., 1998). In contrast, apoptosis is not suppressed by Bcl-2 over-expression in type I cell lines, since they produce sufficient caspase-8 to activate effector caspases independently of mitochondrial perturbation. Experiments with Apaf-1\(^{-/-}\) and caspase-9\(^{-/-}\) knockout mice show that thymocytes (which are type I cells) remain sensitive to anti-CD95 antibody (reviewed in Yeh et al., 1999). This suggests that the apoptosome is not required for CD95-mediated apoptosis in these cells. The classification of type I and type II cells remains controversial, however. Huang et al. (1999) maintain that the type I and type II phenotypes are an artefact resulting from inconsistencies between anti-CD95 antibody- and CD95 ligand-induced apoptosis.

Death-receptor induced apoptosis can be inhibited by FLICE-inhibitory proteins (FLIPs; Irmler et al., 1997), which act as decoy inhibitors to prevent caspase-8 activation (Figure 1.4). FLIPs share significant sequence homology with procaspase-8 and contain two N-terminal DEDs but lack essential catalytic residues. By means of the DEDs they compete with procaspase-8 for binding to FADD. FLIPs also interact with procaspase-8, forming inactive heterodimers thus preventing recruitment of procaspase-8 to the DISC.
1.4.3 Apoptosis induced by cell stress or DNA damage

Cell death induced by stimuli such as DNA-damaging chemicals, ionising radiation and protein kinase inhibitors is mediated by intrinsic apoptotic signalling pathways (Figure 1.5). Intrinsic sensors of DNA damage or cellular stress are not currently well understood, but pro-apoptotic signals likely converge on the mitochondria, possibly mediated by pro-apoptotic members of the Bcl-2 family. Pro-apoptotic signalling to the mitochondria induces cytochrome c release, which oligomerises with Apaf-1 and caspase-9 in the presence of dATP to form a multi-protein caspase-activating apoptosome complex (Li et al., 1997). While Apaf-1 does bind to cytochrome c in the absence of dATP, its presence is required for oligomerisation (Saleh et al., 1999). Procaspase-9 is recruited to the CARD domain of Apaf-1 by homologous interaction, with the direct CARD-CARD interaction giving a 1:1 ratio procaspase-9 to Apaf-1.

A ~1.4 MDa apoptosome complex has been reconstituted from purified recombinant proteins (Saleh et al., 1999; Zou et al., 1999) and a ~700 kDa apoptosome complex has also been reported in dATP-activated cell lysates (Cain et al., 1999). Analysis of caspase-activating activity indicates that the ~700 kDa apoptosome is the physiological form (Cain et al. 2000). Recruitment of multiple procaspase-9 molecules to the complex leads to their activation, probably due to close proximity leading to autoactivation (Salvesen and Dixit, 1999). The apoptosome complex activates a caspase cascade with caspase-9 at its apex. Using in vitro cell lysates to analyse this post-mitochondrial cascade, Slee et al. (1999) show that caspase-9 activates the effector caspases -3 and -7. Caspase-3 then activates caspases -6 and -2 and also feeds back to activate more caspase-9. Caspase-6 may subsequently activate caspases -8 and -10 as well as more caspase-3.
Figure 1.5: Chemical stress- or DNA damage-mediated apoptosis. Signalling pathways between detection of cellular stress or DNA damage and release of cytochrome c are currently unknown, but may be transduced by pro-apoptotic members of the Bcl-2 family. The precise stoichiometry of the apoptosome is currently unknown and is represented as an octamer for aesthetic reasons only.
1.5 THE INVOLVEMENT OF MITOCHONDRIA IN APOPTOSIS

The release of cytochrome c from mitochondria is crucial in many forms of apoptosis (Newmeyer et al., 1994; Green and Reed, 1998) and represents a common convergence point in many of these apoptotic pathways. Cytochrome c is found in quiescent cells in the mitochondrial intermembrane space where it is involved in the process of oxidative phosphorylation. In some circumstances the decision whether the cell undergoes apoptosis or necrosis depends on the supply of ATP from the mitochondria. If the mitochondria of a challenged cell are unable to produce sufficient ATP the cell will undergo cell death by necrosis (Nicotera and Leist, 1997). The inner mitochondrial membrane is impermeable to both solutes and proteins, whereas the outer mitochondrial membrane is fully permeable to ions and proteins below ~5 kDa. As a requirement for the process of oxidative phosphorylation, the mitochondrial transmembrane potential ($\Psi_m$) is hyperpolarised relative to the cytoplasm, due mostly to the unequal distribution of protons across the inner mitochondrial membrane. Reduction of $\Psi_m$ is associated with cytochrome c release in many models of apoptosis (Zamzami et al., 1995). During necrosis the $\Psi_m$ collapses completely and irreversibly, the mitochondria swell and there may be rupturing of both inner and outer mitochondrial membranes (Kroemer et al., 1998). Conversely, reduction of $\Psi_m$ in apoptosis is likely to be a regulated event rather than a gross collapse, as the matrix environment does not equilibrate with the cytoplasm until the terminal stages of the apoptotic program (Zamzami et al., 1995).

There is some controversy over whether the reduction of $\Psi_m$ is required for release of cytochrome c. Release of cytochrome c precedes reduction of $\Psi_m$ and caspase activation in CEM or HeLa cells following UV irradiation or STS treatment (Bossy-Wetzel et al., 1998). In support, Goldstein et al. (2000) report cytochrome c release without reduction of $\Psi_m$ in HeLa cells, and also show that reduction of $\Psi_m$ but not cytochrome c release is dependent upon caspase activation. Studies using caspase-9<sup>−/−</sup> and Apaf-1<sup>−/−</sup> gene knockout mice show that addition of apoptotic stimuli induces the release of cytochrome c but not reduction of $\Psi_m$ (Yeh et al., 1999). This argues for release of cytochrome c due to a change in permeability of the outer mitochondrial membrane rather than changes to the inner mitochondrial membrane. In contrast, in pheochromocytoma-6 cells undergoing apoptosis, reduction of $\Psi_m$ accompanies cytochrome c release (Heiskanen et al., 1999).
The mechanism by which cytochrome c is released from mitochondria is currently not clear. There are several theories as to how this occurs:

i) A ‘megachannel’ called the permeability transition pore complex (PTPC) opens (Green and Reed, 1998; Viera et al., 2000), allowing water and solutes to enter. The mitochondrion swells, and the outer membrane is ruptured, leading to the release of pro-apoptotic intermembrane proteins (Vander Heiden et al., 1997). The PTPC is a large conductance channel that allows molecules smaller than ~1.5 kDa to pass. It consists of the adenine nucleotide translocator (ANT), which is located in the inner membrane, the voltage-dependent anion channel (VDAC; also called mitochondrial porin) in the outer membrane (reviewed in Miller, 1998) and the matrix protein cyclophilin D. These proteins are thought to form the PTPC where the membranes are apposed. The PTPC may also be comprised of Bcl-2 family proteins, which control the opening of the pore by interacting with VDAC (Tsujimoto and Shimizu, 2000). This theory for PTPC opening would involve a direct involvement between reduction of $\Psi_m$ and cytochrome c release.

ii) The outer mitochondrial membrane is destabilised by the insertion of pro-apoptotic Bcl-2 proteins such as Bax, Bak and Bid (Kudla et al., 2000; see section 1.6), thus causing a loss of membrane integrity and release of proteins.

iii) Cytochrome c is released by the formation of a sufficiently large channel that is selective for cytochrome c (Green and Reed, 1997). This would not result in major mitochondrial morphological changes such as swelling and rupture of the outer mitochondrial membrane. Potential candidates for the formation of this pore include pro-apoptotic members of the Bcl-2 family.

Evidence for the role of mitochondrial swelling during apoptosis is equivocal. Electron microscopic examination (Dallaporta et al., 1999) and flow cytometric analysis of apoptotic cells (Vander-Heiden et al., 1997) show some evidence for transient mitochondrial swelling during apoptosis, though other studies monitoring apoptosis by confocal microscopy claim there is no mitochondrial swelling (Kroemer et al., 1998).
Also located in the mitochondrial intermembrane space is apoptosis inducing factor (AIF; Susin et al., 1999b). Upon induction of apoptosis, AIF is translocated to the nucleus where it participates in DNA fragmentation. AIF is also implicated in positive feedback of pro-apoptotic signalling to mitochondria leading to further release of cytochrome c and appears to be an inducer of permeability transition (Susin et al., 1999b). Caspases -2, -3 and -9 have been localised to the mitochondrial intermembrane space (Mancini et al., 1998; Susin et al., 1999a; Zhivotovsky et al., 1999). Translocation of activated caspases resulting from disruption of the outer mitochondrial membrane may be crucial for their subcellular localisation and activation during apoptosis.

Smac (second mitochondrial-derived activator of caspases)/DIABLO (direct IAP binding protein with low pi) (Du et al., 2000; Verhagen et al., 2000) is also localised in the mitochondria and is released during apoptosis. Smac/ DIABLO binds to IAPs and prevents their action as inhibitors of apoptosis, thus relieving the inhibition of caspase activity (Figure 1.5). Smac/ DIABLO appears to be the mammalian homologue of Reaper, Grim and HID, pro-apoptotic proteins found in the fruit fly Drosophila melanogaster (reviewed in Abrams, 1999). The concentration of pro-apoptotic proteins such as Bax and DIABLO and anti-apoptotic proteins such as IAPs set the threshold for the induction of apoptosis and will determine the response to apoptotic stimuli. IAPs inhibit the activity of small quantities of activated caspase-9 and -3 and may thus prevent the ‘accidental’ initiation of apoptosis following the inadvertent release of cytochrome c.

1.6 THE BCL-2 FAMILY

The gene encoding Bcl-2 (B cell lymphoma/leukaemia-2) was first identified in patients suffering from B cell malignancies (Tsujimoto et al., 1985). Bcl-2 protects cells from a wide variety of apoptotic stimuli, including ultraviolet and γ irradiation, growth factor deprivation, heat shock, some cytotoxic lymphokines (e.g. TNF), calcium ionophores, glucocorticoid treatment, viral infection and agents that promote formation of free-radicals (Reed, 1994). To date at least nineteen cellular members of the Bcl-2 family (seven pro-survival members, twelve pro-apoptotic) and five viral homologues have been identified (reviewed in Adams and Cory, 1998; Reed, 1998). The Bcl-2 family can be divided into three
subfamilies: the pro-survival Bcl-2 subfamily (e.g. Bcl-2, Bcl-xL, Mcl-1) and the pro-apoptotic Bax (e.g. Bax, Bcl-xS, Bak) and BH3 subfamilies (e.g. Bid, Bik, Bad).

All members contain at least one of four homology domains, termed BH1 to BH4 (for Bcl-2 homology domains). Deletion mutations suggest that these regions govern protein-protein interactions between the family members. The BH3 domain is essential for pro-apoptotic function as its deletion from proteins of the BH3 subfamily abolishes their pro-apoptotic action and also prevents their dimerisation with pro-survival proteins such as Bcl-2 or Bcl-xL. Most Bcl-2 family proteins, except for several members of the BH3-only subfamily such as Bad and Bid, contain a C-terminal hydrophobic region that anchors them in intracellular membranes. Bcl-2 is embedded in the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum (González-García et al., 1994). In contrast, both Bcl-xL and Bax are mostly cytoplasmic prior to apoptotic stimulation, whereupon Bax and Bcl-xL translocate to membranes (Hsu et al., 1997). Interestingly, the anti-apoptotic Bcl-2 and Bcl-xL appear to be caspase substrates. Caspase digestion in vitro abolishes their anti-apoptotic nature and the resultant processed proteins appear to have a Bax-like pro-apoptotic function (Cheng et al., 1996).

The principal role of the Bcl-2 family appears to be the regulation of cytochrome c release from mitochondria. It is not clear how members of the Bcl-2 family function to enhance or inhibit apoptosis. Extrapolating from C. elegans, however, suggests that Bcl-2 may directly inhibit Apaf-1 oligomerisation and/or mitochondrial perturbation upon apoptotic insult, thus regulating release of cytochrome c from the mitochondria and/or formation of the apoptosome. Bcl-xL exerts its anti-apoptotic effect at least in part by regulating \( \Psi_m \) and volume homeostasis of mitochondria (Vander Heiden et al., 1997).

Many of the Bcl-2 family proteins can dimerise with themselves or one another, with one monomer antagonising or enhancing the function of the other. Due to these interactions between members, the ratio of anti-apoptotic proteins to pro-apoptotic proteins generally determines the reaction of a cell to an apoptotic insult (Oltvai et al., 1993). Phosphorylation of the BH3 protein Bad prevents its interference with Bcl-2 and Bcl-xL, thus removing its pro-apoptotic nature (Zha et al., 1996; Zhou et al., 2000). BH3-only proteins are unable to
homodimerise but by heterodimerisation may activate the pro-apoptotic Bax or inactivate the anti-apoptotic Bcl-2 and Bcl-xL (Wang et al., 1996). Pro-apoptotic members of the Bcl-2 family such as Bax and Bak may induce cytochrome c release by direct interaction with the VDAC or possibly another subunit of the PTP such as ANT. In contrast, BH3-only proteins such as Bid and Bik do not directly target VDAC in the release of cytochrome c (Shimizu, 2001). Bid appears to function at the mitochondrial surface by inducing oligomerisation of other Bcl-2 family proteins such as Bak and Bax, which form pores to release cytochrome c independently of the PTP (Korsmeyer et al., 2000).

*Formation of mitochondrial pores or channels by Bcl-2 family members.* Bcl-2 and several of the anti-apoptotic homologues are multifunctional proteins. One putative mechanism of action is the formation of ion channels or pores. The amino acid sequence of Bcl-xL shares some similarity with pore-forming bacterial toxins, which kill cells by the formation of high-conductance voltage-gated ion channels in the plasma membrane. Other members of the Bcl-2 family in addition to Bcl-xL have been proposed as having some ion channel function (reviewed in Schendel et al., 1998). Bcl-xL (Minn et al., 1997) and Bcl-2 (Schendel et al., 1997) form cation-conducting channels in synthetic lipid membranes. Bax forms ion channels that can conduct both cations and anions (Antonsson et al., 1997; Schlesinger et al., 1997) and formation of these channels is abolished in the presence of Bcl-2. The cleaved form of Bid has also been shown to form channels in planar bilayers and liposomes at physiological pH (Schendel et al., 1999). Interestingly, the uncleaved form of Bid does not form channels, which may explain why caspase cleavage of Bid is required for its pro-apoptotic action. However, it has not been established whether Bcl-2 family proteins form pores *in vivo* and whether ions or proteins pass through the pores. It is also unclear why both pro- and anti-apoptotic family members should have pore-forming capabilities, especially since the characteristics of pores appear do not vary greatly in their specificity and conductance characteristics.

**1.7 Nuclear changes in apoptosis.**

As described in section 1.3, the nuclei of apoptotic cells undergo characteristic morphological changes. Biochemical analysis of DNA by agarose gel electrophoresis shows a laddered appearance, first described by Wyllie (1980). This characteristic DNA laddering is
often used as a marker of apoptosis. Degradation of DNA is performed by the caspase-activated deoxyribonuclease (CAD; Enari et al., 1998), which exists in an inactive complex with a specific chaperone inhibitor of CAD (ICAD) in the cytoplasm of quiescent cells (reviewed in Nagata, 2000). Induction of apoptosis and activation of caspase-3 lead to the cleavage of ICAD and dissociation of the CAD/ICAD complex (Sakahira et al., 1998). CAD is released and translocates to the nucleus where it degrades chromosomal DNA, cutting genomic DNA between nucleosomes to generate nuclear fragments with lengths corresponding to multiples of 180 base pairs. Consistent with the caspase-3-dependent activation of CAD, knockout studies have shown that caspase-3 activation is required for induction of chromatin condensation and DNA degradation during apoptosis (Woo et al., 1998).

While degradation of genomic DNA is not essential for progression of apoptosis, there are several reasons why degradation might occur. Apoptotic cells in vivo might feasibly contain mutated or virally-contaminated DNA that would be a danger to phagocytosing cells and the organism. This potentially harmful DNA is therefore degraded prior to being engulfed by neighbouring cells, and thereby recipient cells can avoid transformation by the activated oncogenes or viral genes. The purpose of DNA cleavage might also be to reduce the immune response, as DNA is a strong autoantigen.

1.8 PHAGOCYTOSIS

Phagocytosis of apoptotic cells by macrophages or ‘amateur’ cleaner cells such as fibroblasts, epithelial cells or vascular smooth muscle cells serves to remove cell corpses and prevent the leakage of contents from dying cells, thus suppressing inflammation. Several cell surface changes mark apoptotic cells for phagocytosis by neighbouring cells or macrophages. The best characterised of these is externalisation of phosphatidylserine (PS). PS is a phospholipid normally localised in the inner leaflet of the plasma membrane that, during apoptosis, is externalised as a result of decreased aminophospholipid translocase activity and activation of a calcium-dependent scramblase (reviewed in Fadok et al., 1998; Savill and Fadok, 2000). The literature is equivocal over whether PS externalisation is mediated by or dependent upon active caspases.
1.9 Comparison between mechanisms of Apoptosis and Necrosis

Apoptosis and necrosis are modes of cell death with distinctively differing morphological and biochemical features, as well as differing effects on surrounding cells. These differences between apoptosis and necrosis are summarised in table 1.1.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological or pathological (subnecrotic damage).</td>
<td>Accidental, always pathological.</td>
</tr>
<tr>
<td>Sporadic incidence; individual cells.</td>
<td>Localised cell death; groups of neighbouring cells affected.</td>
</tr>
<tr>
<td>‘Silent’.</td>
<td>Disruptive.</td>
</tr>
<tr>
<td>Susceptibility tightly regulated.</td>
<td>Unregulated or poorly regulated.</td>
</tr>
<tr>
<td>Intracellular calcium undergoes a moderate rise.</td>
<td>Intracellular calcium undergoes a rapid increase several thousand-fold to equilibrate with the external environment.</td>
</tr>
<tr>
<td>Plasma membrane intact until late.</td>
<td>Early loss of plasma membrane integrity.</td>
</tr>
<tr>
<td>No leakage of cell content; little or no inflammation.</td>
<td>Dispersal of cytoplasmic contents into the extracellular space; inflammation.</td>
</tr>
<tr>
<td>Apoptotic cells and debris are eliminated by phagocytosis.</td>
<td>No phagocytosis.</td>
</tr>
<tr>
<td>Active process.</td>
<td>Passive process.</td>
</tr>
<tr>
<td>Cellular enzymes participate, causing characteristic biochemical and morphological features including chromatin condensation, nuclear fragmentation, protein cleavage by caspases and cell shrinkage.</td>
<td>Biochemical and morphological features include disruption of internal homeostasis, swelling of the entire cytoplasm and critically damaged organelles such as mitochondria with high-amplitude swelling.</td>
</tr>
</tbody>
</table>

Table 1.1 Comparison between the key features of apoptosis and necrosis.

The same cellular insult can induce apoptosis or necrosis, depending on intensity of treatment. In addition, the mechanisms of apoptotic and necrotic cell death have several biochemical features in common, such as proteolytic degradation and inhibition by Bcl-2 or...
Bel-xL. Current evidence suggests, however, that apoptosis and necrosis constitute distinct mechanistic pathways rather than opposing extremes of a continuum (reviewed in Raffray and Cohen, 1997).

1.10 Ionic Changes during Apoptosis

There is a loss of ion homeostasis during necrosis that stems from failure of plasma membrane ion pumps, decreased plasma membrane integrity and eventual membrane disruption (Buja et al., 1993). During apoptotic cell death, however, plasma integrity is maintained until late and there appear to be several regulated changes in intracellular ionic concentrations that have some bearing on the apoptotic program. There have, however, been relatively few investigations into the action of ions compared to the protein-protein interactions during apoptosis. Since electrical potentials and ion fluxes are involved in the regulation of many cellular processes such as division, secretion and signalling, it is highly probable that signalling by ionic flux has a major regulatory role in apoptosis.

1.10.1 The Intracellular Ionic Environment

The internal ionic environment of the cell is different to the external environment. Potassium is at a much higher concentration inside cells compared to the extracellular fluid, whereas the internal sodium concentration is lower than the external (Figure 1.6). Exclusion of sodium from the cytoplasm of cells may have originally been adopted as a means to drive transport of ions and solutes via the electrochemical sodium gradient, leaving potassium to be accumulated by cells in order to develop a negative turgor pressure (Epstein, 1986). The internal calcium concentration of cells is tightly controlled due to its role as a second messenger and the number of calcium-dependent enzymes. There is a ~15,000-fold difference between internal and external concentrations. Internal chloride concentration is lower relative to the external environment as there are many organic anions that maintain electroneutrality. The ionic environment of intracellular organelles may be different again from the cytoplasmic milieu. The ER is thought to act as a calcium store, containing up to 80% of cellular calcium. As already stated, the mitochondrial matrix has a different ionic environment to the cytoplasm and is hyperpolarised relative to the cytoplasm, principally due to proton imbalance that is required for oxidative phosphorylation (Chen, 1988).
Figure 1.6: Ionic environment of the cell. Ionic concentrations are taken from Sperelakis (1998).

Membrane potentials, such as those across the plasma membrane or inner mitochondrial membrane, are defined as the potential of the inner solution minus the outer solution. The cell maintains an ionic environment that is markedly different from the extracellular fluid, be it plasma or culture medium, and it is these ionic differences that set the plasma membrane potential ($\Psi_p$) of resting cells at approximately -80 mV relative to the extracellular medium. The $\Psi_m$ is approximately -165 mV relative to extracellular medium. The plasma membrane potential is created by a 30:1 ratio between intra- and extracellular...
potassium concentration ([K+]_\text{ext}; \sim 150 \text{ vs. } \sim 5 \text{ mM}), selective permeability of the plasma membrane to different ions, electrogenic ion pumps (particularly Na^+\text{,}K^+\text{-ATPase}) and the intracellular synthesis of large non-diffusible ions.

1.10.2 Ionic fluxes during apoptosis

Several ionic species have been implicated as having a role in apoptosis (reviewed in Lang et al., 1998; Gulbins et al., 2000; Yu and Choi, 2000). Cell shrinkage observed during apoptosis is almost certainly associated with efflux of cytoplasmic potassium ions (K^+) and accompanying efflux of water molecules by osmosis. This apoptotic cell shrinkage is an early event relative to other changes during apoptosis. Renal tube epithelial cells microinjected with cytochrome c displayed cell shrinkage 30 to 60 minutes later (Chang et al., 2000). In U937 cells treated with STS, apoptotic cell shrinkage occurred after 1 h, preceding cytochrome c release, caspase activation and DNA laddering (Maeno et al., 2000). Efflux of cytoplasmic K^+ has been described as an important regulatory event in the progression of apoptosis (Bortner and Cidlowski, 1996). The efflux of intracellular K^+ from cells has been demonstrated using flow cytometric assessment of intracellular [K^+] ([K^+]_i) (Barbiero et al., 1995; Bortner et al., 1997; Bortner and Cidlowski, 1999), electron probe X-ray microanalysis (Fernández-Segura et al., 1999), and using the K^+ surrogate ion 86Rb^+ (Gómez-Angelats et al., 2000). The [K^+]_i of apoptotic cells is decreased from \sim 150 \text{ mM} to between \sim 50 \text{ mM} (Barbiero et al., 1995; Hughes et al., 1997) and \sim 65 \text{ mM} (McCartney and Cotter, 1997).

Consistent with an important role for cytoplasmic K^+ in the initiation of apoptosis, there is some evidence that treatment of cells with agents that deplete intracellular K^+ induces apoptosis. The K^+ selective ionophore valinomycin, for example, induces apoptosis in thymocytes (Deckers et al., 1993) and a bone marrow cell line (Furlong et al., 1998). Similarly, K^+ depletion by the K^+ selective ionophore nigericin of lipopolysaccharide-stimulated macrophages (Perregaux and Gabel, 1994) or monocytes (Walev et al., 1995) leads to rapid release of processed interleukin-1\beta, resulting from activation of caspase-1 processing (Cheneval et al., 1998). Although these ionophores would also have other effects on the plasma membrane potential and the state of intracellular organelles, these findings support an important early role for the efflux of K^+ in apoptosis.
A number of other intracellular ions have been implicated in apoptosis. CD95-mediated apoptosis in B cells is associated with an early increase in cytoplasmic magnesium ions (Chien et al., 1999). Consistent with this, Bax-induced release of cytochrome c is highly dependent upon and is potentiated by magnesium ions (Eskes et al., 1998). Chloride ion concentration may have some function in apoptosis since oligonucleosomal, though not high molecular weight, DNA cleavage is abolished if efflux of intracellular chloride is prevented (Rasola et al., 1999). The cystic fibrosis transmembrane regulator (CFTR), a transmembrane anion channel that conducts chloride and bicarbonate, has also been suggested to play a role in apoptosis, as loss of CFTR activity inhibits cycloheximide (CHX) -induced apoptosis in mouse epithelial C127 cells (Gottlieb and Dosanjh, 1996).

Modulation of intracellular calcium can either inhibit or induce apoptosis. The sesquiterpene lactone thapsigargin irreversibly inhibits ER calcium adenosine triphosphatases (ATPases; Thastrup et al., 1990), leading to inhibition of calcium uptake by the ER and a subsequent increase in cytoplasmic calcium concentration. Thapsigargin can inhibit apoptosis in neuronal cells (Lampe et al., 1994) or induce apoptosis (Tsukamoto and Kaneko, 1993). Overbeeke et al. (1999) have shown in a flow cytometric analysis of T cells, however, that fluctuations in intracellular calcium concentrations are distal to PS externalisation and reduction of \( \Psi_m \). As this calcium flux appears to be a relatively late event in the progression of apoptosis, calcium is less likely to regulate apoptotic signalling at an early stage.

Fluctuations in intracellular hydrogen ion concentration (intracellular pH) are also associated with induction of apoptosis. Matsuyama et al. (2000) show that mitochondrial-dependent apoptotic stimuli such as Bax, STS or UV irradiation induce rapid cytoplasmic alkalinisation. In contrast, CD95-mediated apoptosis in Jurkat cells is associated with cytoplasmic acidification (Meisenholder et al., 1996). Somatostatin-induced apoptosis in MCF-7 cells is associated with a caspase-8-dependent decrease in pH that is prior to effector caspase activation and mitochondrial perturbation (Liu et al., 2000). In other apoptotic models, however, cytoplasmic acidification inhibits apoptosis (Tsao and Lei, 1996). There is some evidence for the involvement of Na\(^+\) ions in apoptosis, as the Na\(^+\),K\(^+\)-ATPase inhibitor Bufalin induces apoptosis in several leukaemia cell lines, possibly by causing an influx of sodium ions (Kawazoe et al., 1999). This is supported by Bortner et al. (2001), who show that, following
apoptotic stimulation of Jurkat cells, a rapid increase in intracellular sodium ions with an accompanying depolarisation of the plasma membrane is observed. Since the Na\textsuperscript{+},K\textsuperscript{+}-ATPase catalyses the coupled active transport of Na\textsuperscript{+} and K\textsuperscript{+} ions across the plasma membrane thus maintaining intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations and the plasma membrane potential, this sodium influx and plasma membrane depolarisation upon apoptotic stimulation might be achieved by the early inhibition of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase pump.

1.11 ION CHANNELS IN APOPTOSIS.

1.11.1 Potassium ion channels

Potassium ion channels, which are ubiquitous in eukaryotic cells, are integral membrane proteins that undergo conformational changes to open or close an ion-permeable pore across lipid membranes to mediate the passage of potassium ions down their electrochemical gradient. More than sixty pore-forming subunits of potassium ion channels have been cloned in the last ten years (reviewed in Yan and Yan, 1997) and these can be grouped into three families: the voltage-gated potassium channels, the inward-rectifying potassium channels and the recently discovered “two-pore” potassium channels. There is a wide range of potassium channels in vertebrates, due in part to alternative splicing between different subunits and association of accessory subunits that modify properties of the channel. Due to this large diversity of potassium channels, their pharmacology is not as well understood as that of calcium or sodium channels.

Two-pore potassium channels. This family of potassium-specific ion channels was only recently discovered and their physiological role remains to be elucidated (reviewed in Lesage and Lazdunski, 2000). Two-pore potassium channels (K\textsubscript{2P}) contain four transmembrane segments and are active as dimers. These channels are noninactivating, are active at all membrane potentials and are not inhibited by ‘classical’ potassium channel blockers such as 4-aminopyridine (4-AP), tetraethylammonium (TEA) or Cs\textsuperscript{+}. These opening properties designate them as ‘background’ potassium channels, with the current produced independent of time and voltage. The physiological functions of K\textsubscript{2P} channels include maintenance of resting plasma membrane potential and regulation of K\textsuperscript{+} transport associated
with recycling and secretion (Lesage and Lazdunski, 2000). The gating properties of K₂P channels make them unlikely candidates for involvement in apoptosis.

**Voltage-gated potassium channels.** Voltage-gated potassium channels (Kv) have six transmembrane segments (Figure 1.7) and are intrinsically sensitive to changes in membrane potential, which allows the activity of these channels to be regulated by membrane potential. Changes in membrane potential trigger conformational changes in the channel protein, thereby generating gating currents and causing the channel to be activated for ion permeation. Mammalian voltage-gated potassium channels can be subdivided into six subfamilies named Kv1.x, Kv2.x, Kv3.x, Kv4.x, HERG and maxi K (reviewed in Conley and Brammar, 1999), which correspond to six Drosophila potassium channel genes. Kv channels are inactivated by a ‘ball and chain’ mechanism (Hoshi et al., 1990). An N-terminal hydrophobic region followed by a hydrophilic region forms a ball that is linked to the membrane spanning domain by a hydrophilic chain. Under resting conditions, the channel is closed by the ball occupying the channel pore, thus blocking passage of K⁺ ions.

**Figure 1.7: Transmembrane structure of voltage-gated K⁺ (Kv) channels.** Kv channels contain six membrane-spanning domains, S1-S6. Domains S5 and S6 make up the pore region and there is an intrinsic voltage sensor on the S5 domain. Channel closure is effected by an N-terminal ‘ball’ domain in fast-inactivating Kv channels.
Inwardly rectifying potassium channels. Inwardly rectifying potassium (Kir) channels (reviewed in Isomoto et al., 1997; Nichols and Lopatine, 1997; Reimann and Ashcroft, 1999) allow the passage of potassium ions into the cell more readily than out of the cell at any given driving force (voltage). Kir channels have two transmembrane segments and do not contain intrinsic voltage sensors (Figure 1.8). The majority of Kir channels are gated by a C-terminus ball and chain mechanism, though the channel pore region of a few weak inwardly rectifying potassium channels is blocked in a voltage-dependent manner by magnesium ions or polyamines such as spermine, spermidine and putrescine (Oliver et al., 2000). Kir channels play a significant role in determining the plasma membrane potential of resting cells and set a threshold for excitation, thereby modulating the electrical activity of cardiac and neuronal cells and insulin secretion. Kir channels also play a role in the secretion and absorption of K\(^+\) ions across membranes. Kir channels may be regulated by hormones, neurotransmitters and the internal metabolic status of the cell.

**Figure 1.8:** Transmembrane structure of inward rectifying K\(^+\) (Kir) channels. Kir channels contain two membrane-spanning domains, M1 and M2, which make up the pore region. A segment between these two regions, termed M5 or P, is responsible for the K\(^+\) specificity of the channel. The C' terminus contains 'ball and chain' that controls gating of the channel.
**Chapter One**

KIR channels can be divided into seven subfamilies, referred to as KIR1.x to KIR7.x. KIR1.x channels are expressed predominantly in the kidney and are weak inward rectifiers involved in transepithelial transport (Ho et al., 1993). KIR2.x channels are concerned with controlling the excitability of the heart and brain and are ubiquitously expressed in these tissues (Kubo et al., 1993; Ishii et al., 1994; Périer et al., 1994). KIR3.x channels are G-protein gated and mediate the effects of certain G-coupled receptors on electrical activity in cardiac, neuronal and neurosecretory cells (Karschin et al., 1994; Ferrer et al., 1995). KIR4.x channels maintain K⁺ homeostasis in the kidneys and glia (Ito et al., 1996). The physiological roles of KIR5.x, which is expressed in the brain (Takumi et al., 1995), are currently unknown. KIR6.x, also referred to as ATP-sensitive (K_ATP) channels, are regulated by cytosolic nucleotides. They are found in both the plasma and mitochondrial membranes and link cellular metabolism to electrical activity and K⁺ fluxes (Babenko et al., 1998; Ashcroft and Gribble, 1998). KIR6.x channels play a crucial role in coupling metabolic energy to the membrane potential of cells and are centrally involved in the regulation of pancreatic insulin secretion (Seino et al., 2000). KIR6.x channels also appear to play a role in cell survival following ischaemia (Akao et al., 1997). KIR7.x channels are widely expressed in the brain and are also present in the kidneys and intestine. Their function appears to be setting the resting membrane potential of these tissues (Krapivinsky et al., 1998).

### 1.11.2 Potential candidate ion channels in apoptosis

Several channel subtypes have been implicated as having a role in apoptosis (reviewed in Gulbins et al., 2000). The voltage-gated potassium channel Kv1.3, which is involved in regulatory volume decrease (Deutsch and Chen, 1993), has been implicated in the apoptotic mechanisms initiated by death receptor ligation. Upon induction of CD95-mediated apoptosis, Kv1.3 is down-regulated by tyrosine phosphorylation of the channel protein (Szabó et al., 1996; reviewed in Szabó et al., 1997). The activity of Kv1.3 has also been shown to be down-regulated by ceramide (Gulbins et al., 1997), a lipid metabolite synthesised following CD95 ligation (Cifone et al., 1994).

The membrane potential of resting T cells is strongly influenced by voltage-gated channels, and pharmacological block of these channels is sufficient to depolarise the plasma membrane and prevent activation (Leonard et al., 1992). However, since the gating condition
of Kv channels requires a change in membrane potential, earlier ionic changes would be required to cause opening of this channel. In Drosophila, the Reaper and Grim proteins, activators of caspase-like molecules that may be homologous to mammalian Smac/DIABLO, inactivate voltage-gated channels (Avdonin et al., 1998). This observation suggests that chronic depolarisation of the plasma membrane by sustained inactivation of K+ channels may lead to activation of the caspase family.

Inward rectifying K+ channels appear to be promising candidates for mediating outward flux of K+ during apoptosis. The K_ATP subtype of K_IR channels, in particular, which functions to link the metabolic state of the cell to membrane potential and K+ flux is a good candidate for an early role in apoptosis. Mutations in inwardly rectifying K+ channels have been implicated in the pathogenesis of a number of diseases termed channelopathies (Abraham et al., 1999), though these conditions are not necessarily linked with aberrant induction of apoptosis. In humans, persistent hyperinsulinemic hypoglycaemia of infancy has been linked to disregulation of insulin secretion in pancreatic B cells by defects in K_IR6.2 or associated proteins. Mutation of K_IR3.2 in mice leads to the Weaver phenotype, characterised by abnormal movement and extensive apoptosis in cerebellar neurons (Migheli et al., 1997; Harrison and Roffler-Tarlov, 1998). This mutation removes pore selectivity for K+, thus allowing the passage of Na+ through the K_IR3.2 channel.

1.12 AIMS AND OBJECTIVES OF THE THESIS.

The involvement of K+ in apoptosis was investigated in preference to Ca2+, Na+ or Cl− because an efflux of intracellular K+ associated with the induction of apoptosis appears to be a widespread observation common to many experimental models of apoptosis. These changes in intracellular K+ may be intrinsic to the apoptotic pathway and have a direct role in the regulation of apoptosis rather than mediating other cellular pathways with only secondary role in apoptosis. Since K+ is important in the regulation and setting of plasma membrane potential, it is therefore likely to be involved in any electrical changes at the plasma membrane during apoptosis. The intracellular changes in concentrations of Ca2+, Na+ or Cl− ions following induction of apoptosis and their roles in apoptosis are less clear. Changes in intracellular levels of these ions during apoptosis appear to depend upon the cell line and stimulus investigated.
Calcium has many intracellular roles, with many calcium-sensitive enzymic processes that can affect cell survival, making its role in apoptosis difficult to isolate.

Ion channels are likely to have an important regulatory role in apoptosis and therefore identification of the ion fluxes during apoptosis and the ion channels mediating these fluxes may be important for the development of therapeutic strategies. There has been considerable interest in the development of specific inhibitors of different members of the caspase family. As detailed in section 1.4.1, members of the caspase family differ in their substrate specificity and role in the cell and different tissues, making their specific modulation a possible strategy for treatment of conditions such as ALS, head trauma and Parkinson’s disease (Nicholson, 2000). However, the targeting and modulation of intracellular proteins is difficult. In contrast, ion channels contain an extracellular domain that can be accessed and modulated relatively easily. Ion channels may therefore make excellent targets for the therapeutic treatment of certain pathological conditions.

The aims of the thesis were to:

- characterise inhibition of apoptosis by elevated medium [K⁺] in cerebellar granule neurons (Chapter Three).
- investigate electrical changes in the plasma membrane potential during CD95- and chemical stress-mediated apoptosis in immortalised cell lines and to characterise efflux of intracellular K⁺ in relation to the apoptotic phenotype (Chapter Four).
- investigate how K⁺ ion channels are involved in apoptosis and how their modulation affects the induction of apoptosis; to identify which channel subtypes mediate ionic changes during apoptosis (Chapter Five).
- investigate the inhibition of CD95- and chemical stress-mediated apoptosis in immortalised cell lines by elevated medium [K⁺] and to elucidate the mechanism by which this is achieved (Chapter Six).
- elucidate the intracellular signalling pathways that mediate chemical stress-mediated apoptosis; in particular to investigate the role of acylpeptide hydrolase in apoptosis (Chapter Seven).
Chapter Two. Materials and Methods

2.1 MATERIALS

All chemicals were obtained from Sigma (Poole, U.K.) unless stated otherwise. \(^{86}\)RbCl was from Amersham Pharmacia (Bucks, U.K.). Benzylloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD.fmk) and benzylloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (z-DEVD.AFC) were from Enzyme System Products (CA, U.S.A.). Fluorescein isothiocyanate (FITC) -conjugated Annexin V was from Bender Medsystems (Vienna, Austria). Phycoerythrin (PE) -conjugated Annexin V was from BD PharMingen (CA, U.S.A.). Calcein acetoxyethyl ester (calcein AM), tetramethylrhodamine ethyl ester (TMRE), 3,3’diohexyloxacarbocyanine iodide (DiOC\(_6(3)\)), bis (1,3-dibutylbarbituric acid) trimethene oxonol (DiBAC\(_4(3)\)), potassium-binding benzofuran isophthalate acetoxyethyl ester (PBFI-AM) and Pluronic F-127 were from Molecular Probes (OR, U.S.A.). Carbobenzoxy-Leu-Leu-Leu-CHO (MG132) and glyburide were from Calbiochem (Notts, U.K.). TNF-related apoptosis-inducing ligand (TRAIL) was prepared as described previously (MacFarlane et al., 1997). Media and serum were purchased from Life Technologies Inc. (Paisley, U.K.). Tritiated diisopropylfluorophosphate (DFP) was from Dupont NEN (Hounslow, U.K.). N-acetyl-leucyl-chloromethylketone (ALCK) was synthesised by Dr. B. Reidle, (Bayer AG, Wuppertal, Germany). Organophosphate (OP) compounds were obtained from Greyhound / ChemService (Birkenhead, U.K.). The potassium channel openers diazoxide, P1060 and RP49356 were a kind gift from Professor A. Weston (Manchester, U.K.). Recombinant Margatoxin (rMgTX) was from Alomone Labs (Jerusalem, Israel). 4-Aminopyridine (4-AP) was from Tocris Cookson (Bristol, U.K.).

2.2 ANIMALS

Wistar rat pups were bred by the Biomedical Services Department, University of Leicester. Both male and female pups, aged 6 to 7 days, postnatal were used.
## 2.3 Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Mono/polyclonal</th>
<th>Animal raised in</th>
<th>Dilution</th>
<th>% SDS-PAGE gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apaf-1</td>
<td>R&amp;D Systems Inc. (MN, U.S.A.)</td>
<td>monoclonal</td>
<td>Mouse</td>
<td>1:1,000</td>
<td>13</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Amersham Pharmacia</td>
<td>monoclonal</td>
<td>Mouse</td>
<td>1:2,000</td>
<td>13</td>
</tr>
<tr>
<td>caspase-3</td>
<td>1</td>
<td>polyclonal</td>
<td>Rabbit</td>
<td>1:10,000</td>
<td>13</td>
</tr>
<tr>
<td>caspase-8</td>
<td>2</td>
<td>polyclonal</td>
<td>Rabbit</td>
<td>1:3,000</td>
<td>13</td>
</tr>
<tr>
<td>cytochrome c</td>
<td>Becton Dickinson</td>
<td>monoclonal</td>
<td>Mouse</td>
<td>1:2,000</td>
<td>13</td>
</tr>
<tr>
<td>FADD</td>
<td>Transduction Laboratories (KY, U.S.A.)</td>
<td>monoclonal</td>
<td>Mouse</td>
<td>1:500</td>
<td>13</td>
</tr>
<tr>
<td>GFP</td>
<td>Clontech (CA, U.S.A.)</td>
<td>polyclonal</td>
<td>Rabbit</td>
<td>1:2,000</td>
<td>13</td>
</tr>
<tr>
<td>PARP</td>
<td>3</td>
<td>monoclonal</td>
<td>Mouse</td>
<td>1:10,000</td>
<td>7</td>
</tr>
<tr>
<td>anti-mouse secondary</td>
<td>Sigma</td>
<td>monoclonal</td>
<td>Goat</td>
<td>1:3,000</td>
<td>-</td>
</tr>
<tr>
<td>anti-rabbit secondary</td>
<td>Dako (Cambridge, U.K.)</td>
<td>polyclonal</td>
<td>Goat</td>
<td>1:3,000</td>
<td>-</td>
</tr>
<tr>
<td>anti-mouse FITC secondary</td>
<td>Dako</td>
<td>monoclonal</td>
<td>Goat</td>
<td>1:25</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: Antibodies used for Western blotting (see 2.10).

1. Anti-caspase-3 antibody was obtained from Dr. D. Nicholson (Merck Frosst, Canada).
2. Anti-caspase-8 antibody was obtained from Dr. X-M. Sun (M.R.C., Leicester).
3. The PARP antibody (C-2-10, mouse monoclonal) was provided by Dr. G. Poirier (Laval University, Quebec, Canada).

### 2.4 Preparation and culturing of cerebellar granule neurons

An adapted version of the protocol detailed in Miller and Johnson (1996) was followed. Cerebella from six postnatal day 6-7 Wistar rat pups were dissected and placed in ice-cold dissecting buffer (Hanks buffered saline solution, 100 U/ml penicillin, 100 µg/ml streptomycin (all Gibco; MD, U.S.A.), 500 µM MgSO₄, 0.3 % (w/v) BSA). Meningeal layers and blood vessels were removed manually and then cerebella sliced into ~1 mm
Cubes. Minced cerebella were then placed in 20 ml trypsin solution (dissection buffer plus 0.1 % (w/v) trypsin and 1 U/ml DNAase I) and incubated at 37°C for 15 min. Digestion was stopped by the addition of 20 ml trypsin inhibitor solution (Eagle’s minimal essential media (MEM; Gibco), 300 μM MgSO₄, 80 μg/ml trypsin inhibitor, 1 U/ml DNAase I) and the tissue was then pelleted (700 × g for 2 min). The supernatant fraction was removed and 5 ml trituration buffer (MEM, 10 % (v/v) foetal calf serum (FCS; Life Technologies Inc.), 1.5 mM MgSO₄, 1 U/ml DNAase I) was added. Cell dissociation was performed three times; on each occasion cells were triturated through a fire-polished glass pipette 10 to 15 times, the cell suspension allowed to settle for 4 min and the upper 2 ml of cell suspension then removed to a fresh tube. A further 2 ml of trituration buffer was added to the original cell suspension and the cycle repeated twice more. The triturated cell suspension was centrifuged through 2.5 ml of 4 % (w/v) BSA/Earle’s balanced salt solution (EBSS; Gibco) at 120 × g for 5 min to remove debris and the cell pellet then resuspended in 10 ml complete growth medium (MEM with glutamax (Gibco), 10 % (v/v) FCS, plus 33 mM glucose, 20 mM KCl, 50 μg/ml gentamycin). Cell yield and viability were assessed using trypan blue exclusion in a haemocytometer.

Cells were seeded in poly-L-lysine-coated culture dishes (Becton Dickinson; NJ, U.S.A.) at a density of 3.5-5.0 × 10⁵ cells/ml. For 6- and 24-well culture dishes, 3.5 ml and 1 ml per well were seeded, respectively. Cells were grown at 37°C in a humidified 95 % air, 5 % CO₂ atmosphere. 24 h after plating and again after 4 days in vitro, growth medium was half exchanged and 10 μM cytosine arabinoside added to the wells to prevent division of non-neuronal cells. Poly-L-lysine-coating of culture dishes was performed according to manufacturer’s instructions; a solution of 50 μg/ml poly-L-lysine was made up in distilled water and filter-sterilised at 0.2 μm. 200 μl of solution per well was added to 24-well dishes and 700 μl per well added for 6-well dishes. The dishes were incubated at 37°C for 45 min, the poly-L-lysine solution aspirated off and the dish allowed to dry in the incubator for 2 h.

2.5 ASSESSMENT OF CELL DEATH IN CGN CULTURES

For the quantification of culture viability and cell death, a Zeiss Axiovert 135 microscope with a 50 W mercury lamp (365 nm emission) was used in conjunction with filters suitable for the fluorescent dyes detailed below.
**Calcein-acetoxymethyl ester (-AM):** For the assessment of culture viability, cells were incubated for 40 min with calcein-AM, a vital dye that releases the fluorochrome calcein into the cytoplasm of live cells. Hydrolysis of the esterified groups of calcein-AM is essential for producing the fluorescent calcein molecule, since modification of dye carboxylic acid groups with AM ester groups results in an uncharged molecule that is cell permeable but not fluorescent. Once inside the cell, the lipophilic groups are cleaved by non-specific cell esterases, resulting in a molecule that leaks out of cells far more slowly than the parent compound. Calcein has good cell retention, is pH-insensitive and is released from the cell during cytolysis, making it a good indicator of cell culture viability. The cytoplasm of live CGN cells, including neurite networks, was stained green by this dye, whereas dead cells were not stained.

**Hoechst 33342:** To distinguish between normal and apoptotic cells, the cell permeant bisbenzimide dye Hoechst 33342 was used. Hoechst 33342 binds to the minor groove of DNA at A-T rich sequences and fluoresces bright blue when bound to DNA. Hoechst 33342 was chosen over Hoechst 33258 due to its higher cell permeability.

**Propidium iodide:** Propidium iodide (PI) is a cell impermeant dye that, under UV light, increases in fluorescence 20- to 30-fold when bound to nucleic acids. PI was used to distinguish between live and dead cells as it is only taken up by cells that have lost plasma membrane integrity. Only the nuclei of dead cells are stained.

### 2.6 Tissue Culture of Cell Lines

#### 2.6.1 Leukaemic cell lines

SKW 6.4 B lymphocytic, monocytic U937, Chinese hamster ovary (CHO) and human monocytic THP.1 tumour cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Porton Down, U.K.). Jurkat E6.1 T cells were obtained from Prof. U. Zimmerman (Würzburg, Germany) and CEM-C7H2 lymphoblastoma cells from Dr. C. Dive (University of Manchester, U.K.).

Jurkat, SKW 6.4, U937, CEM-C7H2 and THP.1 cells were cultured in RPMI 1640 (Life Technologies Inc.), 10 % (v/v) FCS, 1 % (v/v) glutamax. Jurkat medium was
supplemented with 1% (v/v) non-essential amino acids and 50 µM 2-mercaptoethanol. CHO cells were cultured in MEM alpha medium with 1% (w/v) glutamax and 10% (v/v) FCS. Cells were maintained at 37°C in a humidified 95% air, 5% CO₂ incubator. Suspension cells were counted before use with a CASY 1 cell counter (Schärfe Systems; Reutlingen, Germany). Cells used in experiments were in logarithmic growth phase.

Suspension cells (Jurkat, SKW6.4, U937, THP.1 and CEM) were routinely split by diluting 1:12 in pre-warmed culture medium to maintain a culture density of between 5 x 10⁵ and 1 x 10⁶ cells/ml. The adherent CHO cell line was split approximately every three days. Cells were rinsed in PBS and then trypsin (0.05%) in EDTA/PBS buffer was added to detach cells from the culture dish. After incubation for 10 min, the cell suspension was transferred to a centrifuge tube and complete medium added to inactivate the trypsin. Cells were pelleted for 5 min at 500 x g, resuspended in complete medium at a dilution of 1:10 and then plated in 6-well culture dishes.

2.6.2 Media of defined ionic composition

Experimental media were made up as detailed in Table 2.2, with NaCl and KCl added to a total concentration of 140 mM. The pH of the media was adjusted to 7.4 with either NaOH or KOH, depending on whether Na⁺ or K⁺ was the major cation in the medium. The osmolarity of the medium was checked in each case using an Advanced Micro-osmometer 3MO plus (Advanced Instruments Inc.; MA, U.S.A.), and was within the range of 275-300 mOsm. Media containing the monovalent organic cation N-methyl-D-glucamine (NMDG) were made up as above, but with NMDG substituting for Na⁺. Dialysis of FCS to remove K⁺ and Na⁺ was performed using Slide-A-Lyzer dialysis cassettes (10,000 molecular weight cut-off; Pierce, IL, U.S.A.) for two periods of 1 h in 1.5 l of dialysis buffer, as detailed in Table 2.2.
### Table 2.2: Comparison of components of RPMI 1640 complete medium, media of defined ionic composition and dialysis buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>RPMI 1640</th>
<th>Ionically-defined medium</th>
<th>Dialysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>103 mM</td>
<td>140 mM combined</td>
<td>-</td>
</tr>
<tr>
<td>KCl</td>
<td>5.4 mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>423 µM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>-</td>
<td>500 µM</td>
<td>500 µM</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>406 µM</td>
<td>500 µM</td>
<td>500 µM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>23.8 mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>5.6 mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose</td>
<td>11 mM</td>
<td>10 mM</td>
<td>40 mM</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1 µg/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HEPES</td>
<td>-</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Phenol red</td>
<td>5 µg/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamax</td>
<td>1 %</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FCS</td>
<td>10 %</td>
<td>10 % (dialysed)</td>
<td>-</td>
</tr>
</tbody>
</table>

2.6.3 Induction of apoptosis

For experiments in standard RPMI 1640 medium, cells at a density of $5-8 \times 10^5$/ml were plated in 6- or 24-well plates (Becton Dickinson), 4 ml or 1 ml culture per well respectively, treated with apoptotic stimuli and incubated at 37°C, 5% CO₂ for the appropriate time. For experiments involving ionic manipulation of culture media, cells were washed once in experimental medium and then re-suspended in experimental medium to a final concentration of $5-8 \times 10^5$ cells/ml. Apoptotic stimuli were added 10 min after re-suspension in experimental media. In order to allow z-VAD.fmK time to permeate cells, cultures were incubated with z-VAD.fmK for 30 min prior to treatment with apoptotic stimuli. Except for anti-CD95 antibody, which was diluted with PBS, stock solutions of apoptotic stimuli and pharmacological agents were made up in DMSO, with cell culture DMSO concentrations not exceeding 1 % (v/v). For control-treated cultures, the vehicle
corresponding to the apoptotic stimulus was used. Where both PBS and DMSO were used in an experiment to dissolve compounds, control cells were treated with DMSO.

2.6.4 Treatment with pharmacological agents

Cells were treated with pharmacological agents (glyburide, 4-AP and ouabain) 15 min prior to addition of apoptotic stimuli to allow time for compounds to be taken up by cells. rMgTX was dissolved in buffer (0.1 % (w/v) BSA, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH adjusted to 7.5). Before applying rMgTX, BSA was added to cultures to a final concentration of 0.01% (w/v) to increase stability of the rMgTX protein.

2.7 Flow cytometry

2.7.1 Principles of flow cytometry

FACScan and FACS (fluorescence activated cell sorter) Vantage flow cytometers were used in conjunction with CellQuest software (both Becton Dickinson). For both the FACScan and the FACS Vantage, samples were assayed at a density of 5-8 x 10^5 cells/ml, 500 µl per assay with 5,000 events recorded per assay. The FACScan uses an air-cooled argon gas laser, 15 mW output, with a fixed wavelength emission of 488 nm. The FACS Vantage uses a 5 W water-cooled UV laser, with an emission wavelength of 352 nm, in addition to an argon gas laser.

Flow cytometers work by focussing a laser beam onto a sample stream of particles (cells) that are hydrodynamically focused into a unicellular flow (Figure 2.1). The particles are illuminated individually as they pass through the laser beam and the signal from the interrogated particle is routed by the collection optics of the FACScan to designated optical detectors. Photomultiplier tubes detect the amount of forward and side scatter from the particle, with the size of the particle determining how much light is scattered in the forward direction and the internal complexity of the particle (usually equating to cell density) determining the side scatter. Any fluorescent molecules in or on the particle will fluoresce as the particle is illuminated by the laser beam, with the intensity of emitted light dependent upon the quantity of fluorescent compound on the particle. The FACScan has three fluorescence detection channels, which simultaneously detect green, orange, and far-red light (termed FL-1 to FL-3, respectively). The FACS Vantage also has a fourth ‘blue’ fluorescence detector (FL-4) to detect emissions elicited by the UV laser, in addition to the
green, orange and far-red fluorescence detectors associated with the argon laser. The signal from scattered light or emitted fluorescence is transduced to a voltage by analogue-to-digital converters, with the 1023 channels of each detector corresponding to 10 mV increments, and routed to a dedicated computer (Power Macintosh G3) running CellQuest software.

Figure 2.1: Optics of the FACScan flow cytometer (simplified). The argon gas laser is directed onto a focused stream of cells. Scattered light and emitted fluorescent light is routed by optics of the FACScan to photomultiplier tubes and converted to a digital signal. This is then processed and displayed by dedicated computer software.
The peak absorbance (excitation) wavelength of fluorophores determined which laser was used to generate optimal fluorescence. Dyes such as FITC, DiBAC$_4$(3) and DiOC$_6$(3), with peak absorptions of 490 nm, 493 nm and 484 nm respectively (Table 2.3), were ideal for the 488 nm wavelength argon laser. The dyes PBFI-AM and Hoechst 33342 with peak absorptions of 369 nm and 350 nm required the UV laser of the FACS Vantage to generate emission of fluorescent light. The emission spectra of dyes determined which fluorescence detection channel was used. The 525 nm peak emission of FITC was detected by the FL-1 channel (515 - 545 nm). The FL-2 channel (564 - 606 nm) detected emissions such as that of PE (570 nm peak emission). The FL-3 detected wavelengths above 650 nm. Although the peak emission of TMRE was within the FL-2 channel (574 nm), the emission spectrum was sufficiently broad to give an equally good signal in the FL-3 channel. The FL-4 channel of the FACSVantage (417.5 - 482.5 nm) was used to detect emissions excited by the UV laser. Since particles are interrogated in series by the argon and UV lasers on the FACSVantage, there was no problem with interference between, for example, FITC (525 nm emission peak) and PBFI-AM (557 nm emission peak).

The FACS Vantage was also capable of cell-sorting, separating cells into different populations based on their properties, but this capability was not used. Instead the samples were discarded following analysis for both the FACScan and the FACS Vantage. Analysis of flow cytometry was performed using CellQuest software. Since all flow cytometer measurements taken were relative, it was not possible to calculate values for cell properties from the data obtained, e.g. cell size in μm from FSC.
2.7.2 Fluorophores used in flow cytometry

<table>
<thead>
<tr>
<th>Dye</th>
<th>Peak absorbance (nm)</th>
<th>Peak emission (nm)</th>
<th>FACS channel</th>
<th>Cell parameter analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V-FITC</td>
<td>490</td>
<td>525</td>
<td>FL-1</td>
<td>PS externalisation</td>
</tr>
<tr>
<td>Annexin V-PE</td>
<td>480</td>
<td>570</td>
<td>FL-2</td>
<td>PS externalisation</td>
</tr>
<tr>
<td>Calcein-AM</td>
<td>494</td>
<td>517</td>
<td>FL-1</td>
<td>Live cells</td>
</tr>
<tr>
<td>DiBAC&lt;sub&gt;4&lt;/sub&gt;(3)</td>
<td>493</td>
<td>516</td>
<td>FL-1</td>
<td>Plasma membrane potential</td>
</tr>
<tr>
<td>DiOC&lt;sub&gt;6&lt;/sub&gt;(3)</td>
<td>484</td>
<td>501</td>
<td>FL-1</td>
<td>Mitochondrial transmembrane potential</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>350</td>
<td>461</td>
<td>FL-4</td>
<td>Nuclear condensation</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>536</td>
<td>617</td>
<td>FL-2</td>
<td>Plasma membrane integrity</td>
</tr>
<tr>
<td>PBFI-AM</td>
<td>369</td>
<td>557</td>
<td>FL-4</td>
<td>Intracellular [K⁺]</td>
</tr>
<tr>
<td>TMRE</td>
<td>549</td>
<td>574</td>
<td>FL-3</td>
<td>Mitochondrial transmembrane potential</td>
</tr>
</tbody>
</table>

Table 2.3: Spectral properties of fluorophores used and cell parameters measured.

FSC/SSC: The size and granularity (density) of acquired events was used to discount debris from analysis (Figure 2.2, A and B) as this could significantly bias data if not removed. The assessment of cell shrinkage as an apoptotic marker was achieved by quantifying the percentage of cells beneath a particular size on a population histogram of forward scatter (Figure 2.2, C and D).
Figure 2.2: Assessment of cell size and density and cell shrinkage by flow cytometry. Jurkat T cells treated with vehicle (PBS; A, C) or anti-CD95 antibody (50 ng/ml; B, D) for 5 h were analysed by flow cytometry. For analysis of data, debris and non-cell events were excluded by the gating region shown (A, B). Only events within this region were included for further analysis. For assessment of cell shrinkage, the histogram of population size distribution for anti-CD95 antibody-treated cells (D) was compared against control (C).
Annexin V-FITC/Annexin V-PE: Annexin V is a Ca\(^{2+}\)-dependent phospholipid with a very strong affinity for phosphatidylserine (PS), a membrane phospholipid that is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis as a phagocytotic marker (Martin et al., 1995). Analysis by flow cytometry of cells incubated with FITC- or PE-conjugated Annexin V was used to quantify the proportion of cells exhibiting PS externalisation.

To assess the proportions of cells in treated cultures that were normal, apoptotic or dead, cells were stained with Annexin V-FITC in conjunction with PI and analysed by flow cytometry. Non-cell events (debris and cell fragments) were discounted from analysis by excluding small events on a size/density plot (Figure 2.2A and B). The degree of PS externalisation of cells that were within the region parameters was then plotted against membrane integrity (Figure 2.3). Normal cells were characterised as those without PS externalisation and with an intact plasma membrane. Apoptotic cells exhibited PS externalisation with no loss of membrane integrity. Cells that exhibited both PS externalisation and an increase in permeability of the plasma membrane were interpreted as dead. It was not possible to distinguish by what mechanism these cells had died. Where stimuli were used that induced apoptosis almost exclusively rather than necrosis, however, it was assumed that PI-including cells had undergone apoptosis and had reached a stage where they would \textit{in vivo} have been phagocytosed by surrounding cells. As no cells capable of phagocytosis were present, these cells progressed into what was termed secondary necrosis, with loss of plasma membrane integrity and a corresponding uptake of PI. Therefore, for quantification of induction of apoptosis by stimuli such as anti-CD95 antibody, the proportion of apoptotic cells was calculated by combining the percentages of cells with externalised PS without loss of membrane integrity, and cells with both externalised PS and loss of membrane integrity. Some events were observed with loss of membrane integrity but without PS externalisation. As loss of membrane integrity would also lead to staining of PS, even without externalisation, it was concluded that these events were not membrane-enclosed and were therefore assumed to be the intact nuclei of lysed cells.
Figure 2.3: Quantification of apoptotic cells by Annexin V-FITC and PI. Jurkat T cells incubated with anti-CD95 antibody (50 ng/ml) for 5 h were stained with Annexin V-FITC and PI. Cells without PS externalisation (low Annexin V-FITC fluorescence) and low membrane permeability (low PI fluorescence) were interpreted as normal. Cells with PS externalisation but low membrane permeability were interpreted as apoptotic. High membrane permeability combined with PS externalisation indicated dead (necrotic or secondary necrotic) cells. Events with high PI fluorescence but no Annexin V-FITC staining were thought to be the intact nuclei of lysed cells and were discounted. The percentage of total events is shown for each quadrant.
The protocol for staining suspension cells with Annexin V-FITC/PI was adapted from the manufacturer's instructions. Cells (~2.5 x 10^5) were pelleted at 400 x g for 4 min at room temperature and then resuspended in Annexin V buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2, pH adjusted to 7.4 with NaOH). 1 μl of Annexin V-FITC was added and cells incubated at room temperature for 7 min. PI was added to a final concentration of 1.2 μg/ml and cells then placed on ice until analysed on the flow cytometer. Staining with Annexin-PE was performed in a similar fashion, except 5 μl Annexin V-PE was added and cells incubated for 15 min.

**DiOC_6(3) (3,3'dihexyloxacarbocyanine iodide):** DiOC_6(3) is a cationic dye that accumulates on hyperpolarised membranes and is translocated into the lipid bilayer and therefore is an effective indicator of mitochondrial transmembrane potential (Ψ_m). To assess the reduction of Ψ_m following induction of apoptosis, the DiOC_6(3) fluorescence of cells was plotted against membrane integrity, non-cell events having been removed by size-exclusion (Figure 2.4). Normal cells were characterised by a relatively hyperpolarised Ψ_m and no loss of plasma membrane integrity. Apoptotic cells had a reduced Ψ_m without loss of membrane integrity. Dead cells displayed loss of plasma membrane integrity and reduction of Ψ_m. For analysis of Ψ_m by flow cytometry, cells were gently mixed with DiOC_6(3) to give a final concentration of 50 nM and incubated 15 min at 37°C. PI was added to a final concentration of 1.2 μg/ml and the cells then placed on ice until analysed.

While DiOC_6(3) was an effective measure of Ψ_m, it has been reported that the endoplasmic reticulum membrane and plasma membrane potential also influence the cellular DiOC_6(3) signal (Bortner and Cidlowski, 1999; Dallaporta et al., 1999). Cellular toxicity and the inhibition of mitochondrial respiration by DiOC_6(3) have also been reported (Rottenberg and Wu, 1997), though cells in this study were not incubated in the presence of the dye long enough for this factor to be significant.
Figure 2.4: Assessment of apoptosis by reduction of mitochondrial transmembrane potential ($\Psi_m$) using DiOC$_6$(3) and PI. Jurkat T cells incubated with vehicle (PBS; A) or anti-CD95 antibody (50 ng/ml; B) for 5 h were stained with DiOC$_6$(3) and PI. Cells containing mitochondria with relatively high $\Psi_m$ (high DiOC$_6$(3) fluorescence) and low membrane permeability (low PI fluorescence) were interpreted as normal. Cells with reduction of $\Psi_m$ (low high DiOC$_6$(3) fluorescence) but low membrane permeability were interpreted as apoptotic. High membrane permeability indicated dead (necrotic or secondary necrotic) cells. The percentage of total events is shown for each region.
DiBAC$_4$(3) (bis (1,3-dibutylbarbituric acid) trimethene oxonol): DiBAC$_4$(3) is a bis-barbituric acid oxonol dye that enters depolarised cells and exhibits enhanced fluorescence when bound to intracellular proteins. Hyperpolarisation results in an extrusion of the anionic dye and hence a decrease in fluorescence. Due to its anionic charge, the dye is excluded from the mitochondria and therefore signal fluorescence is unaffected by reduction of $\Psi_m$ (Mohr and Fewtrell, 1987). Measurement of plasma membrane potential was confirmed by loading Jurkat cells with DiBAC$_4$(3) and depolarising the plasma membrane with varying concentrations of KCl immediately prior to analysis. Population histograms of DiBAC$_4$(3) fluorescence correlated well with plasma membrane depolarisation, with DiBAC$_4$(3) fluorescence increasing with increasing plasma membrane depolarisation by KCl (Figure 2.5). For analysis of plasma membrane potential, cells were gently vortexed with DiBAC$_4$(3) to give a final concentration of 100 nM and incubated 15 min at 37°C. PI was added to a final concentration of 1.2 µg/ml as a counterstain and the cells then placed on ice until analysed on the flow cytometer.

**Figure 2.5: DiBAC$_4$(3) fluorescence increases in response to plasma membrane depolarisation.** Untreated cells were loaded with DiBAC$_4$(3) and then a range of concentrations of KCl added to depolarise the plasma membrane immediately prior to analysis. The final concentration of $K^+$ is given above each histogram. DiBAC$_4$(3) fluorescence increased with membrane depolarisation, indicating that DiBAC$_4$(3) is an effective measure of plasma membrane potential.
**PBFI-AM** (*potassium-binding benzofuran isophthalate acetoxyethyl ester)*: PBFI-AM is a cell permeant $K^+$-indicator that consists of fluorophores bound to the nitrogens of a crown ether with a cavity size that confers specificity to $K^+$. Upon ion binding, the excitation spectrum shifts and fluorescence intensity is enhanced several-fold. The specificity of PBFI-AM measurement for intracellular $[K^+]_i$ was verified by assessing PBFI-AM fluorescence of cells incubated with vehicle or the $K^+$-selective ionophore valinomycin (10 $\mu$M), which would cause equilibration of $[K^+]_i$ with the extracellular environment. Cells incubated with valinomycin for 2 h showed a 44 % decrease in fluorescence compared to control cells (data not shown), indicating a decrease in $[K^+]_i$ by the valinomycin. To aid cell loading, PBFI-AM was added to cultures in a 12.5 % (v/v) solution of Pluronic-F127 in DMSO, to give a final concentration of $<0.5$ % Pluronic-F127. PBFI-AM was added to a final concentration of 5 $\mu$M, mixed gently and incubated at 37°C for 1 h. To remove background fluorescence, cells were pelleted at 400 x g for 4 min and resuspended in RPMI 1640. PI was added to a final concentration of 1.2 $\mu$g/ml and the cells then placed on ice.

The measurement of $[K^+]_i$ by PBFI-AM did present some problems due to the long loading time of the dye, measurement were not quantitative and the dynamic range of the dye is small. In addition, the manufacturer states that fluorescence is affected outside the pH range of 6.5 to 7.5 and the dye is only 1.5 times more specific for $K^+$ than $Na^+$, though this is offset to a degree by the far higher cytoplasmic $[K^+]$ relative to $[Na^+]$ (~150 mM vs. ~12 mM). However, there are currently few alternatives available for the measurement of $[K^+]_i$.

**TMRE** (*tetramethylrhodamine ethyl ester)*: TMRE is a lipophilic cationic rhodamine derivative that is rapidly and reversibly taken up by live cells and accumulates in the mitochondria proportionally with $\Psi_m$. To confirm that TMRE measured $\Psi_m$, cells were treated with the mitochondrial uncoupler m-CCCP (carbonyl cyanide m-chlorophenyl-hydrazone; 50 $\mu$M) for 15 min, stained with TMRE and analysed by flow cytometry. Cells treated with m-CCCP would have a lower $\Psi_m$ than untreated cells, since m-CCCP acts as a protonophore (Felle and Bentrup, 1977), dissipating the proton gradient build-up across the inner mitochondrial membrane. The TMRE fluorescence of m-CCCP-treated cells was less intense (Figure 2.6), confirming that TMRE measures $\Psi_m$. To assess the contribution of plasma membrane potential to TMRE fluorescence, 150 mM KCl was
the contribution of plasma membrane potential to TMRE fluorescence, 150 mM KCl was added immediately prior to analysis to cells loaded with TMRE. TMRE fluorescence was no different from cells in normal [K⁺]_{ext}, indicating that TMRE fluorescence was not affected by changes in plasma membrane potential.

Due to the broad emission spectrum of TMRE, leading to considerable cross-over between channels on the FACScan, no suitable counter-stain was found. The proportion of cells with reduction of Ψ_m was therefore calculated using a population histogram of TMRE fluorescence, with cells exhibiting reduction of fluorescence compared against control cells (Figure 2.7). The protocol for staining cells with TMRE was adapted from experiments detailed in the literature (Scaduto and Grotyohann, 1999; Lavoie et al., 1998). Cells were gently mixed with TMRE to give a final concentration of 100 nM and incubated 15 min at 37°C. Cells were placed on ice until analysed.

**Figure 2.6: TMRE is a measure of mitochondrial transmembrane potential.** Jurkat cells loaded with TMRE were incubated with the mitochondrial uncoupler m-CCCP for 15 min (50 μM; red line), vehicle (DMSO; black line) or KCl (added to a final concentration of 150 mM immediately prior to flow cytometric analysis of TMRE fluorescence; blue line). The TMRE histograms of cell populations subjected to the various treatments were compared to verify that TMRE is a measure of mitochondrial transmembrane potential.
Figure 2.7: Assessment of reduction of $\Psi_m$ by TMRE staining. Jurkat T cells incubated with vehicle (PBS; black histogram) or anti-CD95 antibody (50 ng/ml; red histogram) for 5 h were stained with TMRE. The percentage of cells with reduction of $\Psi_m$ was quantified by comparing the population histogram of $\Psi_m$ of anti-CD95 antibody-treated cells against control.

2.8 $^{86}$Rb$^+$ efflux assay

A suitable radioactive isotope of K$^+$ was not available for the monitoring of intracellular K$^+$ flux; the isotope $^{44}$K$^+$ has a half-life of 12.4 h, a $\beta$-emission of 3.5 MeV and a $\gamma$-emission of 1.5 MeV, which renders it impractical for safe experimentation. The K$^+$ surrogate ion $^{86}$Rb$^+$, which has a half-life of 18.7 days and peak $\gamma$-emission at 1.077 MeV, was therefore used to measure efflux of intracellular K$^+$.

The protocol for loading cells with $^{86}$Rb$^+$ and assessing efflux was adapted from Orlov et al. (1996). Jurkat cells were loaded for 6 h at a cell density of $2.5 \times 10^6$ cells/ml in standard RPMI 1640 medium containing 2 $\mu$Ci/ml $^{86}$RbCl. Cells were then pelleted at 400 $\times$ g for 4 min, washed twice in warmed PBS and then re-suspended at $8 \times 10^5$ cells/ml in RPMI 1640 medium. Cells were plated 1 ml/well in 24-well dishes for experiments. At the
indicated times, cells were spun down at 400 x g for 4 min and the media then transferred into a scintillation tube. The cell pellet was washed once in ice-cold PBS and then re-suspended in PBS and transferred to a scintillation tube. Counting was performed in a Wallac CompuGamma 1282 (PerkinElmer Wallac Inc., MD, U.S.A.) for 50 min per sample. The counting time was chosen to minimise potential errors by increasing the sample size and to decrease the quantity of $^{86}$Rb$^+$ required for each experiment. The CompuGamma measures $\gamma$ emissions with energies of 10 to 2000 keV, which correspond logarithmically to 256 counting channels. Since emissions in each channel were counted in series, the peak of $^{86}$Rb emission energy was assessed to minimise counting time required for each sample. The $\gamma$ emissions from ~10 kBq of $^{86}$Rb were counted over all 256 channels (Figure 2.8) and the channels encompassing the peak emission (channels 224 - 232, corresponding to approximately 1030 - 1220 keV) selected for counting emissions of experimental samples. For each experimental assay, both culture medium and cell pellet activity were recorded. The relative $^{86}$Rb$^+$ activity in the cell pellet was calculated as a percentage of total assay activity and compared to the control value at each time-point. Results are represented as a ratio of experimental against control cell pellet [$^{86}$Rb$^+$], termed $K_R$.

$$K_R = \frac{cell\ pellet\ [^{86}\text{Rb}^+]_{\text{treatment}}}{cell\ pellet\ [^{86}\text{Rb}^+]_{\text{control}}}$$

The background count was negligible and was discounted. Assays were performed at room temperature.

Figure 2.8: Calibration of CompuGamma $\gamma$-counter for peak emissions of $^{86}$Rb$^+$. To calibrate the $\gamma$-counter for the most efficient program, ~10 kBq of $^{86}$RbCl was counted over all 256 energy channels for 1 min per channel. For quantification of $\gamma$-emissions in experiments, the channels encompassing the peak emissions (224 - 232) were selected.
2.9 Bradford Method for Quantification of Protein Content

The Bradford method (Bradford, 1976) was used to determine the protein content of cell lysates. Bradford reagent (protein assay dye concentrate; Bio-Rad, Munich, Germany) was diluted 1:5 with distilled water and aliquoted into spectrometry cuvettes (1 ml each). A Lambda2 spectrometer (PerkinElmer Instruments, MA, U.S.A.) was used to measure absorbance at 595 nm, and was calibrated with cuvettes containing 1, 2, 4, 6 and 8 μg/ml BSA. Cell lysates were added to cuvettes and the spectroscopic absorbance measured. For calculation of protein content, a standard curve was plotted using the BSA standards and sample protein content calculated by linear regression.

2.10 Western Blotting

For Western blotting of immunoreactive proteins, cells (~5 x 10⁵) were pelleted following the required treatments, washed in PBS and resuspended in 50 μl Laemmli buffer (Laemmli, 1970). To increase cell lysis and protein release for immunoblots for PARP, samples were sonicated and 4 M urea added to sample buffer. For sample preparation of cytoplasmic lysates, protein content of lysates was quantified by the Bradford method and then an equal volume of 2 x Laemmli buffer added to 10-15 μg protein. Samples were heated for 3 min in a boiling water bath and then stored at -20°C until required. SDS-polyacrylamide gel electrophoresis (PAGE) immunoblot detection of proteins was performed as detailed in MacFarlane et al. (1997). The following solutions were used:

**Laemmli sample buffer**

- 60 mM Tris
- 15 % (v/v) glycerol
- 5 % (v/v) 2-mercaptoethanol
- 2 % (w/v) SDS
- 0.05 % (w/v) bromophenol blue
- pH adjusted to 6.8 with HCl

**Lower gel buffer**

- 1.5 M Tris
- 0.4 % (w/v) SDS
- pH adjusted to 8.8 with HCl and passed through a 0.45 μm filter.

**Electrode buffer**

- 25 mM Tris
- 192 mM glycine
- 10 % (w/v) SDS

**Transfer buffer**

- 25 mM Tris
- 192 mM glycine
- 20 % (v/v) methanol

**Tris-buffered saline (TBS)**

- 20 mM Tris
- 137 mM NaCl
- pH adjusted to 7.6
**Upper gel buffer**
0.5 M Tris
0.4 % (w/v) SDS
pH adjusted to 6.8 with HCl and passed through a 0.45 μm filter

**Blocking buffer**
1 × TBS
5 % (w/v) Marvel milk powder
1 % (v/v) Tween 20

<table>
<thead>
<tr>
<th>Component</th>
<th>7 % resolving gel</th>
<th>13 % resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>lower gel buffer</td>
<td>6.25 ml</td>
<td>6.25 ml</td>
<td>-</td>
</tr>
<tr>
<td>upper gel buffer</td>
<td>-</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>acrylamide</td>
<td>5.8 ml</td>
<td>10.8 ml</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>12.7 ml</td>
<td>7.95 ml</td>
<td>6.2 ml</td>
</tr>
<tr>
<td>ammonium persulphate</td>
<td>150 μl</td>
<td>150 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED¹</td>
<td>20 μl</td>
<td>20 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>25 ml</strong></td>
<td><strong>25 ml</strong></td>
<td><strong>10 ml</strong></td>
</tr>
</tbody>
</table>

Table 2.4: Constituents of SDS-PAGE gels.

¹ TEMED: N,N,N',N'-tetramethylethylenediamine

Minigel casting kits (Becton Dickinson) were set up according to the manufacturer’s instructions. The resolving gel solution was made up as detailed in Table 2.4, de-gassed for 10 min then poured into the gel set. The resolving gel was left to polymerise with a layer of 70 % (v/v) industrial methylated spirits (IMS) in distilled water covering it to prevent contact with the air. After 15 min, the 70 % IMS was poured off and the de-gassed stacking gel poured on top of the resolving gel. A 10 or 15-well casting comb was inserted and the gel left to polymerise for 15 min. The casting combs were removed, gels were placed in the electrode assemblies and these placed in the tanks filled with electrode buffer.

Samples were heated in a boiling waterbath for 3 min and then loaded into the wells of the resolving gel. Kaleidoscope (Biorad, CA, U.S.A.) and SeeBlue (Invitrogen, CA, U.S.A.) protein standard ladders were loaded to aid estimation of sample band sizes. Gels were run at 100 V until the bromophenol blue dye front had run off the end of the gel (~90 min). The gels were removed from the electrode assembly, the stacking gel discarded and the transfer sandwich constructed with the resolved gel. The transfer sandwich consisted of
the resolved gel and a nitrocellulose membrane (Hybond-C extra, Amersham, Bucks, U.K.)
surrounded on each side by three pieces of chromatography paper (Whatman International
Ltd., Kent, U.K.) and a sponge, held together by a plastic frame. The transfer sandwiches
were placed in a tank of transfer buffer and transferred at 25 V overnight (~15 h).

Protein transfer and equal loading were checked by Ponceau-S staining and
membranes then washed in TBS containing 1 % (v/v) Tween-20 (TBS-T) and blocked for
1 h with blocking buffer. Membranes were washed with TBS-T and probed with the
primary antibody (in TBS-T) for 1 h. Membranes were again washed with TBS-T and
blocked for 1 h, then probed with the appropriate secondary antibody for 1 h. The
membranes were washed in TBS-T and blocked for 1 h, washed again and protein blotting
visualised using chemiluminescence (Supersignal; Pierce) and film (X-Omat; Kodak, NY,
U.S.A.) on an automatic developing machine.

2.11 INVESTIGATION OF SUBCELLULAR FRACTIONS BY WESTERN BLOT

2.11.1 Cytochrome c release

The protocol detailed by Bossy-Wetzel et al. (1998) was used for the analysis of
mitochondrial cytochrome c release. Cells (1.2 × 10^7 per treatment) were collected after 5 h
incubation with vehicle (DMSO), anti-CD95 antibody or etoposide and washed twice in
ice-cold PBS. Cells were re-suspended in cytosol extraction buffer (220 mM mannitol, 68
mM sucrose, 50 mM PIPES (piperazine-N,N'-bis[2-ethane-sulphonic acid]), 50 mM KCl, 5
mM EGTA, 2 mM MgCl_2, plus a protease inhibitor mix (5 mM DTT, 10 µg/ml aprotinin,
20 µg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml pepstatin A),
pH adjusted to 7.4 with KOH) at 10 µl/10^6 cells. Cells were stood on ice for 30 min and
then cells were homogenised with a glass dounce and B pestle for 40 strokes. Cell
homogenates were spun at 14,000 × g for 15 min at 4°C and the supernatant fractions
removed and stored at ~80°C until use. This procedure would result in a subcellular lysate
devoid of mitochondria (Spector et al., 1998). Protein content of cytoplasmic lysates was
determined by the Bradford method (section 2.9). Cytochrome c release was assessed by
separating 15 µg cell homogenates per lane by SDS-PAGE and immunoblotting for
cytochrome c. Western blots were also performed for α-tubulin to demonstrate equal
loading of protein.
2.11.2 Membrane recruitment of FADD and caspase-8

While the preferred method for the analysis of DISC formation during CD95-mediated apoptosis was immunoprecipitation of the DISC and subsequent Western blot analysis of component proteins (Kischkel et al., 1995), the antibody required for precipitation was not available. Instead, a method adapted from Huang et al. (1999) for the analysis of protein recruitment to the plasma membrane fraction of fractionated cells was used.

Jurkat cells (~$10^7$) were treated with anti-CD95 antibody or vehicle (PBS) in either normal or elevated extracellular $[K^+]$ ($[K^+]_{ext}$) and incubated for 1 or 5 h. Cells were spun down at $400 \times g$ for 4 min and washed with ice-cold PBS twice. The cell pellet was then resuspended in ice-cold hypotonic buffer (10 mM Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), 20 mM NaCl, 5 mM DTT, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM PMSF, pH adjusted to 7.5 with HCl) at $10^7$ cells/ml. After 30 min incubation on ice, cells were homogenised with a glass dounce and B pestle for 30 strokes. The cell homogenate was then centrifuged at 1,000 $\times g$ for 10 min at 4°C to remove non-lysed cells, nuclei and larger mitochondria. The supernatant fraction was removed, with care taken not to disturb the pellet and spun at 100,000 $\times g$ for 1 h at 4°C to spin down remaining organelles. The supernatant fraction (predominantly the cytoplasm) was then removed and stored at -80°C until use. The pellet was carefully washed twice in hypotonic buffer and then resuspended at $10^8$ cells/ml in lysis buffer (hypotonic buffer plus 1 % (v/v) Triton X-100, 10 % (v/v) glycerol and stored at -80°C. For analysis of recruitment of proteins to the plasma membrane, an equal volume of 2 $\times$ Laemmli buffer was added to the 100,000 $\times g$ pellet and $10^6$ cells/lane separated by 13 % SDS-PAGE. FADD and caspase-8 protein quantities were analysed by Western blotting.

2.12 Gel filtration chromatography

2.12.1 Preparation of cell lysates

Jurkat and THP.1 cell lysates (100,000 $\times g$ supernatant fractions) were prepared using a protocol adapted from Lazebnik et al. (1993). Cell cultures were pelleted by centrifugation at 400 $\times g$ for 5 min and then washed twice in ice-cold PBS. After the final spin, all supernatant fraction was removed and the pellet resuspended in PIPES buffer (50
CHAPTER TWO

mM PIPES, 2 mM EDTA, 0.1 % (w/v) CHAPS, 5 mM DTT, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM PMSF, pH adjusted to 6.5 with KOH). 166 μl of PIPES buffer was added per 10⁸ cells. Cells were transferred to screw-top microtubes and lysed by three freeze-thaw cycles: tubes were placed in liquid nitrogen for 3 min, then thawed rapidly in a 37°C water bath. The microtubes were then centrifuged at 20,000 x g for 30 min at 4°C to remove large cell debris, non-lysed cells and nuclei. The supernatant fraction was carefully removed and organelles then removed from the cell lysate by centrifugation at 100,000 x g for 45 min at 4°C. The supernatant fraction was then transferred to microtubes and a sample taken for protein content determination by the Bradford method. Cell lysates were diluted to 10 mg/ml protein content with assay buffer (0.1 % (w/v) CHAPS, 10 mM DTT, 100 mM HEPES, 10 % (w/v) sucrose, pH adjusted to 7.0 with NaOH) and then frozen at -80°C until use.

The lysates (10 mg/ml) were activated by the addition of 2 mM dATP/MgCl₂ and incubated at 37°C for 1 h. THP.1 lysates contained low but detectable levels of cytochrome c and further addition was unnecessary (Cain et al, 2000). Jurkat lysates, however, required addition of exogenous cytochrome c (0.5 mg/ml) for activation. After incubation the caspase activity of the lysate was measured.

2.12.2 Fluorimetric assay of caspase activity

The DEVDase activity (primarily a measure of caspase-3 and -7 activity) of lysates or column fractions was measured using the fluorogenic substrate DEVD.AFC (Cain et al., 2000). Assays were carried out at 37°C in a 96 well plate in a total volume of 200 μl assay buffer plus 20 μM DEVD.AFC. The plate was assayed for 10 cycles using a Wallac Victor² 1420 Multilabel counter at an excitation wavelength of 405 nm and an emission wavelength of 510 nm. The cleavage rate of the fluorogenic substrate was determined by linear regression and activities were expressed as either pmol/min/mg protein or pmol/min/fraction.

2.12.3 Chromatographic methods

Lysates were separated by size-exclusion chromatography as described previously (Cain et al., 2000) using a fast protein liquid chromatography protein purification system on analytical (16/60), preparative (26/60) Hi-Prep S300 Sephacryl high-resolution and

57
Superose 6 HR 10/30 columns (Amersham Pharmacia). The columns were calibrated by resolving a number of known protein standards (Amersham Pharmacia) including blue dextran (2 MDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (158 kDa) and ovalbumin (43 kDa). The Sephacryl column was equilibrated with column buffer (5 % (w/v) sucrose, 0.1 % (w/v) CHAPS, 20 mM HEPES, 5 mM DTT, pH 7.0) and run at a constant flow rate of 0.5 ml/min. Samples were applied to the column and eluted proteins were detected by measuring the absorbance at 280 nm and 2 ml fractions were collected. The Superose 6 column was equilibrated with column buffer supplemented with 50 mM NaCl, at a constant flow rate of 0.4 ml/min and 0.5 ml fractions were collected. Caspase assays and SDS-PAGE/Western blotting was carried out on the collected fractions.

2.12.4 Assay of apoptosomal effector caspase activating activity

A simple in vitro assay, as described in Cain et al. (2000), was developed to assess the ability of the ~1.4 MDa and ~700 kDa apoptosome complexes to process and activate effector procaspases. Basically, 100 μl of the column fractions obtained from a control, dATP-activated or dATP-activated pretreated with 50 mM KCl Superose 6 column run were incubated with 25 pg of procaspases for 30 min at 37°C. The procaspases were obtained following fractionation of control lysates on a preparative (26/60) Hi-Prep S300 Sephacryl column. Fractions 18-20 were concentrated in Vivaspin 4 (Vivascience; Lincolnshire, U.K.) concentrators (10kDa cut-off) to a final concentration of 8-14 mg/ml. These fractions contained procaspase-3, -7 and -9 and when added to an already oligomerised Apaf-1-containing complex were proteolytically processed. To determine the ability of the two complexes to activate the procaspases the DEVDase activity of the fractions was measured.

2.13 Molecular Biology

2.13.1 cDNA and Plasmids

Rat Kᵢᵣ6.1 and mouse Kᵢᵣ6.2ΔKpnI cDNAs were obtained from Prof. K. Takata (University of Gunma, Japan). An internal KpnI restriction site was removed from the Kᵢᵣ6.2 sequence by a single base change to facilitate ligation and sub-cloning of cDNA. The amino acid sequence was unchanged. Kᵢᵣ2.3 cDNA was obtained from Grant Dewson.
(University of Leicester). The pCMV/myc/mito/GFP and pcDNA3.1(−)/Myc-HisB vectors were from Invitrogen.

2.13.2 Bacterial strains and growth media

SURE ("stop unwanted rearrangement events"), Supercompetent X-gal-blue and Supercompetent XL1 strains of *Escherichia coli* bacteria were used for preparation of cDNA (Stratagene, CA, U.S.A.). All bacterial stocks were stored at -20°C in the form of glycerol stocks, made up by adding 0.6 ml overnight culture to 0.4 ml sterilised 75% (v/v) glycerol. The following bacterial growth media were used: L-Broth (Luria-Bertani medium; 10 gm bacto-tryptone, 5 gm bacto yeast extract (both DIFCO, MI, U.S.A.), 10 gm NaCl in 1 l sterile water) and SOC (20 gm bacto-tryptone, 5 gm bacto yeast extract, 0.5 gm NaCl, 20 mM glucose in 1 l sterile water). LB agarose was obtained from core stocks.

2.13.3 Preparation of plasmid DNA

*Small-scale preparation of plasmid DNA.* The Wizard plasmid miniprep kit was used (Promega; WI, U.S.A). Bacterial cultures were pelleted and the supernatant fraction poured off. Pellets were completely resuspended and then lysis solution added. Alkaline protease solution was then added and the tube incubated at room temperature for 5 min. Neutralisation solution was added and the tube centrifuged at 13,000 × g for 10 min to remove precipitated protein and cell debris. The cleared lysate was transferred to a clean Miniprep spin column assembly and centrifuged at 13,000 × g for 1 min. The flow-through liquid was discarded and wash solution added to the spin column assembly. The tube was then centrifuged at 13,000 × g for 1 min. The flow through was discarded and the wash procedure repeated. The spin column was transferred to a clean tube and DNA eluted in sterile water.

*Medium scale preparation of plasmid DNA.* The Concert maxi-prep column kit was used (Gibco). Bacterial cultures (~1.5 l) were pelleted and resuspended in suspension buffer and then incubated with lysis buffer for 5 min to lyse bacteria. Neutralisation buffer was added and the preparation centrifuged at 13,000 × g for 5 min. Filter columns were washed with equilibration buffer and then the supernatant fraction was pipetted into the filter columns. The columns were allowed to drain and were then washed through with wash buffer. Flow-through was discarded and the DNA eluted with elution buffer. DNA
was precipitated with an equal volume of isopropanol and pelleted by centrifugation at 13,000 × g for 30 min at 4°C. The supernatant fraction was poured off, DNA washed with 70 % (v/v) ethanol in distilled water and the pellet air-dried for 5 min, then resuspended in sterile water.

**Extraction of DNA from agarose gels.** The QIAQuick (Qiagen Ltd., Surrey, U.K.) gel extraction kit was used to extract DNA from bands of interest on agarose gels. Bands were excised from the gel and 300 μl of binding buffer was added to each gel slice in a spin filter assembly. The tube was heated to 55°C for 5 min to melt the gel and then centrifuged for 15-30 sec to transfer the liquid to the catch tube, leaving the DNA in the spin filter. The catch tube was emptied and 500 μl wash solution added to the spin filter, which was centrifuged spun again for 30 sec to wash the DNA. This wash step was then repeated. The catch tube was emptied and the tube spun for 1 min to dry the DNA pellet. 150 μl elution solution added and the DNA resuspended by flicking the tube. The tube was then spun for 30 sec to transfer the DNA into a clean catch tube. The DNA was ethanol-precipitated (as in section 2.13.4) and resuspended in 20 μl sterile water.

2.13.4 Precipitation of oligodeoxynucleotide primers

Oligodeoxynucleotide primers used in polymerase chain reaction (PCR) reactions and sequencing were prepared by the Protein and Nucleic acid laboratory (PNACL; University of Leicester) and supplied in ammonia solution. Two volumes of 100 % ethanol at -20°C (600 μl) and 1/5 th volume of 2 M sodium acetate (60 μl) were added to 1 volume primer stock solution (300 μl). The solution was mixed and then centrifuged at 13,000 × g for 5 min at 4°C. The supernatant fraction was aspirated off and the pellet washed with 400 μl cold 70 % ethanol in sterile distilled water. The pellet was then resuspended in 500 μl sterile distilled water.

2.13.5 Quantification of nucleic acid concentration by spectrophotometry

*Double-stranded DNA (dsDNA):* The concentration of DNA in aqueous solution was determined by measuring the absorbance at 260 nm (A_{260}) of a dilution of the DNA solution in a quartz cuvette. An aliquot of DNA solution was diluted 1:100, the spectrophotometer zeroed at 260 nm using sterile water and the absorbance of the diluted
DNA solution measured. The concentration of DNA was then calculated using the following equation:

\[ \text{dsDNA concentration (µg/ml)} = A_{260} \times 100 \times \text{dilution} \times 50 \]

**Oligodeoxynucleotide primers**: The concentration of primers was determined using the following equation:

Primer concentration (pmol/µl) = \(A_{260} \times \text{dilution} \times \frac{100}{1.5 N_A + 0.71 N_C + 1.2 N_G + 0.84 N_T} \)

where \(N\) is the number of residues of base A, C, G or T.

### 2.13.6 DNA agarose gels

To separate DNA by size, samples were mixed with gel-loading buffer (50 % (v/v) sterile water, 50 % (v/v) glycerol, 1 mM EDTA, 2.5 mg/ml bromophenol blue) and then loaded on agarose gels. Gels consisted of 1-3 % (w/v) agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), with the percentage agarose depending on the size of the band of interest. To avoid the need for staining the gel after running, 0.5 µg/ml ethidium bromide was added to agarose gels. DNA samples were run at 50-100 V for the required time. To visualise DNA bands, gels were photographed under UV light.

### 2.13.7 Enzymic manipulation of DNA

**Restriction enzyme digestion.** Restriction enzymes and their 10× reaction buffer were obtained from Stratagene.Digests were performed according to the manufacturer's instructions. A typical digest contained 5 µg DNA, 1 µl 10× reaction buffer, 0.5 µl 100× BSA, 2 µl restriction enzyme (equivalent to ~20 Units) and 6.5 µl sterile water. Reactions were incubated at 37°C for periods ranging from 1 h to overnight.

**Polymerase chain reaction (PCR).** A typical PCR reaction was performed in a volume of 100 µl containing 250 µM of each nucleotide (dNTP), 1 µM of forward and reverse primers, 50-100 ng of template DNA, 10µl of 10× reaction buffer (100 mM Tris, 500 mM KCl, 15 mM MgCl₂, pH adjusted to 8.3 with HCl), and 2 µl (1 Unit) of Advantage Taq (Boehringer Mannheim, Sussex, U.K.) or PfuI DNA polymerase (Stratagene). PCR reactions were cycled 20 times with denaturation at 94°C for 30 s,
annealing at 54°C for 60 s, and primer extension at 72°C for 2 mins. PCR products were then analysed on a 1 % agarose gel.

**Ligation.** The T4 DNA ligase enzyme was used to insert cDNA into expression vectors. A typical ligation reaction contained 50-100 ng of vector DNA, 25-50 ng insert DNA, 1 μl of 10× ligase buffer, 1 μl ATP supplement (to a final concentration of 1 mM), 1 μl T4 DNA ligase (1 unit; Boehringer Mannheim) in a total volume of 10 μl. Ligation reactions were incubated overnight at 4°C.

### 2.13.8 Mutagenesis of Kir6.1 and Kir6.2

To produce a 61 amino acid C-terminal truncation of Kir6.1 and a 36 amino acid C-terminal truncation of Kir6.2, reverse and complement PCR primers were used to introduce a stop codon into the sequence at the appropriate point. The basic procedure uses a double-stranded cDNA template and synthetic oligonucleotide primers containing the desired mutation (stop codon). The primers, each complimentary to opposite strands of the vector, are extended during PCR cycling, generating linear copies of the vector that incorporate the mutation/stop codon.

**Oligonucleotide design**

Oligonucleotide primers were designed to generate the truncation constructs (see Figure 5.9). Both constructs contained a NotI site at the start (3' end) and XbaI site at the 5' end to allow for subcloning. To create the truncated ion channel proteins upon translation, a stop-codon was introduced at residue I363 for Kir6.1 and at residue L355 for Kir6.2. The primers detailed below were used (5' to 3'). Prefixes are sequential names of primers and are not connected in any way with primer function.

**NUC-K:** all rKir6.1 Forward:

GCGGCCGCAATGTTGGCCAGGAAGAGCATC

**NUC-M:** rKir6.1CA61:

TCTAGATTAGGAAGGCTTCTCATCCAGCTCCC

**NUC-L:** Reverse and complement for rKir6.1 full length:

TGATTCTGATGGGCACTGGTTTC

**NUC-N:** all mKir6.2 Forward:

GCGGCAGGCCGCAATGCTGTCCCGAAAGGCAATTATCCCTGAG
NUC-P: mKir6.2Δ36:

GGCTCTAGATTAGCTGCGGTCCTCATCAAGCTGGCGGGCTGTGCAG

NUC-Q: Reverse and complement for mKir6.2 full length:

AGCATCTCTCCAGATTCCTTGTCCTGA

For mutagenesis of Kir6.x cDNAs, PCR reactions were performed in a volume of 100 µl containing 500 µM of each dNTP, 2 µM of forward and reverse primers, 100 ng of template DNA, 10µl of 10× reaction buffer (100 mM Tris, 500 mM KCl, 15 mM MgCl₂, pH adjusted to 8.3 with HCl), and 0.5 µl of PfuI DNA polymerase (Boehringer). All solutions except the enzyme and reaction buffer were added to the tube and then a wax plug overlaid before the enzyme and buffer were added. This was to prevent degradation of DNA and primers before the reaction was ready to start. The reaction was cycled 30 times with denaturation at 94 °C for 15 sec, and annealing and primer extension at 68°C for 3 min.

Sequencing of Kir6.x truncations

Primers WAT-E and WAT-F were used in conjunction with NUC-K and NUC-N to sequence the Kir6.1 and Kir6.2 truncation mutants, respectively.

WAT-E: CAATCTGCCCTTTCGAAAGATC

WAT-F: CACCTACTCAGACAATGCGATGC

The Big Dye termination mix (PE Biosystems, CA, U.S.A.) was used, obtained from PNACL, plus 3.2 pmol primer, 500 ng template and sterile water. The reaction was cycled 50 times with denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and primer extension at 60°C for 4 min. Products were ethanol-precipitated, dried and then given to PNACL for sequencing.

2.13.9 Transformation

Frozen competent E. coli cells were thawed on ice for 5 min and gently mixed. 50 µl aliquots were placed in pre-chilled polypropylene tubes. Bacteria were incubated on ice for 10 min before adding 2 µl 2-mercaptoethanol and 20 µl ligation mix (see section 2.13.7) to the transformation reaction. The transformation reaction was mixed gently and incubated on ice for a further 30 min on ice. The transformation reactions were heat-pulsed for 45 sec at 42°C and immediately returned to ice for 2 min. 360 µl of SOC was added to
each reaction and incubated at 37°C for 1 h (shaking at 250 revolutions per minute in an orbital shaker). Using a flame-sterilised spreader, 80 µl of each transformation reaction was spread onto separate LB-ampicillin (100 µg/ml) agarose plates. Plates were incubated at 37°C overnight and then transformants picked. To check successful ligation and transformation, small-scale cultures of transformed bacteria were grown in 5 ml L-Broth containing 100 µg/ml ampicillin and grown overnight at 37°C in an orbital shaker at 225 – 250 r.p.m. DNA was extracted (see section 2.13.3) and restriction digests performed. The products were run on a 1 % agar gel to check that the size of restriction fragments was correct.

2.13.10 Preparation of cDNA stocks (Maxipreps)

200 ml L-broth containing 100 µg/ml ampicillin was placed in each 2.5 L flask. 4 ml of successfully transformed culture was added to each and then incubated overnight at 37°C. Cultures were pelleted by centrifugation at 1000 × g for 8 min, L broth removed and pellet resuspended in resuspension buffer. DNA was prepared using maxi-prep columns (see section 2.12.3).

2.13.11 In vitro transcription and translation

Reactions were set up with in vitro transcription/translation to produce radiolabelled recombinant peptides from cDNA using the Promega TNT lysate coupled transcription/translation kit. Reactions were set up according to manufacturer’s instructions and contained the following solutions:

- Rabbit reticulate lysate 12.5 µl
- Reaction buffer 1 µl
- T7 RNA polymerase 0.5 µl
- amino acid mixture minus methionine (1mM) 0.5 µl
- 35S-methionine (1,000 Ci/mmol) at 10 mCi/ml (NEN, MA, U.S.A.) 2 µl
- RNAsin ribonuclease inhibitor (40 U/µl) (Promega) 0.5 µl
- DNA template 0.5 µg
- Sterile water to give total of 25µl
**Protocol.** Reagents were removed from the -80°C freezer and placed on ice. The reticulate lysate was rapidly thawed by hand warming, the other reagents thawed at room temperature and then placed on ice. The components listed above were assembled and gently mixed. The reaction was incubated at 37°C for 2 h and then products analysed by autoradiograph (see section 2.13.13).

### 2.13.12 Caspase cleavage of $^{35}$S-labelled peptides

Recombinant caspase-3 (7.5 mg/ml protein content) was obtained from Dr. K. Cain (M.R.C., Leicester). Recombinant caspase-8 (6 mg/ml protein content) was obtained from Dr. X-M. Sun (M.R.C., Leicester). Peptides labelled with $^{35}$S were produced by the TNT reticulate lysate system from cDNA. To investigate cleavage by caspases, 1 μl of reaction mix from 2.13.11 was incubated for 1 h at 37°C with 1 μl of a range of dilutions of recombinant caspase-3 or -8 in 8 μl reaction buffer (10 mM HEPES, 2 mM EDTA, 0.1 % (w/v) CHAPS, pH adjusted to 7.5 with KOH, containing protease inhibitors (as 2.11.1)). Cleavage products were analysed by autoradiograph (see section 2.13.13).

### 2.13.13 Autoradiography

For analysis of $^{35}$S-labelled peptides, samples were separated on 13 % SDS-PAGE gels and run at 40 mA for 1 h. Gels were placed in fixing solution (30 % (v/v) methanol, 3 % (v/v) glycerol, 10 % (v/v) acetic acid, 57% distilled water for 20 min and then washed with scintillant for 20 min (NEN Enlightening, Zaventem, Belgium). Gels were dried on a vacuum gel drying machine (Speedgel SG200; Savant Instruments Inc., NY, U.S.A.) and then placed in an exposure cassette with a sheet of Kodak film. The film was developed after 12 h to 2 days.

### 2.13.14 Expression of cDNA in mammalian cell lines

Transfection of cDNA into mammalian cell lines was carried out using FuGENE 6 reagent (Boehringer Mannheim), according to the manufacturer’s instructions. For each transfection reaction, 100 μl of serum-free MEM alpha medium was mixed with 4 μl FuGENE 6 reagent, mixed and left for 10 min. This was then added to 2 μg cDNA, mixed, left for 15 min and then added drop-wise to cultured cells. After 12 - 24 h, cultured cells were examined under a fluorescence microscope for GFP-expression.
2.14 **CONFOCAL MICROSCOPY**

For visualisation of stained cells, a Leica TCS 4D confocal microscope equipped with UV and argon/krypton lasers was used, in conjunction with a Leitz DM IRBE inverted microscope. For confocal microscopy, cells were used at a density of 1.5-2.5 x 10^6 cells/ml. Cells were treated and stained, and then mounted in PBS on slides with zero gauge coverslips. The mitochondrial marker Mitotracker was used at a final concentration of 20 nM, loaded for 20 min at 37°C.

2.15 **ACYLPEPTIDE HYDROLASE AND SERINE HYDROLASE ASSAYS.**

2.15.1 **Measurement of acylpeptide hydrolase activity**

Cell pellets (containing approximately 10^6 cells) were washed twice in ice-cold PBS before lysis in 150 µl of 1 % (v/v) Triton X-100 in water. Acylpeptide hydrolase (ACPH) activity was measured using the chromogenic substrate N-acetyl-alanyl-p-nitroanilidealanyl-p-nitroanilide (AANA). Lysates (100 µl) were added to 1 ml of 0.2 M Tris/HCl, 1 mM DTT, pH 7.4 containing 4 mM AANA. The rate of hydrolysis was determined by the release of p-nitroaniline (ε₄₀₅=7530 M⁻¹ cm⁻¹) at 37°C.

2.15.2 **Calculation of IC₅₀ values**

Samples of horse-liver ACPH (Roche, Lewes, U.K.) were incubated in 50 mM Tris (pH adjusted to 7.4 with HCl) at 37°C with varying concentrations of ALCK. ALCK was diluted from stock solutions in acetone just prior to use (final acetone concentration <1 %). Residual enzyme activity was recorded after 20 min and the concentration of inhibitor producing 50 % inhibition was calculated from a plot of log (% activity remaining) vs. ALCK concentration. IC₅₀s from OP compounds were taken from a previous reference (van der Staay et al., 1996).

2.15.3 **Analysis of the inhibition of DFP labelling**

Cell lysates (50 µl) were incubated with 5 µl of 120 µM tritiated-DFP (specific activity 310 GBq/mmol) for 2 h at 37 °C, after which 20 µl of 4 × Laemmli sample loading buffer was added. 10 µl of the samples were resolved on a 10 % NuPAGE gel (Novex, Frankfurt, Germany) using a 2-[N-morpholino]ethanesulphonic acid buffer system and blotted onto polyvinylidene difluoride membranes following the manufacturer's
instructions. To image the low amount of tritium on the blot, a prototype detection system (Lees and Richards, 1999) was used that employs radioisotope-free, low noise microchannel plates developed for photon counting in X-ray astronomy. The current equipment has the ability to quantitatively image samples in real-time with high sensitivity, low background and a spatial resolution of 70 μm. A linear correlation between activity loaded on the gel and events recorded by the detector is found down to at least 4 fmol tritium per protein band (i.e. less than 0.2 ng of a 50 kDa protein). The linearity of detector response is 6 orders of magnitude, therefore areas of high and low activity within a sample can be resolved on a single image.

2.16 Data handling and repeats

Data in bar charts are from at least three independent experiments, unless stated otherwise. Error bars shown in bar charts are standard error of the mean (S.E.M.). Western blots shown in figures are representative of at least three independent experiments.
Chapter Three. Influence of extracellular $[K^+]$ on the induction of apoptosis in mature cerebellar granule neuron cultures

3.1 Introduction

Apoptosis is critical for the functional development of the central nervous system. About half of the cells produced within the developing vertebrate nervous system ultimately die by apoptosis in order to sculpt the gross anatomy of the brain (Oppenheim, 1991; Johnson and Deckwerth, 1993). Neurons that are inappropriately or not synaptically connected will undergo apoptosis to eliminate them from the brain. The culture of primary (ex vivo) cerebellar granule neurons (CGN) from rat and mice pups provides a useful model for the study of neuronal apoptosis (D'Mello et al., 1993; Miller and Johnson, 1996). CGNs are the most abundant neuronal cell population, yielding twenty million granule cells per animal (Miller and Johnson, 1996), and provide a highly homogenous cell culture. CGN cultures require a depolarising extracellular concentration of $K^+$ ($[K^+]_{\text{ext}}$) of 25 – 30 mM for survival and differentiation in vitro (Gallo et al., 1987; reviewed in Franklin and Johnson, 1992). Combined reduction of $[K^+]_{\text{ext}}$ and serum withdrawal after maturation of the culture (6 - 8 days in vitro (DIV)) induces cell death in >95 % of cells within 96 h (D'Mello et al., 1993; Miller and Johnson, 1996).

Several hypotheses have been proposed to explain this inhibition of apoptosis by elevation of $[K^+]_{\text{ext}}$:

i) Elevated $[K^+]_{\text{ext}}$ is associated with increased $Ca^{2+}$ influx. Elevated $[K^+]_{\text{ext}}$ stimulates the opening of voltage-gated $Ca^{2+}$ channels by depolarising the plasma membrane (Courtney et al., 1990). This influx of $Ca^{2+}$ and subsequent increase in intracellular $Ca^{2+}$ concentration ([Ca$^{2+}$]) may stimulate intracellular pro-survival signalling pathways (Franklin and Johnson, 1992). According to this hypothesis, depolarising $[K^+]_{\text{ext}}$ may mimic innervation of developing neurons (Gallo et al., 1987), providing the survival signals neurons would receive in vivo when functionally innervated. Conversely, reduction of $[K^+]_{\text{ext}}$ may initiate the apoptotic program by mimicking the removal of afferent innervation or removal of trophic support.
ii) **Potential influence of \([K^+]_{\text{ext}}\) on phosphorylation status.** Elevated \([K^+]_{\text{ext}}\) has also been shown to indirectly stimulate the activity of phosphatidylinositol 3-kinase (PI-3-K) (Miller et al., 1997; Shimoke et al., 1997), a lipid kinase whose products are involved in many cellular processes (reviewed in Kapeller and Cantley, 1994). One of the downstream targets of PI-3-K is the serine-threonine kinase c-Akt (Franke et al., 1995; Dudek et al., 1997), which phosphorylates Bad (Datta et al., 1997; del Peso et al., 1997), a pro-apoptotic member of the Bcl-2 family (Yang et al., 1995). In its phosphorylated state, Bad binds to the cytosolic docking protein 14-3-3\(t\) (Morrison, 1994; Burbelo and Hall, 1995) in preference to anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-\(x_L\) (Zha et al., 1996; Zhou et al., 2000). This allows for the normal function of Bcl-2 and Bcl-\(x_L\), thereby increasing the resistance of the cell to apoptotic insult. Thus elevated \([K^+]_{\text{ext}}\) may inhibit apoptosis by increasing cellular levels of unsequestered anti-apoptotic Bcl-2 proteins.

iii) **Elevated \([K^+]_{\text{ext}}\) decreases driving force of \(K^+\) ions out of apoptotic cells.** Several studies in non-neuronal cell lines have demonstrated efflux of intracellular \(K^+\) during apoptosis (Bortner et al., 1997; Fernandez-Segura et al., 1999; Gómez-Angelats et al., 2000). Inhibition of apoptosis has been achieved by preventing this efflux by pharmacological blockade of ion channels (Dallaporta et al., 1999; Maeno et al., 2000). The total driving force of an ion across a membrane is the sum of two forces: an electrical force (the negative potential inside a cell will tend to draw in cations) and a diffusion force, based on the concentration gradient of the ion. Depolarisation of the plasma membrane potential by pharmacological agents or reduction of the concentration gradient by elevation of \([K^+]_{\text{ext}}\) will decrease the driving force of \(K^+\) ions out of the cells, and therefore elevated \([K^+]_{\text{ext}}\) may inhibit apoptosis in CGN cultures by preventing efflux of intracellular \(K^+\).

Experiments were performed to confirm the induction of apoptosis by reduction of \([K^+]_{\text{ext}}\), and the time dependence and apoptotic characteristics of this were recorded. For the investigation of the role of elevated \([K^+]_{\text{ext}}\) on voltage gated \(Ca^{2+}\) channels and \([Ca^{2+}]_i\) in CGN apoptosis, inorganic mercury (Hg\(^{2+}\)) was used. At low concentrations Hg\(^{2+}\) induces apoptosis by facilitating entry of \(Ca^{2+}\) into the cell by altering the conductance of L-type \(Ca^{2+}\) channels, thus interfering with regulation of \([Ca^{2+}]_i\) (Rossi et al., 1997). The set-point hypothesis (Koike
et al., 1989; Koike and Tanaka, 1991) states that neurons at optimal levels of \([\text{Ca}^{2+}]_i\) (~240 nM) can survive independently of trophic support. This calcium-promoted cell survival appears to be mediated chiefly by calcium- and calmodulin-dependent protein kinases (Hack et al., 1993; Boutillier et al., 1999). Excessive influx of \(\text{Ca}^{2+}\), however, may raise \([\text{Ca}^{2+}]_i\) to toxic levels by directly affecting \(\text{Ca}^{2+}\)-dependent catabolic processes such as protease activation and mitochondrial dysfunction.

To investigate the role of intracellular \([\text{K}^+]_i\) \(([\text{K}^+]_i)\) during apoptosis and the effect of disregulation of \([\text{K}^+]_i\), several pharmacological \(\text{K}^+\) channel openers (KCOs) were used (Figure 3.1). Some KCOs affect plasma membrane \(K_{\text{ATP}}\) channels, and \(I_K\)-like currents. The conductance and gating properties of these channels make them candidates for mediating efflux of intracellular \(\text{K}^+\). If the efflux of intracellular \(\text{K}^+\) is a critical stage in the apoptotic program then inducing efflux of \(\text{K}^+\) by treatment with pharmacological agents might affect the onset or progression of apoptosis. KCOs have also been reported to have a protective role in ischaemia by opening mitochondrial \(K_{\text{ATP}}\) channels (Garlid et al., 1996). This class of compound has also been considered for treatment of hypertension. If these compounds were to influence the initiation or progression of apoptosis this could affect their clinical utility.

![Figure 3.1 Structure of potassium channel openers.](image-url)
The aims of this chapter were:

i) to set up a consistent model of neuronal apoptosis.

ii) to optimise assessment of apoptosis.

iii) to define the mechanism(s) by which apoptosis is inhibited by elevated \([K^+]_{\text{ext}}\).

### 3.2 Results

#### 3.2.1 Assessment and quantification of cell death in CGN cultures by fluorescence microscopy

The method for preparation and culturing of CGNs was developed and techniques for assessing culture viability and cell death were optimised, as detailed in Methods (sections 2.4 and 2.5). After plating, cultures were incubated for 7 days to mature and differentiate. Newly-plated cells formed aggregates that appeared to increase cell viability, possibly by providing cell-cell contact or necessary survival factors. Solitary cells did not develop or differentiate. Plated cells flattened and adhered to the culture dish and an extensive neurite network was observed in healthy cultures. (Figure 3.2A).

The induction of apoptosis and necrosis in CGN cultures were assessed by viewing cells stained with a combination of the fluorescent nuclear dyes Hoechst 33342 (bisbenzimide) and propidium iodide (PI) by UV microscopy (Figure 3.2B). The nuclei of normal cells were dimly stained by Hoechst 33342, whereas apoptotic nuclei were a more intense blue colour and were condensed or fragmented. PI was only taken up by cells that had lost plasma membrane integrity; therefore nuclei that stained red were dead or late-stage apoptotic (secondary necrotic) cells. Since there were very few or no cells in the culture capable of phagocytosing apoptotic cells, dead and dying cells remained in the culture.
Figure 3.2: Staining and photomicroscopy of mature CGN cultures (7 DIV). Photomicrographs show an untreated culture stained with calcein-AM/PI (A) and Hoechst 33342/PI (B). Culture medium was switched from 25 mM K\(^+\) complete medium to 5 mM K\(^+\) serum-free medium for 24 h. Hoechst 33342 and PI staining clearly showed decreased culture viability (C), with nuclear fragmentation and pyknosis characteristic of apoptosis (D).
3.2.2 Induction of apoptosis in CGN cultures by reduction of $[K^+]_{\text{ext}}$ and withdrawal of serum

The effects of reduction of $[K^+]_{\text{ext}}$ and withdrawal of serum on mature CGN cultures were investigated by switching culture medium. Control cultures (conditioned medium replaced after washing) showed very low levels of apoptosis (<3 %), with only a small decrease in culture viability over 96 h (Figure 3.3). Reducing $[K^+]_{\text{ext}}$ from 25 to 5 mM induced apoptosis over 48 h, though by 96 h almost all susceptible cells had already undergone apoptosis, as <4 % of cells were apoptotic at this time. However, as conditioned 5 mM K+ complete medium was not available, this treatment contained fresh serum. Consequently induction of cell death by this treatment was complicated by an increased glutamate concentration, which may itself have induced excitotoxic cell death by continuous stimulation of NMDA receptors (Ankarcrona et al., 1995; Du et al., 1997). Alternatively, the excitatory action of the increased glutamate concentration may have compensated for the reduction in $[K^+]_{\text{ext}}$ (Yan et al., 1994), thus leading to an underestimation of the induction of apoptosis by reducing $[K^+]_{\text{ext}}$.

Reduction of $[K^+]_{\text{ext}}$ without serum withdrawal is therefore a poor experimental paradigm as there are multiple factors beyond experimental control involved in the induction of cell death. Withdrawal of serum while keeping $[K^+]_{\text{ext}}$ at 25 mM induced apoptosis to a greater extent than decreasing $[K^+]_{\text{ext}}$ alone, with maximal induction of apoptosis after 72 h. This relatively slow induction of cell death by serum withdrawal contrasts with Miller and Johnson (1996), who show induction of cell death with $t_{50}$ of 4 h. Unfortunately, the 96 h culture for this treatment became infected, though it is likely that the same trend as other treatments was followed, with a decreased proportion of apoptotic cells at 96 h. Withdrawal of serum in conjunction with reducing $[K^+]_{\text{ext}}$ induced apoptosis to the greatest extent, with apoptosis peaking after 48 h (27.2 %) and a corresponding decrease in culture viability and increase in dead cells. Almost all cells (>97 %) were dead after 96 h. While reducing $[K^+]_{\text{ext}}$ alone did not induce high levels of apoptosis, combined withdrawal of serum and reduction of $[K^+]_{\text{ext}}$ clearly induced more apoptosis than withdrawal of serum alone (27.2 % vs. 16.2 % apoptosis after 48 h). This further demonstrated the effect on induction of cell death by a reduction in $[K^+]_{\text{ext}}$, and also suggested that the growth factors present in serum could partially compensate for a reduction in $[K^+]_{\text{ext}}$. Examination of cultures by light microscopy revealed
that induction of cell death by the various treatments led to rapid disintegration of the neurite networks, shrinkage and rounding of cells and detachment of some cells from the culture dish.

It has been suggested that there are several apoptotic mechanisms in the $[K^{+}]_{\text{ext}}$ reduction-serum withdrawal model of apoptosis. Separate subpopulations of cells respond to the two apoptotic stimuli by different kinetics (Miller and Johnson, 1996), with a more rapid induction of cell death in cells susceptible to serum withdrawal. Data has also been presented showing that cells die by a combination of both apoptosis and necrosis after combined $[K^{+}]_{\text{ext}}$ reduction-serum withdrawal (Villalba et al., 1997). In addition, mature CGN cultures are vulnerable to the high concentration of glutamate present in fresh serum (Ankarcrona et al., 1995), with the mode of cell death determined by the intensity of insult, the induction of necrosis over apoptosis increasing with glutamate concentration (Du et al., 1997). While combined $[K^{+}]_{\text{ext}}$ reduction and serum withdrawal in CGN cultures is a highly complex and non-homogeneous model of apoptosis, ex vivo cell cultures give a better approximation of the physiological behaviour of cells than immortalised cell lines. This particularly applies to neuronal cells since terminally differentiated neurons do not divide, so immortalised neuronal cell lines bear little physiological resemblance.
Figure 3.3: Reduction of $[K^+]_{ext}$ and serum withdrawal induces apoptosis in CGN cultures. The growth medium of mature CGN cultures (7 DIV) was replaced with conditioned complete medium (control; ○), 25 mM $[K^+]_{ext}$ medium without serum (□), 5 mM $[K^+]_{ext}$ with fresh serum (●) or 5 mM $[K^+]_{ext}$ without serum (■). Cultures were stained with Hoechst 33342 and PI and viewed under a UV microscope. Cells were quantified from three representative fields for each treatment. This experiment was only performed once.
3.2.3 *Inorganic Hg^{2+} induces both apoptosis and necrosis in CGN cultures.*

Inorganic Hg^{2+} was selected as a positive control for the induction of apoptosis since low concentrations of Hg^{2+} interfere with the regulation of [Ca^{2+}]_i (Rossi et al., 1997) and thus is relevant to the role of [Ca^{2+}]_i in induction of apoptosis by reducing [K]^\text{ext}. Mature CGN cultures were incubated with a range of concentrations of Hg^{2+} for 30 h (Figure 3.4). Although control cultures had a relatively high proportion of dead cells, induction of apoptosis was minimal. Treatment with 100 μM Hg^{2+} exclusively induced necrosis, characterised by PI-stained nuclei without pyknosis or nuclear fragmentation (Figure 3.5C), with no induction of apoptosis, whereas high levels of both necrosis and apoptosis were induced by 10 μM Hg^{2+}. In contrast, 1 μM Hg^{2+} induced apoptosis, characterised by intense staining with Hoechst 33342 of fragmented and condensed nuclei (Figures 3.5A and B), with little necrosis above control levels. Concentrations of Hg^{2+} below 1 μM did not induce detectable levels of apoptosis over 30 h.

![Figure 3.4: Induction of apoptosis and necrosis in CGN cultures by Hg^{2+} is concentration-dependent.](image)

Mature CGN cultures (7 DIV) were incubated for 30 h with a range of concentrations of Hg^{2+}. Cultures were stained with Hoechst 33342 and PI and viewed under a UV microscope. Apoptotic and dead cells were quantified from three representative fields for each treatment. This experiment was only performed once.
Figure 3.5: Induction and assessment of apoptosis in mature CGN cultures (7 DIV). Incubation with 1 μM Hg$^{2+}$ for 48 h induced apoptosis in CGN cultures (A). Apoptotic nuclei exhibited characteristic fragmentation and pyknosis (B). Treatment of CGN cultures with 100 μM Hg$^{2+}$ for 8 h induced extensive necrosis (PI-positive nuclei without fragmentation or condensation) with little or no apoptosis (C). Treatment with 500 μM diazoxide for 24 h induced moderate levels of both apoptosis and necrosis (D).
3.2.4 Pharmacologically-active concentrations of potassium channel openers do not induce apoptosis in CGN cultures

Having established an experimental model and characterised the induction and assessment of apoptosis, the effects of the KCOs diazoxide, R49356 and P1060 on CGN cultures were investigated. As previously discussed (section 3.1), KCOs target ion channels which are candidates for the efflux of intracellular K\(^+\) during apoptosis, and the aim of these experiments was to investigate whether causing efflux of intracellular K\(^+\) by opening these channels would induce apoptosis. Since it was not possible to directly monitor the action of these compounds on ion channels by patch clamping, a range of concentrations was used that have previously been shown to be effective. Diazoxide has been shown to be pharmacologically active at 100 pM (Fujimara et al., 1997), P1060 at 30 μM (Guillemare et al., 1994) and RP49356 at 100 μM (Lebrun et al., 1991).

Mature CGN cultures treated with vehicle alone showed a high background level of cell death (~15 %), with a gradual but slight decrease in culture viability over 72 h (Figure 3.6). Treatment of cultures with diazoxide or RP49356 induced a time- and concentration-dependent induction of cell death, with a corresponding decrease in culture viability over time. Morphological features of cell death, including neurite disintegration and rounding of cells, were observed by light microscopy in cultures treated with high concentrations of diazoxide and RP49356. However, the mode of cell death was a mixture of both apoptosis and necrosis, as assessed by Hoechst 33342 and PI (Figure 3.5D), rather than distinct induction of apoptosis as seen with 1 μM Hg\(^{2+}\) (Figure 3.5A). Treatment of cultures with P1060 did not induce cell death to a significant extent above control.

While high concentrations (5 mM) of the KCOs did induce cell death, lower but still pharmacologically active concentrations (50 - 100 μM) did not effectively induce apoptosis, leading to the conclusion that either these KCOs had no effect on cellular K\(^+\) channels or that direct opening of K\(^+\) channels did not induce apoptosis in CGN cells. This result was unexpected in light of previous reports of induction of apoptosis in CGN cells by the KCO cromakalim (Yu et al., 1997).
Figure 3.6: Treatment of CGN cultures with KCOs does not induce apoptosis at pharmacologically active concentrations. Mature CGN cultures (7 DIV) were treated with a range of concentrations of KCOs, as shown in chart legends. Cultures were stained with Hoechst 33342 and PI and viewed under a UV microscope. Cells were quantified from three representative fields for each treatment. This experiment was only performed once.
3.3 DISCUSSION

Mechanisms of apoptosis induced by withdrawal of K\(^+\) are complex. The CGN apoptotic model was set up to investigate the role of K\(^+\) and ion channels in neuronal apoptosis. The induction of apoptosis by the reduction of \([K^+]_{\text{ext}}\) and withdrawal of serum that has been previously reported (D'Mello et al., 1993; Miller and Johnson, 1996) was reproducible in this system (Figure 3.3). Mature CGN cultures underwent extensive apoptosis, characterised by nuclear condensation and fragmentation, in a time-dependent fashion when medium [K\(^+\)] was reduced from 25 to 5 mM or when serum was withdrawn from the medium. The mechanisms of apoptotic induction appeared to be complex, as it was extremely difficult to reduce \([K^+]_{\text{ext}}\) without withdrawing serum. This withdrawal of serum or addition of fresh serum added extra complexity to the system. The consequences of serum-withdrawal and \([K^+]_{\text{ext}}\)-reduction are difficult to separate, though it has been reported that CGN cultures do not require growth factors after ~5 DIV (Gallo et al., 1987). A recent study has implicated the activation of p38 mitogen-activated protein (MAP) kinase as mediating apoptosis induced by reduction of \([K^+]_{\text{ext}}\) (Yamagishi et al., 2001). p38 MAP kinase phosphorylates c-Jun, which is a necessary event in neuronal apoptosis (Watson et al. 1998). This indicates that apoptosis induced by reduction of \([K^+]_{\text{ext}}\) may result from a down-regulation of pro-survival signalling. The involvement of caspases in apoptosis induced by withdrawal of K\(^+\) has been demonstrated (Eldadah et al., 1997), with increasing caspase-3 activity following serum and potassium withdrawal (Eldadah et al., 2000). This Chapter has also demonstrated the induction of apoptosis by Hg\(^{2+}\), with nuclear morphology characteristic of apoptosis. However, experiments were not continued to a point where the influence of Ca\(^{2+}\) disregulation on CGN cultures could be thoroughly investigated. Combined treatment of Hg\(^{2+}\) with reduction of \([K^+]_{\text{ext}}\) might have been useful for investigation into the role of \([Ca^{2+}]_{i}\) in inhibition of apoptosis by K\(^+\).

Treatment of CGN cultures with KCOs does not induce apoptosis. Experiments investigating the action of KCOs demonstrated that, in my hands, diazoxide, RP49356 and P1060 did not induce apoptosis at concentrations that have been shown to be pharmacologically active in other systems (Lebrun et al., 1991; Guillemare et al., 1994; Fujimara et al., 1997). Higher concentrations of diazoxide and RP49356 did induce cell death that was a mixture of apoptosis and necrosis. While it was not possible to demonstrate the
pharmacological action of these agents by patch clamping or the instigation of $K^+$ efflux, the
data suggested that these agents induced cell death by a mechanism other than induction of $K^+
$ efflux, such as cell-stress. It therefore appears that decreasing intracellular $[K^+]_i$, $(K^+_i)$ alone
did not initiate apoptosis in CGN cultures. This interpretation is supported by the conclusions
of Bortner et al. (1997), who show that reducing $[K^+]_i$ alone is not sufficient to induce
apoptosis. The exact mode and site of action of KCOs is not known, however, and the
proposal that opening of certain $K^+$ channels would result in a decrease in $[K^+]_i$ may have
been incorrect. Alternatively, KCOs may have a secondary effect on the cell other than
causing a gross efflux of $K^+$.

**Does KCO treatment potentiate induction of apoptosis?** An interesting problem that
was not approached experimentally was the potentiation of apoptosis by KCOs. While the
action of KCOs on CGNs did not directly induce apoptosis, their action may potentiate the
initiation of apoptosis by other agents such as $Hg^{2+}$ or the kinase inhibitor staurosporine. This
would be consistent with the demonstration in a non-neuronal cell line that $[K^+]_i$-depleted cells
are far more susceptible to apoptotic stimuli (Bortner et al., 1997). The induction of apoptosis
in cultured neocortical neurons by the KCO cromakalim (500 μM) has been reported (Yu et
al., 1997), apparently mediated by the enhanced efflux of intracellular $K^+$. In support of this,
Holmuhamedov et al. (1998) showed that the KCOs cromakalim, levocromakalim and pinacidil
induced cytochrome c release and depolarisation of isolated mitochondria, an important event
in apoptosis, as discussed in Chapter One (section 1.5). However, a cytoprotective role for
cromakalim (100 μM) has also been reported (Lauritzen et al., 1997), inhibiting glutamate-
induced excitotoxicity in hippocampal neurons. A protective effect has also been reported in
cardiac ischaemia by KCO action on mitochondrial $K_{ATP}$ channels (Garlid et al., 1997).

**Conclusions.** While the CGN model is a good approximation of physiological
apoptosis and does illustrate the importance of $K^+$ in apoptosis, it was decided to change to a
simpler system to follow up the findings of these experiments. The mechanism of the $[K^+]_{ext}$
reduction/serum withdrawal model appeared to be mediated by multiple mechanisms and the
culture population is reportedly not completely homogeneous (Miller and Johnson, 1996). The
adherent and fragile nature of the CGN cells and neurite networks meant that a number of
useful analytical techniques such as flow cytometry, assessment of cell size, transfection and
patch clamping were technically very difficult or not feasible. In addition, the complexity and difficulty of culture preparation limited the number of cells available and meant that fewer experiments and repeats were possible.

The three directions for further research that were raised by this initial investigation were the roles in apoptosis of \([\text{Ca}^{2+}]_i\), the transduction of survival pathways by PI-3-K and efflux of intracellular K+. All of these three mechanisms are complex in nature and it was decided to concentrate on one experimental approach in a more accessible model of apoptosis. Since the roles of Ca\(^{2+}\) and PI-3-K in apoptosis are likely to be more specific to the CGN model, it was therefore decided to investigate the role of K\(^+\) in apoptosis using a range of leukocyte cell lines.

3.4 SUMMARY

- Reduction of extracellular [K\(^+\)] from 25 to 5 mM induced apoptosis in CGN cultures, as characterised by nuclear condensation and fragmentation (Figures 3.2C and D and 3.4).
- Low concentrations of inorganic Hg\(^{2+}\) induced apoptosis in CGN cultures, though higher concentrations induced necrotic cell death fragmentation (Figures 3.4 and 3.5A and B).
- The KCOs diazoxide, RP49356 and P1060 did not induce apoptosis at concentrations that have previously been shown to cause K\(^+\) channel opening (Figure 3.6).
Chapter Four. Induction of apoptosis in Jurkat T cells is associated with plasma membrane depolarisation and efflux of intracellular K⁺.

4.1 INTRODUCTION

A well-documented cellular change during apoptosis is cell shrinkage. This feature led Kerr (1971) to refer to apoptosis as ‘shrinkage necrosis’ and was one of the characteristic changes in apoptotic cells that was noted by Kerr et al. (1972). Apoptotic cell shrinkage is almost certainly associated with efflux of the principal cytoplasmic ion potassium (K⁺) and osmotic efflux of water molecules. Efflux of cytoplasmic K⁺ has been described as an important event in the progression of apoptosis (Barbiero et al., 1995; Bortner et al., 1997; McCarthy and Cotter, 1997; Fernández-Segura et al., 1999; Gómez-Angelats et al., 2000), and the intracellular [K⁺] ([K⁺]ᵢ) of apoptotic cells is decreased from ~150 mM to between ~50 mM (Barbiero et al., 1995; Hughes et al., 1997) and ~65 mM (McCartney and Cotter, 1997).

Prevention of this efflux of intracellular K⁺ by pharmacological block of ion channels has been reported to inhibit death receptor- and chemical-induced apoptosis (Dallaporta et al., 1999; Maeno et al., 2000). Efflux of K⁺ may be a prerequisite for apoptosis as the normal physiological range of cytoplasmic [K⁺] is not compatible with the progression of apoptosis; normal [K⁺], inhibits some of the key enzymic events involved in the execution phase of apoptosis (Walev et al., 1995; Hughes et al., 1997; Dallaporta et al., 1998). These include activation of caspases, a family of cysteine proteases that cleaves many structural and enzymic substrates during apoptosis (Cohen, 1997), and endonucleases, which digest chromatin to produce oligonucleosomal DNA fragments (Wyllie, 1980).

Chapter Three briefly discussed the functional role of ion channels in the plasma membrane, plasma membrane potential and K⁺ ions in apoptosis. Experiments indicated that medium [K⁺] might have both an electrical and concentration-dependent role in the regulation of apoptosis. Since membrane potentials are set by ionic concentration gradients across them, changes in ionic concentrations will almost certainly be associated with changes in the
membrane potential. Therefore monitoring of the plasma and mitochondrial membrane potentials will give information as to the movement of ions during apoptosis.

The aims of this chapter were:

i) to characterise apoptosis in immortalised cell lines.

ii) to investigate whether there is any change in the plasma membrane potential of apoptotic cells and, if so, the relationship with other apoptotic events.

iii) to investigate whether \([K^+]_i\) changes during apoptosis and examine the relationship of this with other apoptotic events.

Flow cytometric analysis of properties of suspension cells treated with apoptotic stimuli provided a very powerful tool for the examination of ionic and electrical changes during apoptosis. The plasma membrane potential and \([K^+]_i\) of cells treated with apoptotic stimuli were examined in relation to apoptotic markers such as cell shrinkage, PS externalisation, reduction of mitochondrial transmembrane potential (\(\Psi_m\)) and caspase activation.

4.2 RESULTS

4.2.1 The plasma membrane of apoptotic cells is depolarised relative to normal cells

The plasma membrane potential is set by the potential of the intracellular fluid (i.e. the cytoplasm) minus the potential of the extracellular fluid (in this case culture medium). Variations in the intracellular environment will therefore be reflected in the plasma membrane potential. Changes in plasma membrane potential often accompany cell signalling, principally due to the opening and closing of multiple classes of ion channels. The efflux of intracellular \(K^+\) during apoptotic cell shrinkage will lead to changes in plasma membrane potential, and the loss of plasma membrane integrity late in apoptosis will lead to equilibration of the cytoplasm with the extracellular environment and dissipation of the plasma membrane potential. Changes prior to cell shrinkage and loss of plasma membrane integrity, however, might indicate early signalling events in the apoptotic program.
Figure 4.1: Assessment of apoptosis and plasma membrane depolarisation by DiBAC₄(3) and PI. Jurkat T cells were incubated with vehicle (DMSO; A) or anti-CD95 antibody (50 ng/ml; B) for 5 h and then stained with DiBAC₄(3) and PI. Cells with low membrane permeability and a relatively hyperpolarised plasma membrane (low DiBAC₄(3) fluorescence) were characterised as normal; cells with low membrane permeability and a relatively depolarised plasma membrane (high DiBAC₄(3) fluorescence) were characterised as apoptotic; cells with loss of membrane integrity (high PI) were interpreted as dead.

The plasma membrane potential of Jurkat T cells treated with vehicle or anti-CD95 antibody for 5 h was analysed by flow cytometry using DiBAC₄(3). Control cultures showed a homogenous population of cells with a hyperpolarised plasma membrane (i.e. low DiBAC₄(3) fluorescence) and low membrane permeability (low PI fluorescence; Figure 4.1A). Cultures in which apoptosis had been induced by treatment with anti-CD95 antibody (Nagata, 1997) exhibited a population of cells with a plasma membrane potential comparable to the control culture and also a population of cells with increased uptake of the anionic DiBAC₄(3) (Figure 4.1B), indicative of depolarisation of the plasma membrane. This population of cells with a relatively depolarised plasma membrane potential was not observed in control cultures and
was interpreted as apoptotic. There was also a small proportion of cells in the anti-CD95 antibody-treated culture and to a lesser extent in the control culture with increased plasma membrane permeability and depolarised membrane potential. These were consistent with the characteristics of dead cells.

To confirm the correlation of plasma membrane depolarisation with induction of apoptosis, externalisation of PS, a phagocytotic marker of apoptosis (Martin et al., 1995), was examined in conjunction with plasma membrane potential in cells treated with vehicle or anti-CD95 antibody. In control cultures 3.4% of cells displayed externalisation of PS and there was little variation in plasma membrane potential (Figure 4.2A). Cultures treated with anti-CD95 antibody showed a high proportion (58.4%) of cells with PS externalisation (Figure 4.2B). Of those cells with PS externalisation, the minority with a highly depolarised plasma membrane (2.7%) were interpreted as dead cells that had lost membrane integrity, which would be associated with the collapse of plasma membrane potential. The larger population of cells exhibiting externalisation of PS (55.7%; Figure 4.2B) had a plasma membrane potential that was depolarised relative to the normal cells, though not dissipated as in the dead cells. These cells were interpreted as apoptotic, consistent with Dallaporta et al. (1999), who report plasma membrane depolarisation in apoptotic thymocytes.
Figure 4.2: Plasma membrane depolarisation correlates with PS externalisation in apoptotic cells. Jurkat T cells were incubated with vehicle (PBS; A) or anti-CD95 antibody (50 ng/ml; B) for 5 h and then stained with Annexin-PE and DiBAC₄(3).

To investigate the extent to which plasma membrane depolarisation reflected induction of apoptosis, cells were treated with the death-receptor stimuli anti-CD95 antibody, TRAIL (MacFarlane et al., 2000) or TNF-α in combination with cycloheximide (CHX; Kull and Cuatrecasas, 1981), or the chemical stimuli etoposide (Chen et al., 1984) or staurosporine (STS; Jacobson et al., 1993). At the indicated times, plasma membrane potential was analysed in conjunction with two measures that assess different features of the apoptotic program, namely Annexin V-FITC to measure PS externalisation and DiOC₆(3) to measure \( \Psi_m \). Control cells showed a relatively low level of apoptosis (<12 %) as assessed by the three methods of analysis (Figure 4.3). Notably, some disparity existed between the three parameters, with assessment by plasma membrane depolarisation giving a far higher figure compared to that from PS externalisation (11.7 % compared to 2.7 %). All apoptotic stimuli induced apoptosis above control levels, though TNF-α in combination with CHX induced relatively little apoptosis (15.1 - 18.7 % by the three parameters). The three methods of analysis gave very
similar estimates of the percentage of apoptotic cells for the death receptor stimuli (TRAIL, anti-CD95 antibody and TNF-α/CHX). For the chemical inducers of apoptosis (etoposide and STS), estimates of the percentage of apoptotic cells by the three parameters were more disparate, with plasma membrane potential having a wider range than the other two measures of apoptosis. Despite this, the correlation between quantification of apoptosis by assessment of PS externalisation, plasma and reduction of $\Psi_m$ suggested that plasma membrane depolarisation might be considered part of the apoptotic phenotype and used as a measure of apoptosis.

Figure 4.3: Assessment of apoptosis by DiBAC$_4$(3)/PI is comparable with Annexin V/PI and DiOC$_6$(3)/PI. Jurkat T cells were incubated with vehicle (DMSO), TRAIL (1 μg/ml), anti-CD95 antibody (50 ng/ml), TNF-α/CHX (10 ng/ml, 1 μM respectively), etoposide (50 μM) or STS (1 μM) for 5 h before quantification of apoptosis by PS externalisation (Annexin V-FITC; hatched bars), plasma membrane depolarisation (DiBAC$_4$(3); open bars) and reduction of $\Psi_m$ (DiOC$_6$(3); solid bars). This experiment was only performed once.
Having established the occurrence of plasma membrane depolarisation during apoptosis prior to a loss in plasma membrane integrity, the order of this event in relation to other apoptotic markers was investigated. An early occurrence might suggest an important signalling role for depolarisation in the initiation stages of apoptosis, whereas a late depolarisation would be consistent with cell shrinkage. Analysis of the plasma membrane potential of cells exhibiting PS externalisation showed a very close correlation between depolarisation and PS externalisation (Figure 4.2), suggesting that these events are simultaneous or overlapping. There was not a discernable third population of cells displaying plasma membrane depolarisation without PS externalisation, or vice versa, that would have indicated that one event occurred before the other.

As a more suitable method for ordering apoptotic events, PS externalisation, plasma membrane potential and cell shrinkage were assessed at the times indicated following treatment, up to 8 h. This was performed in Jurkat cells treated with vehicle, anti-CD95 antibody or etoposide. Control cells showed a consistently low level of apoptosis (<8 % at 8 h) for all methods of quantification of apoptosis (Figure 4.4). Both anti-CD95 antibody and etoposide induced apoptosis in a time-dependent manner. Anti-CD95 antibody-treated cells showed good correlation between cell shrinkage and PS externalisation, with a greater proportion of plasma membrane depolarisation from 4 h onwards. Since the percentages of apoptotic cells at 8 h as assessed by the three methods were not consistent, it was difficult to draw any conclusions about the order of these events. While plasma membrane depolarisation did appear to precede cell shrinkage and PS externalisation in anti-CD95 antibody-induced apoptosis, the percentage of cells at 8 h was also higher. It appeared, therefore, that measurements of plasma membrane depolarisation overestimated the extent of apoptosis (or the other two measures underestimated the extent of apoptosis) rather than plasma membrane depolarisation preceding PS externalisation and mitochondrial depolarisation. Etoposide-treated cells showed good correlation between the three measures with none of the markers appreciably preceding the others. It was noted for both apoptotic stimuli that plasma membrane depolarisation did not correlate exactly with cell shrinkage. This argues against the hypothesis that the plasma membrane depolarisation observed during apoptosis was a reflection of a change in ionic balance during cell shrinkage, and is instead a separate event.
Figure 4.4: Plasma membrane depolarisation accompanies PS externalisation and cell shrinkage. Jurkat T cells were incubated with vehicle (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 μM). At the times shown, assays were performed for PS externalisation (■; Annexin V-FITC/PI), cell shrinkage (▲) and plasma membrane depolarisation (○; DiBAC₄(3)/PI). This experiment was only performed once.
Taken together, these data demonstrate the depolarisation of the plasma membrane in apoptotic cells prior to loss of plasma membrane integrity and therefore initiated by the controlled movement of ions in or out of the cell. This depolarisation correlated well with apoptotic cell shrinkage, PS externalisation and reduction of \( \Psi_m \) and therefore could be considered as part of the apoptotic phenotype.

4.2.2 Plasma membrane depolarisation is upstream of caspase activation in etoposide- but not anti-CD95 antibody-induced apoptosis.

In order to determine the relationship between plasma membrane depolarisation and caspase activity, Jurkat cells stimulated with vehicle, anti-CD95 antibody or etoposide were treated with the broad-spectrum caspase inhibitor z-VAD.fmk (25 \( \mu \text{M} \); Fearnhead et al., 1995). Control cultures were relatively homogenous for cell size, PS externalisation and plasma membrane potential (Figure 4.5, A to C and 4.6, A to C, grey fill). In contrast, cells treated for 5 h with anti-CD95 antibody or etoposide alone showed cell shrinkage relative to control cultures, high proportions of cells with PS externalisation and depolarisation of the plasma membrane (Figure 4.5, A to C and 4.6, A to C, red line). In control cells, treatment with z-VAD.fmk had no effect on the three measures of apoptosis, whereas z-VAD.fmk completely inhibited expression of all apoptotic markers in anti-CD95 antibody-treated cells, with cell size, PS externalisation and plasma membrane potential comparable to untreated cells (Figure 4.5, D to F). In contrast, z-VAD.fmk did not inhibit etoposide-induced plasma membrane depolarisation or cell shrinkage, though PS externalisation was completely blocked (Figure 4.6, D to F). This suggested that, while anti-CD95 antibody-induced depolarisation of the plasma membrane was dependent upon initiator caspase activation, both etoposide-induced plasma membrane depolarisation and cell shrinkage were independent of or prior to caspase activation.
Figure 4.5: z-VAD.fmk inhibits anti-CD95 antibody-induced apoptosis upstream of cell shrinkage, PS externalisation and plasma membrane depolarisation. Jurkat T cells were incubated with either vehicle (PBS; grey fill) or anti-CD95 antibody (50 ng/ml; red line) for 5 h in the absence (A - C) or presence (D - F) of z-VAD.fmk (25 μM). Cell shrinkage was assessed by decrease in forward scatter, PS externalisation by Annexin V-FITC/PI staining and plasma membrane potential by DiBAC₄(3)/PI.
Figure 4.6: z-VAD.fmk inhibits etoposide-induced PS externalisation but not cell shrinkage or plasma membrane depolarisation. Jurkat T cells were incubated with either vehicle (DMSO, grey fill) or etoposide (50 µM; red line) for 5 h in the absence (A - C) or presence (D - F) of z-VAD.fmk (25 µM). Cell shrinkage was assessed by decrease in forward scatter, PS externalisation by Annexin V-FITC/PI staining and plasma membrane potential by DiBAC₄(3)/PI.
4.2.3 Apoptotic cells have a decreased \([\text{K}^+]_i\) relative to resting cells

Several studies have demonstrated the efflux of intracellular \(\text{K}^+\) during apoptosis, though it is not clear whether this efflux is an important early event (Bortner and Cidlowski, 1996) or a later event (Dallaporta et al., 1998). Both cell shrinkage and plasma membrane depolarisation during apoptosis are almost certainly mediated by the movement of ions across the plasma membrane, though are not necessarily the same event or involve the same ionic species. It was therefore decided to investigate \([\text{K}^+]_i\) during induction of apoptosis.

---

**Figure 4.7: Reduction of \(\Psi_m\) correlates with PS externalisation.** Jurkat T cells were incubated with anti-CD95 antibody (50 ng/ml) for 5 h then stained with Annexin V-PE and DiOC\(_6\)(3).

PS externalisation and reduction of \(\Psi_m\) were used as apoptotic markers against which to correlate a decrease of \([\text{K}^+]_i\) in apoptotic cells. Several studies have shown that reduction of \(\Psi_m\) is a key event in apoptosis induced by diverse stimuli such as UV and \(\gamma\) irradiation, growth
factor withdrawal, DNA damage, chemical stress, Bax over-expression and ligation of death receptors (Petit et al., 1996). In order to characterise these markers of apoptosis, the mitochondrial membrane potential of Jurkat cells stimulated with anti-CD95 antibody was compared against PS externalisation (Figure 4.7). All cells with PS externalisation exhibited reduction of Ψm compared to those without PS externalisation. There was, however, a population of cells without PS externalisation that exhibited reduction of Ψm, which implied that the latter was upstream of PS externalisation, in agreement with previous studies (Overbeeke et al., 1999; Dallaporta et al., 1999).

In order to examine [K+]i of apoptotic cells, cultures of Jurkat cells stimulated with anti-CD95 antibody for 5 h were stained with the K⁺ indicator PBFI-AM, DiOC6(3) and PI. Using cell size and density to gate out non-cellular events (Figure 4.8A), Ψm was examined in conjunction with plasma membrane integrity in order to characterise cells as normal, apoptotic and dead (Figure 4.8B). The [K⁺]i of these three populations was then examined (Figure 4.8, C and D). Normal cells, as characterised by high Ψm and intact plasma membrane, had a higher [K⁺]i than apoptotic cells, as characterised by reduction of Ψm and intact plasma membrane. This was indicated by the PBFI-AM peak emission at a higher fluorescence intensity for the normal cells (Figure 4.8C, blue line) compared to the apoptotic cell PBFI-AM fluorescence intensity (Figure 4.8C, green line). Dead cells, as characterised by loss of plasma membrane integrity, had a much lower [K⁺]i than both apoptotic and normal cells (Figure 4.8C, red line). This is in agreement with Dallaporta et al. (1999), who show that decreased [K⁺]i was only observed in cells with reduction of Ψm. The decrease in [K⁺]i of apoptotic cells was only slight in comparison with the decrease in [K⁺]i of dead cells. The greatly lowered [K⁺]i of PI-including cells may have been due to the cytoplasmic environment equilibrating with the culture medium, leading to a [K⁺]i of ~5 mM. Alternatively, the dye may have leached from cells that had lost membrane integrity, or the lack of esterase activity in dead cells resulted in no dye being activated by cleavage of the acetoxy methyl group.
Figure 4.8: Cells with reduction of $\Psi_m$ have decreased $[K^+]_i$. Jurkat T cells were incubated with anti-CD95 antibody (50 ng/ml) for 5 h and then stained with DiOC$_6$(3), PBFI-AM and PI. A dot-plot of size against density (FSC vs. SSC) was used to exclude debris and non-cell events (A). Cells were characterised as normal, apoptotic and dead according to $\Psi_m$ and membrane integrity (B). A histogram of $[K^+]_i$ for the individual cell populations showed a clear difference between the three (C), which was also seen in a contour plot of $\Psi_m$ against $[K^+]_i$ (D).
Figure 4.9: Cells with PS externalisation have decreased \([K^+])_i\). Jurkat T cells were incubated with anti-CD95 antibody (50 ng/ml) for 5 h and then stained with Annexin V-FITC, PBFI-AM and PI. A dot-plot of size against density (FSC vs. SSC) was used to exclude debris and non-cell events (A). Cells were characterised as normal, apoptotic and dead according to PS externalisation and membrane integrity (B). A histogram of \([K^+])_i\) for the individual cell populations (C) showed a clear difference between the three, which was also seen in a contour plot of PS externalisation against \([K^+])_i\) (D).
Investigation of \([K^+]_i\) using PS externalisation as an apoptotic marker also indicated that the \([K^+]_i\) of apoptotic cells was decreased relative to normal cells. Non-cellular events were removed from analysis by a plot of size against density (Figure 4.9A), and cells were characterised as normal, apoptotic and dead according to PS externalisation and plasma membrane integrity (Figure 4.9B), as detailed in Methods (section 2.7.2). Examination of the \([K^+]_i\) of these three populations revealed that apoptotic cells had decreased \([K^+]_i\) relative to normal cells (Figure 4.9C, green and blue lines, respectively), with dead cells having a very low PBFI-AM signal intensity (Figure 4.9C, red line), consistent with equilibration of the cytoplasmic \(K^+\) with the culture medium (~5 mM).

**4.2.4 Decreased \([K^+]_i\) in anti-CD95 antibody-induced apoptosis is dependent upon caspase activation**

Following on from the data in section 4.2.2, which showed that plasma membrane depolarisation was dependent upon caspase activation in anti-CD95 antibody- but not etoposide-induced apoptosis, the effect of inhibition of caspase-activity by z-VAD.fmk (25 μM) on the decrease in \([K^+]_i\) following induction of apoptosis was investigated. As shown previously (Figures 4.5 to 4.9), treatment of Jurkat cells with anti-CD95 antibody or etoposide resulted in cell shrinkage, PS externalisation and lowered \([K^+]_i\) compared to control cells (Figure 4.10, A to C and Figure 4.11, A to C). Consistent with previous results (Figure 4.5, D and E), z-VAD.fmk completely inhibited anti-CD95 antibody-induced cell shrinkage and PS externalisation (Figure 4.10, D to E), and also completely inhibited loss of \([K^+]_i\) (Figure 4.10F). Complete inhibition of cell shrinkage, PS externalisation and \([K^+]_i\) decrease by z-VAD.fmk was also observed in etoposide-treated cells (Figure 4.11, D to E). This contradicted the findings of section 4.2.2 and also those of Sun et al. (1999), that z-VAD.fmk did not inhibit etoposide-induced cell shrinkage. The data are therefore questionable, despite this result being achieved in two independent experiments, and it could not be discerned by this method whether decreased \([K^+]_i\) in etoposide-induced apoptosis was inhibited by z-VAD.fmk.
Figure 4.10: z-VAD.fmk inhibits anti-CD95 antibody-induced apoptosis upstream of cell shrinkage, PS externalisation and $K^+$ efflux. Jurkat T cells were incubated with vehicle (PBS; grey fill) or anti-CD95 antibody (50 ng/ml; red line) for 5 h in the absence (A - C) or presence (D - E) of z-VAD.fmk (25 μM). Cell shrinkage was assessed by decrease in forward scatter, PS externalisation by Annexin V-FITC/PI staining and $[K^+]_i$ by PBFI-AM. Data are representative of 2 independent experiments.
Figure 4.11: *z*-VAD.fmk inhibits etoposide-induced apoptosis upstream of cell shrinkage, PS externalisation and K⁺ efflux. Jurkat T cells were incubated with vehicle (DMSO; grey fill) or etoposide (50 μM; red line) for 5 h in the absence (A - C) or presence (D - E) of *z*-VAD.fmk (25 μM). Cell shrinkage was assessed by decrease in forward scatter, PS externalisation by Annexin V-FITC/PI staining and [K⁺]ᵢ by PBFI-AM. Data are representative of 2 independent experiments.
4.2.5 \( K^+ \) efflux accompanies the increase in PS externalisation, cell shrinkage and reduction of \( \Psi_m \) during the induction of apoptosis

While many previous studies have used DiOC\(_6\)(3) to assess reduction of \( \Psi_m \) during apoptosis (such as Dallaporta et al., 1998; Sun et al., 1999), this dye is also influenced by the membrane potentials of the plasma membrane and of membrane enclosed organelles such as the ER (Dallaporta et al., 1999; Bortner and Cidlowski, 1999). Any signal of reduction of \( \Psi_m \) is therefore potentially contaminated by depolarisation of other organelles. DiOC\(_6\)(3) has also been shown to interfere with mitochondrial bioenergetic function thereby inducing necrotic cell death (Rottenberg and Wu, 1997). For these reasons the rhodamine-derivative dye TMRE was used for further investigation of reduction of \( \Psi_m \) during apoptosis, as this dye is not influenced by plasma membrane potential (see Figure 2.6).

Analysis of the decrease in \([K^+]_i\) during apoptosis by PBFI-AM was problematical. As detailed in Methods (section 2.7.2), PBFI-AM is only 1.5-times more specific for \( K^+ \) than for \( Na^+ \) ions, is pH-sensitive (only reliable between pH 6.5 - 7.5) and takes \(~1\) h to be loaded into cells. In addition, the dynamic range is extremely small, as demonstrated by the small changes in population fluorescence between normal and apoptotic cells in Figures 4.8 and 4.9. The time required for calibration of both lasers of the FACS Vantage was excessive and added extra complication to experiments. A more robust, sensitive and \( K^+ \)-specific method was therefore required, and the \(^{86}\)Rb\(^+\) efflux assay was developed and adapted to monitor efflux of intracellular \( K^+ \) from apoptotic cells.

Induction of apoptosis in Jurkat cells was analysed by three measures of apoptosis, which assess different features of the apoptotic phenotype, namely Annexin V-FITC fluorescence to measure PS externalisation, decrease in TMRE fluorescence as an indicator of reduction of \( \Psi_m \) and decrease in forward light scatter as a measure of cell shrinkage. In control cells, spontaneous apoptosis was \(~5\%)\, as assessed by these three parameters (Figure 4.12 A). 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 (Figure 4.12, B and C).
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, cells were pre-loaded with \(^{86}\text{Rb}^+\) and at the indicated times following treatment with the different apoptotic stimuli, \([K^+]_i\) was determined indirectly by measuring the retention of \(^{86}\text{Rb}^+\) in the cell pellet. The change in \([K^+]_i\) was represented by a ratio of experimental against control cell pellet \([^{86}\text{Rb}^+]\), termed \(K_R\) (see section 2.8). Induction of apoptosis led to a decrease in \(K_R\) (Figure 4.12, B and C), which indicated a decrease in \([K^+]_i\). This \(K^+\) efflux accompanied PS externalisation, cell shrinkage and reduction of \(\Psi_m\) (Figure 4.12, B and C), which suggested that loss of \([K^+]_i\) might also be utilized as an indicator of apoptosis in some cellular systems. The decrease in \(K_R\) was not due to cell lysis as at all times <3% of cells stained positive for PI.

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

To clarify the data discussed in section 4.2.4, the effect of caspase inhibition by \(z\text{-VAD.fmk}\) (20 \(\mu\text{M}\)) on anti-CD95 antibody- and etoposide-induced efflux of intracellular \(K^+\) was investigated using \(^{86}\text{Rb}^+\). In control cells, \(z\text{-VAD.fmk}\) did not affect any of the four measures of apoptosis (Figure 4.12D), whereas it completely inhibited the changes in the apoptotic measures induced by anti-CD95 antibody (compare Figures 4.12, B and E). In contrast, in cells treated with etoposide, \(z\text{-VAD.fmk}\) completely blocked externalisation of PS, whereas reduction of \(\Psi_m\) and efflux of intracellular \(K^+\) were only partially inhibited and cell shrinkage was unaffected (Figure 4.12F). Taken together, these data are compatible with the hypothesis that \(z\text{-VAD.fmk}\) 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, reduction of \(\Psi_m\) and \(K^+\) efflux, were either upstream or independent of post-mitochondrial caspase activation.
Figure 4.12: Efflux of intracellular K⁺ from apoptotic cells is concomitant with PS externalisation, reduction of \( \Psi_m \) and cell shrinkage. Jurkat T cells, either loaded with \(^{86}\text{Rb}^+\) or sham-loaded, were incubated with vehicle (DMSO; A, D), anti-CD95 antibody (50 ng/ml; B, E) or etoposide (50 \( \mu \)M; C, F) for 5 h in standard RPMI medium. At the times shown, assays were performed for \(^{86}\text{Rb}^+\) efflux (●), PS-externalisation (■), cell shrinkage (□) and reduction of \( \Psi_m \) (○). Experiments were also performed in the presence of z-VAD.fmk (20 \( \mu \)M; D-F). The right-hand y-axis displays cell pellet \(^{86}\text{Rb}^+\) as a ratio of control \( K_R \). Data are representative of 3 independent experiments.
4.3 DISCUSSION

*Induction of apoptosis is associated with plasma membrane depolarisation.* The results presented in this chapter demonstrate depolarisation of the plasma membrane in both death receptor- and chemically-induced apoptosis, with similar proportions of cells exhibiting PS externalisation and cell shrinkage (Figures 4.1 to 4.3). This suggested that plasma membrane depolarisation occurred in most, if not all, cells undergoing apoptosis and was part of the apoptotic phenotype under the conditions tested. The lack of correlation between plasma membrane depolarisation and cell shrinkage (Figure 4.4) suggests that these events were unrelated, with separate ionic fluxes mediating these events. Plasma membrane depolarisation was not an early event, but rather was synchronous with PS externalisation and cell shrinkage (Figure 4.4) and, as such, was not consistent with an early signalling role for plasma membrane depolarisation in apoptosis. However, it is not inconceivable that there could be early changes in the plasma membrane potential that were below the resolution of my system. Bortner et al. (2001) have recently presented evidence that CD95-mediated apoptosis involves an early increase in intracellular $\left[ Na^+ \right]$ ($\left[ Na^+ \right]_i$) and depolarisation of the plasma membrane that precedes cell shrinkage.

In anti-CD95 antibody-induced apoptosis, plasma membrane depolarisation was dependent upon caspase activation (Figure 4.5), which was consistent with an early requirement for initiator caspase activation in CD95-mediated apoptosis. For etoposide-induced apoptosis, plasma membrane depolarisation was not prevented by treatment with z-VAD.fmk (Figure 4.6), which implied a mechanism that was either upstream or independent of caspase activation. Although the ion flux mediating plasma membrane depolarisation was not identified, the difference in plasma membrane potential between dead and apoptotic cells suggested that depolarisation of the plasma membrane during apoptosis was a controlled event rather than the equilibration between cytoplasmic contents and the external environment leading to a collapse of plasma membrane potential, as occurred in dead cells.

A problem encountered with the DIBAC$_4$(3) dye was the low dynamic range of the signal, with little change in signal intensity between normal and apoptotic cells. Comparing signal intensities between control and apoptotic cells (Figure 4.5, B and C), there is a ~30-fold intensity increase in Annexin-FITC fluorescence and only a ~4-fold increase in DiBAC$_4$(3)
fluorescence. This low signal to noise ratio made it more difficult to distinguish between populations of cells with normal and depolarised plasma membranes and may have introduced a degree of error into estimates of the extent of apoptosis. Acquisition of data using a non-logarithmic scale was considered but this led to populations of cells on the dot-plot being more diffuse and thus harder to distinguish between populations.

**Intracellular [K+] decreases during apoptosis, as indicated by two independent measures.** Monitoring [K+]\textsubscript{i} during apoptosis using the K\textsuperscript{+}-sensitive dye PBFI-AM showed a decrease in [K\textsuperscript{+}]\textsubscript{i} that correlated with two independent markers of apoptosis, namely reduction of Ψ\textsubscript{m} (Figure 4.8) and PS externalisation (Figure 4.9). Inhibition of caspase activation prevented the observed decrease in [K\textsuperscript{+}]\textsubscript{i}, in addition to preventing PS externalisation and cell shrinkage in both CD95- and chemical-mediated apoptosis. The inhibition of a decrease in [K\textsuperscript{+}]\textsubscript{i} in CD95-mediated apoptosis (Figure 4.10) was consistent with data presented in Figure 4.5, with complete inhibition of cell shrinkage and PS externalisation. Investigating the effect on [K\textsuperscript{+}]\textsubscript{i} of caspase-inhibition on etoposide-treated cells showed, however, that all apoptotic markers were inhibited (Figure 4.11), which was inconsistent with data presented in Figure 4.6 and previously published data (Sun et al., 1999). It was therefore concluded that the data showing that caspase inhibition prevented a decrease in [K\textsuperscript{+}]\textsubscript{i} in etoposide-treated cells was not reliable. Monitoring [K\textsuperscript{+}]\textsubscript{i} using PBFI-AM was unsatisfactory due to a number of difficulties in using the dye, as discussed previously (section 4.2.5). The K\textsuperscript{+} surrogate ion \textsuperscript{86}Rb\textsuperscript{+} was therefore used as a measure of intracellular K\textsuperscript{+} to corroborate and expand on the results obtained with PBFI-AM.

Induction of apoptosis in Jurkat cells elicited a time-dependent efflux of intracellular K\textsuperscript{+}, as assessed by \textsuperscript{86}Rb\textsuperscript{+}, that was concomitant with other markers of apoptosis including PS externalisation, cell shrinkage and reduction of Ψ\textsubscript{m} (Figure 4.12). The data did not support the recent suggestions that efflux of K\textsuperscript{+} is a very early event (Bortner and Cidlowski, 1996; Hughes et al., 1997; Maeno et al., 2000; Nietsch et al., 2000) nor a late-stage secondary event, but did indicate that K\textsuperscript{+} efflux contributes to the apoptotic phenotype. Efflux of intracellular K\textsuperscript{+} closely reflected cell shrinkage, suggesting they might be related, with water leaving the cell as an osmotic consequence of an efflux of K\textsuperscript{+} ions. The effect of inhibition of caspase activity by z-VAD.fmK on the efflux of intracellular K\textsuperscript{+} was consistent with the data for
plasma membrane depolarisation (Figure 4.5), demonstrating that CD95- but not chemical-mediated efflux of intracellular K⁺ was dependent upon caspase activity, further emphasising the different mechanisms for induction of apoptosis by these different stimuli (Figure 4.11).

Certain apoptotic markers are independent of caspase activation in chemical- but not CD95-mediated apoptosis. The finding that cell shrinkage, plasma membrane depolarisation and efflux of intracellular K⁺ were dependent upon caspase activation in anti-CD95 antibody- but not etoposide-induced apoptosis is in agreement with and further extends previous studies (Bortner and Cidlowski, 1999; Sun et al., 1999), and accentuates differences in execution of the apoptotic program induced by death receptors and chemical agents. The data are consistent with CD95-mediated apoptosis requiring the activation of caspase-8 at the DISC, which then activates the effector proteins of apoptosis in order to carry out apoptotic changes such as cell shrinkage, plasma and reduction of Ψₘ (see Figure 1.4). Caspase activation is likely a later event in etoposide-induced apoptosis, with more intermediate events between initial apoptotic insult and activation of caspases (see Figure 1.5). Following from this, several aspects of the apoptotic phenotype, such as plasma membrane depolarisation and efflux of intracellular K⁺, are upstream or independent of caspase activation. Some apoptotic events such as PS externalisation, however, are still dependent upon caspase activation.

One caveat to accompany the differences between CD95- and chemical-mediated apoptosis following z-VAD.fmk treatment is that different initiator caspases are involved in the two mechanisms of apoptosis, with caspase-8 being the apical caspase in CD95-mediated apoptosis (Medema et al., 1997; Muzio et al., 1997) and caspase-9 in chemical-mediated apoptosis (Sun et al., 1999). The proximity of caspase-8 to the plasma membrane may make it more susceptible to inhibition by z-VAD.fmk than the cytoplasmic caspase-9, thereby leading to complete inhibition of caspase-8 and subsequent apoptotic markers, but inefficient inhibition of caspase-9 leading to expression of some but not all apoptotic markers, giving the false impression that the markers not inhibited are caspase-independent.

As stated in Chapter One (section 1.10.2), investigation of the movement of Cl⁻ ions during apoptosis was considered outside the scope of this thesis. It is almost certain, however, that an efflux of intracellular Cl⁻ will accompany movement of K⁺ and water out of the cell
during apoptosis to preserve electroneutrality. Inhibition of CD95-mediated apoptosis by chloride channel blockers has been demonstrated, which implies the opening of chloride channels in response to CD95 stimulation (Szabó et al., 1998). Manipulating ionic Cl⁻ flux, however, might only affect the progression of apoptosis by the consequent effect on K⁺ flux, assuming Cl⁻ efflux is commensurate with K⁺ efflux to preserve electroneutrality.

Conclusions. The induction of both death receptor- and chemical-mediated apoptosis has been characterised in the Jurkat T cell line. Depolarisation of the plasma membrane has been demonstrated during apoptosis, which has recently been confirmed by Bortner et al. (2001). Also demonstrated was the time-dependent efflux of intracellular K⁺ during apoptosis. From investigations of the relationships between different aspects of the apoptotic program, several unresolved questions remain:

i) What is the function of plasma membrane depolarisation during apoptosis?

ii) How important is plasma membrane depolarisation in the apoptotic program? If depolarisation is inhibited will apoptosis still progress?

iii) The ordering of plasma membrane depolarisation in relation to other apoptotic events has not been firmly established.

The use of specific inhibitors of different apoptotic events, such as inhibition of mitochondrial perturbation by Bcl-2 overexpression, or specific pharmacological inhibitors of membrane depolarisation, might provide data as to the function and importance of plasma membrane depolarisation. The possible mechanism of plasma membrane depolarisation has not as yet been addressed. Since this depolarisation might feasibly be caused by an influx of cations or efflux of anions, the examination of Na⁺ ions during the course of apoptosis might yield interesting answers. Examination of ²²Na⁺ flux in a similar fashion to ⁸⁶Rb⁺ would elucidate how [Na⁺]ᵢ changes during apoptosis. However, the 1.3 MeV γ-emission energy and 2.6 year half-life of ²²Na would make experimentation difficult to perform safely. An alternative would be the use of the Na⁺-specific fluorochrome sodium-binding benzofuran isophthalate-AM (SBFI-AM), though this dye would suffer from many of the problems encountered with PBFI-AM. An alternative approach to monitoring the flux of ions would be to attempt to identify, examine and manipulate candidate ion channels that might mediate the flux of ions during apoptosis, a subject that will be addressed in Chapter Five.
4.4 SUMMARY

• Depolarisation of the plasma membrane occurred as part of the apoptotic phenotype in both death receptor- and chemical-mediated apoptosis.

• Plasma membrane depolarisation of apoptotic cells was synchronous with PS externalisation and cell shrinkage.

• Efflux of intracellular K⁺, as demonstrated by two independent techniques, occurred following induction of death receptor- or chemical-mediated apoptosis.

• Efflux of intracellular K⁺ was concomitant with cell shrinkage, reduction of Ψₘ and PS externalisation.

• Both plasma membrane depolarisation and efflux of intracellular K⁺ were dependent upon caspase-activation in CD95- but not etoposide-mediated apoptosis.
Chapter Five. Localisation of $K^+$ efflux following induction of apoptosis

5.1 INTRODUCTION

From the data in Chapter Four demonstrating depolarisation of the plasma membrane during apoptosis, several interesting questions were raised regarding ion fluxes during apoptosis that could not be answered by flow cytometry. These were: i) the identity of the ion channels that mediate the apoptotic efflux of intracellular $K^+$; ii) how the initiation of this efflux is signalled; iii) the factors that caused the differences observed between chemical and death-receptor mediated apoptosis, in particular caspase-dependency of $K^+$ efflux in anti-CD95 antibody- but not etoposide-induced apoptosis. An efflux of intracellular $K^+$ during apoptosis that was synchronous with PS externalisation, cell shrinkage and mitochondrial perturbation was demonstrated in Chapter Four. It was difficult to ascertain a precise order between apoptotic events by assessing these apoptotic markers directly by flow cytometry or $^{86}$Rb$^+$ efflux due to the lack of sensitivity of the analytical systems and the heterogeneous nature of the cell culture. It was therefore decided to investigate the questions outlined above by inhibition of the efflux of intracellular $K^+$ during apoptosis, thereby attempting to further discern the relative importance of $K^+$ efflux during apoptosis. Initiating efflux of intracellular $K^+$ in isolation might also clarify the importance of this event in the apoptotic program, clarifying whether it is a requisite or secondary event.

There have been several studies investigating pharmacological blockade of ion channels during apoptosis, but due to the wide range of different sub-types of $K^+$ channel protein, the ion channels involved in apoptosis have yet to be fully resolved (reviewed in Lang et al., 1998; Yu and Choi, 2000). The non-selective $K^+$ channel blockers DIDS$^1$, NPPB$^2$, phloretin and Ba$^{2+}$ block apoptosis induced by staurosporine (STS) and tumour necrosis factor-$\alpha$ (TNF-$\alpha$) in combination with cycloheximide (CHX) in four cell lines (Maeno et al., 2000). Tetrapentylammonium, which blocks $K^+$-selective plasma membrane channels and leads to collapse of the plasma membrane potential, prevents induction of apoptosis in thymocytes by the glucocorticoid dexamethasone (Dallaporta et al., 1999).

---

1 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid

2 5-nitro-2-(3-phenylpropylamino)-benzoate
UV-induced apoptosis, which is mediated by aggregation of CD95 receptors (Rehemtulla et al., 1997), is inhibited by the non-selective K⁺ channel blocker 4-aminopyridine (4-AP) in myeloblastic leukaemia cells (Wang et al., 1999), though 4-AP does not block etoposide-induced apoptosis in this cell line. Glyburide inhibits CD95-mediated apoptosis, apparently by inhibiting an open rectifying chloride channel (ORCC; Szabó et al., 1998), though this channel has not yet been cloned and no molecular details of its role in apoptosis are known. In addition to its inhibition of ORCC, glyburide also strongly inhibits K⁺-selective ion channels. Treatment of mice with quinine inhibits lipopolysaccharide-induced apoptosis (Gantner et al., 1995). In contrast, 4-AP induces apoptosis in hepatoblastoma cells by blockade of K⁺ channels, causing plasma membrane depolarisation and activation of voltage-sensitive Ca²⁺-permeable non-selective channels, which induces influx of Ca²⁺ and initiates the apoptotic program (Kim et al., 1999). Overall, studies using a wide variety of apoptotic stimuli, cell lines and K⁺ channel blockers suggest that inhibition of apoptosis by K⁺ channel block is specific to stimulus and cell line.

**Ion channels as caspase substrates.** A caspase-dependent efflux of intracellular K⁺ was demonstrated in CD95-mediated apoptosis in Chapter Four (section 4.2.4). Following on from this, a hypothesis could be made that K⁺ efflux resulted from caspase cleavage, with cleaved channels having a much higher conductance for K⁺. As described in Chapter One (section 1.11.1), many classes of K⁺ channel are gated by a 'ball and chain' mechanism (Hoshi et al., 1990). An N' or C'-terminal hydrophobic region followed by a hydrophilic region forms a ball that is linked to the membrane spanning domain by a hydrophilic chain. The channel is closed under resting conditions by the ball occupying the channel pore, thus preventing passage of K⁺ ions. Removal of the ball by caspase cleavage would result in a constitutively open channel that would mediate outward efflux of K⁺ ions. Experiments were designed to test the hypothesis that efflux of intracellular K⁺ during apoptosis was mediated by caspase cleavage of K⁺ ion channels.

**Identification of K⁺ channels potentially involved in apoptosis.** As detailed in Chapter One (section 1.11.1), there is a large variety of K⁺-selective channel proteins. It was therefore necessary to identify possible candidates for involvement in apoptosis before proceeding with experiments. Chapter Four demonstrated a close correlation between cell shrinkage and efflux of intracellular K⁺ (see section 4.2.5), with associated plasma
membrane depolarisation (see section 4.2.1). Kv1.3 is a key channel in volume regulatory decrease and has also been implicated in several apoptotic models (Szabó et al., 1997). Kv1.3 can therefore be considered a possible candidate for mediation of apoptotic flux of ions.

The involvement of Kir6.1x (K<sub>ATP</sub>) channels in apoptosis was investigated because these channels have a pivotal role in secretion and absorption of K<sup>+</sup> ions across the cell membrane (Ashcroft and Gribble, 1998). They also link cellular metabolism to K<sup>+</sup> fluxes and couple metabolic energy to the membrane potential of cells (Yan and Yan, 1997). Kir6.1 mRNA expression is ubiquitous in rat tissue (Erginel-Unaltuna et al., 1998) and Kir6.1 is localised primarily in the mitochondria of cells (Suzuki et al., 1997). Kir6.1 and Kir6.2 may be involved in apoptosis as they have been implicated as having a cytoprotective role in cardiac ischaemia (Akao et al., 1997). Kir6.2 dominant negative transgenic mice suffer from hypoglycemia and excessive apoptosis in pancreatic β cells (Miki et al. 1997; Seino et al., 2000). Although this does not indicate a vital role for Kir6.2 in apoptosis, both Kir6.1 and Kir6.2 are potential candidates for mediating the efflux of intracellular K<sup>+</sup> during apoptosis.

Role of mitochondrial ion channels in apoptosis. In addition to their role in the plasma membrane, ion channels regulate the function of many organelles. In particular, ion channels have been demonstrated to regulate matrix volume and respiration rate of mitochondria (Garlid, 1996; Holmuhamedov et al., 1998). Mitochondria play a pivotal role during apoptosis, as detailed in Chapter One (section 1.5). In addition to cytochrome c, whose release may form a commitment point of the cell to undergo apoptotic cell death (Kluck et al., 1997; Green and Reed, 1998), the intermembrane space of mitochondria contains proteins such as Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000), apoptosis inducing factor (AIF) (Susin et al., 1999b) and caspases -2, -3 and -9 (Krajewski et al., 1999; Zhivotovsky et al., 1999). Depolarisation of the inner mitochondrial membrane during apoptosis has been well documented (Zamzami et al., 1995), but it is not clear whether this is preliminary to mitochondrial membrane permeability transition and release of apoptotic intra-mitochondrial proteins.

Studies have described a functional role for the mitochondrial ATP-sensitive K<sup>+</sup> channel (mitoK<sub>ATP</sub>) as regulating the osmotic status of the mitochondrial matrix in addition
to respiration and ATP generation (Garlid, 1996; Garlid, 1998). However, sustained opening of mitoK\textsubscript{ATP} leads to increased matrix volume and swelling in isolated mitochondria, leading in turn to a change in permeability of the outer membrane to molecules resident in the intermembrane space (Holmuhamedov et al., 1998). From 'resting' mitochondrial transmembrane membrane potentials ($\Psi_m$) of $-180 \pm 15\ mV$ in isolated mitochondrial preparations, Holmuhamedov et al. (1998) demonstrated that the mitoK\textsubscript{ATP} openers cromakalim and pinacidil induce reduction of $\Psi_m$. This is associated with increases in the mitochondrial respiration rate and mitochondrial matrix volume, a decrease in the rate of ATP synthesis and release of the intermembrane proteins adenylate kinase and cytochrome c.

The most likely mechanism by which $\Psi_m$ is reduced during apoptosis is the influx of either H$^+$ or K$^+$ ions from the cytoplasm. Since mitochondrial volume is highly dependent upon ion content of the matrix, influx of K$^+$ would lead to swelling. Swelling of mitochondria during apoptosis has been observed in some studies (Susin et al., 1997; Vander Heiden et al., 1997; Dallaporta et al., 1999), though not others (Bossy-Wetzel et al., 1998). The possibility was considered that sustained opening of mitoK\textsubscript{ATP} could account for reduction of $\Psi_m$ and release of proteins from the intermembrane space during apoptosis.

A hypothetical model for the release of pro-apoptotic proteins from mitochondria is by rupture of the outer membrane (Figure 5.1). A pro-apoptotic signal originating from the cytoplasm or plasma membrane triggers the opening of the mitoK\textsubscript{ATP} channel in the inner mitochondrial membrane (Figure 5.1A), possibly by phosphorylation of a subunit of mitoK\textsubscript{ATP}. K$^+$ ions flow down the electrochemical gradient from the cytoplasm into the mitochondrial matrix with accompanying movement of water molecules by osmosis (Figure 5.1B). This influx of water causes swelling. Due to the complex invagination of the inner mitochondrial membrane, the inner mitochondrial membrane can expand to a greater extent that the outer membrane. The swelling of the mitochondrion results in rupture of the outer membrane, releasing the contents of the intermembrane space into the cytoplasm (Figure 5.1C), where cytochrome c interacts with Apaf-1 and caspase-9 to form the caspase-activating apoptosome complex (Li et al., 1997), and the release of AIF leads to nuclear changes associated with apoptosis.
Figure 5.1: Hypothetical release of mitochondrial proteins by rupture of the outer mitochondrial membrane. Gating of mitoK$_{ATP}$ embedded in the inner mitochondrial membrane by phosphorylation (A) results in an influx of K$^+$ ions, with associated influx of water (B). This leads to swelling of the matrix and rupture of the outer membrane, releasing the intermembrane pro-apoptotic proteins (C).
Functional $K_{\text{ATP}}$ channels consist of a heteromultimer of sulphonylurea receptors (SUR), a member of the ATP-binding cassette superfamily (reviewed in Higgins, 1995) and either $K_{\text{IR}}6.1$ or $K_{\text{IR}}6.2$ (reviewed in Babenko et al., 1998). These form a heteromultimer of $(\text{SUR}/K_{\text{IR}}6.x)_4$. The $K_{\text{IR}}6.x$ are the pore-forming subunits, while the SURs act as regulators of $K_{\text{ATP}}$ activity and also confer sensitivity to pharmacological agents such as sulphonylureas and potassium channel openers (KCOs). The various subtypes of SURs and $K_{\text{IR}}6.x$ and different molar ratios of $K_{\text{IR}}6.1$ and $K_{\text{IR}}6.2$ in different tissues result in a wide diversity of $K_{\text{ATP}}$ channel types. The structure of the mito$K_{\text{ATP}}$ channel is currently unknown, but is thought to consist of a heterotetramer of $K_{\text{IR}}6.1$ and/or $K_{\text{IR}}6.2$ and SUR2A/SUR2B (Garlid et al., 1996; Babenko et al., 1998). A mito$K_{\text{ATP}}$ channel consisting of $K_{\text{IR}}6.1$/SUR2B appears most likely from the literature. mito$K_{\text{ATP}}$ has been shown as a target for diazoxide but since SUR2B and not SUR2S subunits are activated by diazoxide (Garlid et al., 1996), it would appear that mito$K_{\text{ATP}}$ contains SUR2B. Considering the evidence discussed above, the mito$K_{\text{ATP}}$ channel is a good candidate for mediating mitochondrial flux of $K^+$ during apoptosis.

The aims of this chapter were:

i) to investigate the consequence of pharmacological ion channel block on death-receptor and chemical-mediated apoptosis. By using a panel of pharmacological ion channel blockers with different target specificities it might be possible to identify the class or classes of $K^+$ ion channel involved in apoptosis.

ii) to investigate whether intracellular $K^+$ efflux is mediated by caspase cleavage of ion channels.

iii) the involvement of $K^+$ flux in the reduction of $\Psi_m$ was investigated by confocal microscopic examination of mitochondrial $K^+$ concentration in apoptotic and normal cells.

iv) to examine whether the gating of mitochondrial $K^+$ channels is involved in the initiation of apoptotic signalling by expression of constitutively open ion channels targeted to the inner mitochondrial membrane.

5.2 Results

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in CEM-C7H2 lymphoblastic cells to analyse which ion channel mRNAs were expressed and thus identify which $K^+$ ion channels were present in this cell line. This would help
eliminate or indicate potential candidate ion channels for a role in apoptosis. Data obtained were poor, however, and no firm conclusions could be made (data not shown).

Sensitivity of Jurkat cells to potassium channel openers (KCOs). Following on from experiments investigating the effect of KCOs on CGN cultures in Chapter Three (section 3.2.4), the response of Jurkat cells to the KCO diazoxide was investigated to examine whether the efflux of intracellular K\(^+\) in isolation would induce apoptosis and whether cells treated with diazoxide would be more susceptible to apoptotic stimuli. No induction of apoptosis by diazoxide treatment alone was observed, even at a very high concentration (500 \(\mu\)M, compared to 100 \(\mu\)M used in Fujimura et al., 1997; data not shown). Potentiation of anti-CD95 antibody-induced apoptosis by diazoxide was investigated but none was observed (data not shown). This lack of KCO toxicity contrasted with previous findings (Holmuhamedov et al., 1998), where the KCOs cromakalim and pinacidil induced release of cytochrome c from isolated mitochondria. However, this study investigated the effect of KCOs on whole cells rather than isolated mitochondria.

5.2.1 Effect of K\(^+\) ion channel block on apoptosis

To further identify candidate ion channels that are involved in apoptosis, the effect of a number of K\(^+\) channel blockers on the induction of CD95- and chemical-mediated apoptosis was investigated. These were 4-aminopyridine (4-AP), glyburide (also called glibenclamide) and rMargatoxin (rMgTX) (Figure 5.2). Although K\(^+\) ion channels subtypes differ in their sensitivity to 4-AP, this compound cannot be considered a specific blocker of any particular sub-type of K\(^+\) channel. Glyburide is also relatively non-selective and blocks certain classes of chloride channel in addition to K\(^+\) channels, though its major effect is the inhibition of K\(_{\text{ATP}}\) channels (Sturgess et al., 1988), which results in plasma membrane depolarisation and influx of Ca\(^{2+}\). In contrast, rMgTX is a recombinant peptide originally purified from the venom of the scorpion Centruroides margaritatus (Garcia-Calvo et al., 1993) that selectively blocks Kv1.3 (Leonard et al., 1992), probably by a simple plugging of the channel pore. Cells were also treated with ouabain, an inhibitor of the Na\(^+\),K\(^+\)-ATPase antiporter, in conjunction with the apoptotic stimuli anti-CD95 antibody or etoposide. Dose-response experiments were performed for 4-AP and glyburide to determine the induction of cell death by these compounds in isolation (data not shown). For experiments in conjunction with anti-CD95 antibody or etoposide treatment, the highest concentrations of 4-AP or glyburide were selected that did not induce cell death.
over the time investigated (5 h). As rMgTX was not toxic at all concentrations tested (1 to 25 nM), the highest concentration was calculated so that the stock would be sufficient for all the experiments planned. This concentration used (25 nM) was five-fold above that shown to be effective by Leonard et al. (1992). Since it was not possible to assess directly the efficacy of the compounds used in blocking K$^+$ channels their action on cells was not confirmed. There is, however, a substantial literature on the efficacy of these compounds in blocking K$^+$ channels and assay data existed for each if the compounds from the respective manufacturers.

Figure 5.2: Chemical structures of the pharmacological agents used and amino acid sequence of recombinant Margatoxin (rMgTX).
Co-treatment of anti-CD95 antibody-stimulated Jurkat cells with 4-AP, rMgTX or ouabain had no effect on the induction of apoptosis (Figure 5.3). Glyburide potentiated anti-CD95 antibody-induced apoptosis, however, with apoptosis increased from 23.4 % to 32.5 %. 4-AP and rMgTX had no effect on etoposide-induced apoptosis, but co-treatment with glyburide or ouabain inhibited apoptosis, from 34.8 % to 22.9 % and 16.0 % respectively. Cells treated with the pharmacological agents alone did not exhibit induction of apoptosis above untreated cells (<4.5 %).

![Figure 5.3: Etoposide-induced apoptosis is inhibited by co-treatment with glyburide or ouabain.](image)

Figure 5.3: Etoposide-induced apoptosis is inhibited by co-treatment with glyburide or ouabain. Jurkat T cells were pre-treated for 15 min with vehicle (DMSO), 4-AP (100 µM), glyburide (100 µM), rMgTX (25 nM) or ouabain (1 µM) and then treated with vehicle (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 µM). After incubation for 5 h, apoptosis was assessed by PS externalisation. Data are mean and S.E.M. of 4 independent experiments.

A possible mechanism by which K⁺ channel blockers modulate apoptosis is by the prevention of intracellular K⁺ efflux (Dallaporta et al., 1999; Maeno et al., 2000). In order
to test this possibility, treated cultures were assessed for cell shrinkage in parallel with PS externalisation. In control cultures, 4-AP, glyburide and rMgTX did not cause any change in cell size (Figure 5.4). Ouabain caused cell shrinkage in 33.9 % of cells, as cytoplasmic [Na⁺] and [K⁺] would start equilibrating with the medium without functional Na⁺/K⁺ exchange, and thus water would follow efflux of K⁺ by osmosis. Induction of apoptosis by anti-CD95 antibody or etoposide caused cell shrinkage. Addition of 4-AP, rMgTX or, surprisingly, ouabain to anti-CD95 antibody-treated cells had no effect on apoptotic cell shrinkage, but glyburide potentiated CD95-mediated cell shrinkage. In etoposide-treated cells, addition of 4-AP, glyburide or rMgTX caused no change in the percentage of shrunken cells. Despite causing a decrease in cells with PS-externalisation, addition of ouabain to etoposide-treated cells resulted in a high proportion of cells with shrunken phenotype (24.2 % shrunken cells with etoposide alone vs. 47.9 % shrunken in cultures treated with both etoposide and ouabain). This suggested that inhibition of etoposide-induced apoptosis by ouabain was not by the prevention of cell shrinkage.

Figure 5.4: Inhibition or potentiation of apoptosis by pharmacological agents does not correlate with cell shrinkage. Jurkat T cells were pre-treated for 15 min with vehicle (DMSO), 4-AP (100 μM), glyburide (100 μM), rMgTX (25 nM) or ouabain (1 μM) and then treated with vehicle, anti-CD95 antibody (50 ng/ml) or etoposide (50 μM). Cell shrinkage was assessed after 5 h. Data are mean and S.E.M. of 4 independent experiments.
The inhibition of etoposide-induced apoptosis by glyburide is indicative of an important role for the efflux of intracellular $K^+$ during chemical-mediated apoptosis. However, the potentiation of anti-CD95 antibody-induced apoptosis is hard to explain. One possibility is that inhibition of $K^+$ channels by glyburide potentiated CD95-mediated inhibition of $K^+$ channels (Szabó et al., 1996), thereby potentiating the induction of apoptosis.

5.2.2 Sensitivity of $\text{K}_{\text{IR}6.x}$ ion channels to caspase digestion

A multitude of proteins is cleaved by effector caspases during the execution phase of apoptosis, as detailed in Chapter One (section 1.4.1). A possible explanation for the caspase-dependent efflux of $K^+$ during CD95-mediated apoptosis demonstrated in section 4.2.6 is that caspases directly cleave ion channels in the plasma membrane, causing an increase in $K^+$ conductance and a subsequent outward movement of $K^+$ ions. Alternatively, the gating mechanisms (such as channel protein phosphorylation) for $K^+$ efflux may be independent of caspase activation.

As described previously (section 5.1), $\text{K}_{\text{IR}6.1}$ and $\text{K}_{\text{IR}6.2}$ are potential candidates for mediating efflux of intracellular $K^+$ during apoptosis. Since antibodies were not available to $\text{K}_{\text{IR}6.1}$ or $\text{K}_{\text{IR}6.2}$, analysis of *in vivo* protein cleavage by Western blotting of protein extracts from apoptotic cells was not possible. Instead, $^{35}$S radiolabelled peptides were expressed using a reticulate lysate system. Cleavage of these peptides was then investigated by incubation with recombinant caspase-3 or -8. The amino-acid sequences of both $\text{K}_{\text{IR}6.1}$ and $\text{K}_{\text{IR}6.2}$ were analysed for caspase cleavage motifs. The IXXD substrate motif selected is a degenerate motif common to many caspase substrates (Thornberry et al., 1997). $\text{K}_{\text{IR}6.1}$ contains an IXXD motif at residues 248 to 251 and $\text{K}_{\text{IR}6.2}$ at residues 384 to 387 (Figure 5.5). Since cleavage at the IXXD sequences for both $\text{K}_{\text{IR}6.1}$ and $\text{K}_{\text{IR}6.2}$ would result in the removal or disruption of the channel-closing ‘ball’ without affecting transmembrane regions, cleavage at this site is likely to result in a constitutively open channel. Radiolabelled $\text{K}_{\text{IR}2.3}$ was used as a positive control, since this channel is unlikely to be involved in apoptosis, having a physiological role in controlling the excitability of cardiac and neuronal cell-types (Kubo et al., 1993). However, analysis of the $\text{K}_{\text{IR}2.3}$ sequence revealed two possible IXXD cleavage sites. From these putative caspase cleavage sites, the sizes of cleavage products were predicted for $\text{K}_{\text{IR}2.3}$, $\text{K}_{\text{IR}6.1}$ and $\text{K}_{\text{IR}6.2}$ (Table 5.1).
Figure 5.5: Representation of $K_{IR2.3}$, $K_{IR6.1}$ and $K_{IR6.2}$ expressed in reticulate lysate. Hydrophobic transmembrane regions M1 and M2 are shown in yellow. Caspase digestion motifs (IXXD) are present at amino acids 247 - 250 and 429 - 432 (out of 445 residues) for r$K_{IR2.3}$, 248 - 251 (out of 424 residues) for r$K_{IR6.1}$ and residues 384 - 387 (out of 391 amino acids) in m$K_{IR6.2}$.

No cleavage by caspase-8 of the $K_{IR2.3}$, $K_{IR6.1}$ or $K_{IR6.2}$ labelled proteins was observed (Figure 5.6), which demonstrated that either the ion channel proteins tested were not substrates of caspase-8 or that the caspase-8 protein used was enzymically inactive. While incubation with concentrated caspase-3 did yield cleavage products for $K_{IR2.3}$, $K_{IR6.1}$ and $K_{IR6.2}$, more dilute (1:10 and 1:100) caspase-3 did not yield any cleavage products. There was some discrepancy between predicted full-length peptide sizes and the expressed peptides, with the $K_{IR2.3}$ peptide larger than predicted and $K_{IR6.1}$ smaller (Table 5.1). This may have been due to errors in the translation and transcription of cDNA or possibly the apparent size of peptides on the acrylamide gel may have been affected by post-translational modifications of the peptides. Comparisons of the size of peptide cleavage products against the predicted values also showed little correlation. While $K_{IR2.3}$ fragments were close to the predicted sizes (29 and 19 kDa compared to predicted 27.9 and 20.1 kDa fragments), neither $K_{IR6.1}$ nor $K_{IR6.2}$ digestions showed correlation with the predicted fragment sizes.

120
Figure 5.6: Candidate ion channels are not substrates of dilute recombinant caspase-3 or -8. $^{35}$S-labelled $K_{IR2.3}$, $K_{IR6.1}$ or $K_{IR6.2}$ were incubated for 1 h at 37°C with vehicle (assay buffer) or a range of concentrations of recombinant caspase-3 or -8. Proteins were separated by SDS-PAGE and imaged by autoradiography. The position of a SeeBlue protein standard ladder (Invitrogen) is shown on the left of each autoradiograph.
### Table 5.1: Predicted and observed sizes of expressed peptides and caspase cleavage products.

Fragment sizes were predicted by analysis of peptide sequences using MacVector software. Observed sizes were measured from Figure 5.6.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acids</th>
<th>Predicted peptide size (kDa)</th>
<th>Observed size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsubscript{IR}2.3</td>
<td>ORF\textsuperscript{3}: 445</td>
<td>49.9</td>
<td>~58</td>
</tr>
<tr>
<td></td>
<td>Fragments: 247</td>
<td>27.9</td>
<td>~34</td>
</tr>
<tr>
<td></td>
<td>182</td>
<td>20.1</td>
<td>~29</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.2</td>
<td>~19</td>
</tr>
<tr>
<td>K\textsubscript{IR}6.1</td>
<td>ORF: 424</td>
<td>47.9</td>
<td>~36</td>
</tr>
<tr>
<td></td>
<td>Fragments: 248</td>
<td>28.1</td>
<td>~32</td>
</tr>
<tr>
<td></td>
<td>177</td>
<td>19.9</td>
<td>~17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.6</td>
<td>~10</td>
</tr>
<tr>
<td>K\textsubscript{IR}6.2</td>
<td>ORF: 391</td>
<td>43.6</td>
<td>~43</td>
</tr>
<tr>
<td></td>
<td>Fragments: 388</td>
<td>43</td>
<td>~35</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.6</td>
<td>~29</td>
</tr>
</tbody>
</table>

Considering all the data together, it appeared that the ion channels investigated were not substrates of caspases -3 or -8 \textit{in vitro}. While cleavage may have occurred at IXXD motifs, there was clearly cleavage of the peptides at other sequences, as more fragments than predicted were observed. Due to the lack of site preference to give the predicted fragments by cleavage at IXXD sites, it is concluded that apoptotic efflux of intracellular K\textsuperscript{+} is not mediated by the caspase cleavage of K\textsubscript{IR}6.1 or K\textsubscript{IR}6.2 ion channels.

### 5.2.3 Possible changes in mitochondrial matrix K\textsuperscript{+} concentration during apoptosis

Mitochondria in quiescent cells are hyperpolarised relative to the cytoplasm (~180 mV across the inner mitochondrial membrane compared with ~70 mV for the plasma membrane), thus forming the theoretical basis for the measurement of $\Psi_m$ using cationic dyes such as TMRE and DiOC\textsubscript{6}(3). The factors that mediate reduction of $\Psi_m$ during apoptosis are currently unknown, but a strong candidate is K\textsuperscript{+} ions moving down an electrochemical gradient from the cytoplasm into the mitochondrial matrix. To investigate

\textsuperscript{3} 'open reading frame' of cDNA expressed in reticulate lysate system
whether flux of $K^+$ into the mitochondrial matrix mediated reduction of $\Psi_m$ during apoptosis, the subcellular distribution of the $K^+$-indicator PBFI-AM was monitored by confocal microscopy in CEM C7H2 cells treated with anti-CD95 antibody.

Examination of control cells stained with PBFI-AM showed a relatively uniform distribution, with what appeared to be the nucleus stained more intensely (Figure 5.7A, green cell). In contrast, cells treated with anti-CD95 antibody for 3 h showed a punctate dye distribution (Figure 5.7, B to D, green cells). This punctate pattern was consistent in size and distribution with mitochondria. Simultaneous staining of cells with the mitochondrial marker Mitotracker Orange revealed a punctate distribution within both control and anti-CD95 antibody-treated cells (Figure 5.7, A to D, red cells). The similar distributions of PBFI-AM and Mitotracker in cells treated with anti-CD95 antibody led to the conclusion that the two fluorochromes were staining the same cellular structures and these were consistent with being mitochondria (Figures 5.7, B to D, compare red and green cells).
Figure 5.7: The mitochondrial [K⁺] of anti-CD95 antibody-treated cells is increased relative to the cytoplasm. CEM cells were incubated with vehicle (PBS; A) or anti-CD95 antibody (50 ng/ml; B-D) for 3 h and then stained with Mitotracker (red) and PBFI-AM (green). Cells were visualised by confocal microscopy. Images are taken from 2 independent experiments.
Alternative explanations for the accumulation of PBFI-AM in mitochondria were considered. Although the peak emission wavelengths of PBFI-AM and Mitotracker Orange are very close (557 nm and 551 nm respectively), the excitation spectra of the two dyes are different (369 nm and 576 nm for PBFI-AM and Mitotracker Orange, respectively). Since the two dyes are excited by the different lasers (PBFI-AM by the UV laser, Mitotracker Orange by the argon-krypton laser), emitted light would have been detected separately by the confocal microscope. It was therefore discounted that coincidence of dye fluorescence was an artefact resulting from proximity of their emission spectra. AM dyes have been reported to spuriously accumulate in membrane-bound organelles, since cleavage of AM groups from fluorochromes results in a cationic moiety. This may accumulate in hyperpolarised membrane-bound organelles such as the mitochondria. Control cells, however, did not display the coincident staining of Mitotracker Orange and PBFI-AM (Figure 5.7A) that was seen in anti-CD95 antibody-treated cells (Figure 5.7, B to D). The possibility that PBFI-AM might bind to intracellular proteins and cause the staining pattern observed was also discounted because control patterns were different from those observed in anti-CD95 antibody-stimulated cells. Discrepancies in staining patterns between Mitotracker and PBFI-AM fluorescence argues against dye interference artefacts, such as the dyes binding to one another or overlap of spectral emissions. The possibility could not be discounted, however, that the punctate staining was a result of poor plasma membrane integrity and leaching of PBFI-AM out of the cytoplasm but remaining in the mitochondria.

In conclusion, a small proportion of anti-CD95 antibody-stimulated cells displayed a changed $K^+$ distribution relative to control cells that was consistent with increased mitochondrial $[K^+]$ relative to the cytoplasm. Increased mitochondrial $K^+$ concentration was not observed in control cells. This pattern of staining was only see in minority of anti-CD95 antibody treated-cells, which suggested that this only occurred in a small proportion of cells undergoing apoptosis, or that elevation of mitochondrial $K^+$ in apoptotic cells was transient.

5.2.4 Expression of mitochondrially-targeted constitutively open $K_{ir}6.x$ channels

From the confocal data showing elevated $[K^+]$ in the mitochondria of cells stimulated with anti-CD95 antibody and from the demonstration that opening of mito$K_{ATP}$ in isolated mitochondria led to release of cytochrome c (Holmuhamedov et al., 1998), the
mitoK\textsubscript{ATP} channel was identified as a likely candidate for involvement in apoptosis. The consequence of effecting mitochondrial K\textsuperscript{+} flux similar to that described (Holmuhamedov et al., 1998) was investigated by creating mutated K\textsubscript{IR}6.1 and K\textsubscript{IR}6.2 ion channels with C-terminal truncations. Transgenic mice with a dominant negative K\textsubscript{IR}6.2 channel (K\textsubscript{IR}6.2G132S) have been shown to have high frequency of apoptotic β-cells in the pancreas (Miki et al., 1997), which is supportive of a role for K\textsubscript{IR}6.2 in cell survival. As K\textsubscript{IR}6.1 and K\textsubscript{IR}6.2 are the pore-forming subunits of K\textsubscript{ATP} channels and because it is not known which are present in mitoK\textsubscript{ATP} channels, truncation mutations were created for both K\textsubscript{IR}6.1 and K\textsubscript{IR}6.2. Ion channel mutants were synthesised with C-terminal truncations to remove the channel-closing ‘ball’ (Figure 5.8). The truncation mutations could therefore be considered constitutively open. While transfection of cells with wild-type K\textsubscript{IR}6.2 requires co-transfection and expression of associated SUR subunits for functional channel formation and pore opening, the K\textsubscript{IR}6.2\Delta C36 truncation mutant does not. (Tucker et al., 1997).

---

**Figure 5.8: Design of truncation mutations of K\textsubscript{IR}6.x ion channels.** A mitochondrial targeting sequence (MTS) and green fluorescent protein (GFP) were added to the N' terminus. Truncation of both K\textsubscript{IR}6.1 and K\textsubscript{IR}6.2 was at the C' terminus, removing the channel-closing ‘ball’.
Constructs were N-terminally tagged with GFP (Figure 5.8) to demonstrate transfection of cells, to show the subcellular localisation of the expressed protein by confocal studies and to allow for flow cytometric analysis. A mitochondrial targeting sequence (MTS) was added to insert the expressed channel into the inner mitochondrial membrane. The constructs, Kir6.1ΔC61/GFP/MTS and Kir6.2ΔKpnIΔC36/GFP/MTS (Figure 5.9) were created by PCR mutagenesis as detailed in Methods (section 2.13.8). Sequencing of constructs revealed that Kir6.1ΔC61/GFP/MTS had two amino acid changes from the predicted sequence at proline 25 to leucine and proline 276 to serine, but these changes were considered unlikely to cause any change in the expressed peptide. The Kir6.2ΔKpnIΔC36/GFP/MTS sequence was as predicted. Constructs were transfected into Chinese hamster ovary (CHO) cells and expression examined by fluorescence microscopy and flow cytometry. Intriguingly, no GFP expression was seen in cells transfected with the truncation mutations, though the empty vector was highly expressed (37% of cells positive for mitochondrially-targeted GFP).

The possibility was considered that, since the sequence of cDNAs was essentially as predicted and DNA was not degraded (as demonstrated by compact bands of the correct size on agarose gels), expression of the truncated ion channels was lethal to cells. Western blots of cells transfected with Kir6.1ΔC61/GFP/MTS probed for GFP showed a very faint band (Figure 5.10), although this band was not the correct size for the entire construct (~34 kDa compared with the predicted size of ~82 kDa). The band was of a similar size to GFP

![Figure 5.9: Representation of Kir6.x truncation mutations. Kir6.1 was truncated by 61 amino acids by introduction of a stop codon at I363. Kir6.2 was truncated by 36 amino acids by introduction of a stop codon at L355.](image-url)
alone, suggesting possible breakage of the translated construct or incomplete translation. These data suggested, however, that cells were expressing the construct at a very low level, which supports the hypothesis that transfection was lethal.

To further investigate whether transfection of the truncation mutations was lethal to CHO cells, transfections were performed with \( \text{K}_{\text{IR}6.1}\text{ΔC61/GFP/MTS} \) or \( \text{K}_{\text{IR}6.2}\text{ΔKpnIΔC36/GFP/MTS} \) in combination with an empty eGFP-expressing vector. Transfected cells were then examined by flow cytometry to quantify GFP-expressing cells. If expression of the truncations was lethal, co-transfected cultures would be predicted to contain very few GFP-transfected cells compared to a culture transfected with GFP alone. Transfection with eGFP alone using 1 \( \mu \)g of cDNA gave \( \sim 20\% \) GFP-positive cells. To maintain similar transfection conditions, 0.5 \( \mu \)g each of eGFP and \( \text{K}_{\text{IR}6.x} \) truncation cDNA were used to give a total of 1 \( \mu \)g DNA. Co-transfection resulted in \( \sim 10\% \) GFP-positive cells for both \( \text{K}_{\text{IR}6.1} \) and \( \text{K}_{\text{IR}6.2} \). This decrease in GFP-expression was almost certainly due to the decreased quantity of eGFP DNA used, rather than induction of cell death by \( \text{K}_{\text{IR}6.x} \) transfection. Combined transfections of eGFP and wild-type \( \text{K}_{\text{IR}6.x} \) were also performed. This yielded fewer GFP-positive cells than the co-transfection of eGFP with the \( \text{K}_{\text{IR}6.x} \) truncations (\( \sim 7\% \)), which was likely due to competition for cDNA expression, if it is assumed there was expression of wild-type channels but no expression of truncations.

![Figure 5.10: Western blot of CHO cell pellets 36 h after transfection with eGFP or \( \text{K}_{\text{IR}6.1}\text{ΔC61} \), probed for GFP.](image)
Transfections with the truncated ion channels were also performed in the presence of the broad-spectrum caspase inhibitor z-VAD.fmk. If the constructs did induce apoptosis upon expression, caspase inhibition might delay or inhibit cell death sufficiently for visualisation of expressed GFP. However, no GFP expression was observed under fluorescence microscopy in this instance. To examine whether the cDNA sequences and primers were intact, the truncation mutation cDNAs were expressed in a reticulate lysate system and radiolabelled with $^{35}$S-methionine. Both $K_{ir}6.1\Delta C61$ and $K_{ir}6.2\Delta KpnI\Delta C36$ produced peptides of roughly the correct size (~34 kDa compared to a predicted size of 41.3 kDa for $K_{ir}6.1\Delta C61$ and ~33 kDa compared to a predicted size of 40.0 kDa for $K_{ir}6.2\Delta KpnI\Delta C36$), demonstrating that the DNA was not significantly degraded and that there were no base changes in the sequences that would prevent translation.

In summary, investigations into the consequences of effecting mitochondrial depolarisation and mitochondrial $K^+$ influx were not successful because transfection of CHO cells with the $K_{ir}6.x$ truncations led to no observable changes. This may have been due either to unsuccessful construction of the truncations or defective expression of the GFP marker protein.

5.3 DISCUSSION

Sensitivity of apoptosis to pharmacological modulation of $K^+$ channels. Induction of apoptosis by etoposide in Jurkat cells was inhibited by the non-selective $K^+$ channel blocker glyburide and the Na$^+$,K$^+$-ATPase inhibitor ouabain (Figure 5.3). This inhibition might have been achieved by i) preventing the efflux of intracellular $K^+$ ii) depolarisation of plasma membrane iii) preventing the normal cell volume changes associated with apoptosis. Cell shrinkage and efflux of intracellular $K^+$ are part of the apoptotic phenotype and pharmacological block of $K^+$ channels might prevent either or both of these. Blockade or inhibition of open $K^+$-channels would be predicted to result in membrane depolarisation due to a reduction in the ‘hyperpolarising’ effect that basal $K^+$-conductances exert on the resting membrane potential. Decreasing $K^+$ conductance will thus reduce this hyperpolarising effect. The inhibition of etoposide-induced apoptosis by ouabain was difficult to interpret. Inhibition of the Na$^+$, K$^+$-ATPase pump would lead to equilibration of cytoplasmic concentrations of $K^+$ and Na$^+$ with the culture medium, resulting in efflux of intracellular $K^+$ and influx of Na$^+$. The plasma membrane would also be depolarised by this equilibration of the internal and external environments. Assessment of cell shrinkage
showed that cells treated with ouabain had undergone cell shrinkage in the control and etoposide-treated cultures (Figure 5.4), demonstrating that inhibition of apoptosis by ouabain was not by the prevention of cell shrinkage. It was not possible, however, to resolve whether glyburide inhibited apoptosis by prevention of intracellular K⁺ efflux or depolarisation of the plasma membrane.

The induction of apoptosis by anti-CD95 antibody was potentiated by glyburide treatment. CD95-mediated apoptosis might be less susceptible to inhibition by the prevention of K⁺ efflux since caspase activation is upstream of K⁺ efflux (as demonstrated in section 4.2.6), whereas efflux of intracellular K⁺ during etoposide-induced apoptosis is prior to (or independent of) caspase activation. Since caspase activation is a commitment point in the apoptotic pathways, the pharmacological block of K⁺-selective ion channels might only inhibit apoptosis prior to this commitment by caspase activation. The finding that CD95-mediated apoptosis was unaffected by ouabain-treatment was in disagreement with subsequent studies that demonstrate potentiation of CD95-mediated cell shrinkage and apoptosis by ouabain (Nobel et al., 2000; Bortner et al., 2001).

It was surprising that rMgTX, a specific blocker of Kv1.3, had no effect on the induction of apoptosis or apoptotic cell shrinkage. This suggested that efflux of intracellular K⁺ was mediated by a mechanism other than regulatory volume decrease mechanisms. Investigating the effects of more ion channel blockers on apoptosis would have been interesting. Agents such as those used in Maeno et al. (2000) (DIDS, NPPB, phloretin and Ba²⁺) or a greater range of peptide blockers are available but it was not economically feasible to extend the range of K⁺ channel modulators used. Many Na⁺, Cl⁻ or Ca²⁺ channel blockers are also available but these were considered outside the scope of this study.

*Kir6.x channels are not caspase substrates.* Although caspase-3 did cleave the candidate ion channels Kir6.1 and Kir6.2, there was little correlation between predicted fragment sizes for cleavage at caspase motifs and the fragments observed. Caspase-3 also cleaved Kir2.3, a potassium channel primarily involved in regulating the excitability of the heart and brain (Kubo et al., 1993) and it is therefore unlikely to play a role in apoptotic K⁺ flux. These data demonstrate the substrate promiscuity of caspase-3. While it was difficult to compare the concentration of recombinant caspases used with physiological
concentrations, the concentration of caspase-3 that induced cleavage was almost certainly far greater than that found in vivo. It can therefore be concluded that efflux of intracellular K⁺ during apoptosis does not occur as a result of cleavage of KᵢR6.1 or KᵢR6.2. In support of this, Chapter Four (section 4.2.6) demonstrated that, although efflux of intracellular K⁺ in CD95-mediated apoptosis was dependent upon initiator caspase activation, chemical-mediated K⁺ efflux was not. Assuming that efflux of intracellular K⁺ occurs by a similar mechanism for both apoptotic stimuli, K⁺ efflux was not mediated by the caspase digestion of ion channels. Nobel et al. (2000) considered the possibility that Na⁺/K⁺-ATPase is a caspase substrate, but demonstrated that apoptotic inhibition of Na⁺/K⁺-ATPase function is reversible in vitro and the protein does not appear to be cleaved during apoptosis.

**Apparent elevation of mitochondrial K⁺ concentration during apoptosis.** Using a death receptor model of apoptosis in a T cell line, a marked redistribution of cellular K⁺ in apoptotic cells was observed. The K⁺-specific fluorochrome PBFI-AM co-localised with the mitochondrial marker Mitotracker Orange. Increased mitochondrial PBFI-AM fluorescence might have become visible because cytoplasmic K⁺ concentration decreased during the progression of apoptosis. Alternatively, this decrease in cytoplasmic [K⁺] might have been caused by a loss of plasma membrane integrity. A simple way to check this would be co-incubation of cells with PI as a marker of membrane integrity. Despite these caveats, the data suggest that, following apoptotic stimulation, cytosolic K⁺ moved into the mitochondria. At the time of observation, the cells did not show any obvious morphological characteristics of apoptosis such as blebbing or chromatin condensation, suggesting that increased mitochondrial [K⁺] was a relatively early event. Raised potassium concentration in the mitochondria relative to the cytoplasm of apoptotic cells is consistent with transient gating of mitoKᵦT channels that permit regulated K⁺ flux into the mitochondria across the inner membrane. These results suggest that metabotropic opening of mitoKᵦT located in the inner mitochondrial membrane may be an event intrinsic to the apoptotic pathway. The influx of K⁺ into the mitochondrial matrix thus depolarising the inner membrane may be a possible cause of the reduction of Ψᵦ, seen during apoptosis that leads to mitochondrial permeability transition.

**Insertion of constitutively open K⁺-selective channels into mitochondria.** Evidence was examined for the correct construction of the KᵢR6.2ΔKpnIΔC36/GFP/MTS and KᵢR6.1ΔC61/GFP/MTS truncation mutant constructs and found to be contradictory.
Sequencing showed that the \( K_{IR}6.2 \Delta Kp nI \Delta C36/\)GFP/MTS cDNA was as predicted and \( K_{IR}6.1 \Delta C61/\)GFP/MTS only had two amino acid changes that were unlikely to cause change in protein folding or function. The size of construct cDNAs was as predicted, as assessed by both agarose gels of DNA excised from the vector by restriction digest and amplified by PCR. Western blotting for GFP of transfected cell lysates showed a faint band for GFP, indicating a low expression in transfected cells, though this band was inconsistent with the predicted mobility for the combined construct and GFP. Although proteins were successfully expressed in the reticulate lysate system from the cDNAs, these were not of the predicted size. This may have been due to post-translational events or incomplete translation of the peptide from the vector cDNA. Most significantly, however, GFP fluorescence was not observed under fluorescence microscopy following transfection of Chinese hamster ovary (CHO) cells with either construct. Co-transfection with eGFP to check whether truncations were lethal showed that expression of eGFP was unaffected.

There are several possible explanations for the results observed:

i) \( K_{IR}6.x \) truncations were not expressed.

ii) \( K_{IR}6.x \) truncations were expressed. Cells were viable but GFP was not functional – other protein interactions or the low pH of the mitochondrial matrix may have affected GFP fluorescence.

iii) \( K_{IR}6.x \) truncations were expressed correctly but were immediately lethal to expressing cells. Although very low levels of GFP were expressed, insufficient was present for visualisation by UV microscopy.

In summary, the objective of studying the effect on cell survival and induction of apoptosis of expressing constitutively open ion channels targeted to the inner mitochondrial membrane was not achieved.

5.4 SUMMARY

- The \( K^+ \)-channel blocker glyburide inhibited chemical-mediated apoptosis but potentiated CD95-mediated apoptosis. Ouabain inhibited chemical-mediated apoptosis. This demonstrates a role for \( K^+ \) channels and possibly the \( Na^+/K^+ \)-ATPase cotransporter in apoptosis, but no further conclusions can be made as to the channel types involved.

- \( K_{IR}6.1 \) and \( K_{IR}6.2 \), two candidate ion channels for mediating the efflux of intracellular \( K^+ \) during apoptosis, were not genuine caspase substrates. This
suggests a mechanism for gating efflux of $K^+$ other than caspase cleavage of ion channel proteins.

- Although there were several caveats accompanying the observations of mitochondrial $K^+$ flux in apoptosis, cells treated with anti-CD95 antibody showed an apparent increase in mitochondrial matrix $K^+$ concentration.

- Truncation of ion channels that were hypothesised to constitute the mito$K_{ATP}$ channel gave uninterpretable results. Insertion of constitutively open $K^+$ channels into the inner mitochondrial membrane may have been lethal to transfected cells, but no firm conclusions were possible.
Chapter Six. Mechanism of inhibition of apoptosis by elevated extracellular [K+]  

6.1 INTRODUCTION

The inhibitory effect on apoptosis of the ion channel blockers glyburide and ouabain (section 5.2.1) suggests an early involvement of ion channels in chemical-mediated apoptosis. As inhibition was only partial in etoposide-treated cells, their involvement may be indirect. In contrast to the weak inhibition of apoptosis by pharmacological block of ion channels, elevation of extracellular [K+] ([K+]ext) to between 102.7 mM (Bortner et al., 1997) and 150 mM (Chacon-Cruz et al., 1998) mM has been shown to almost completely inhibit apoptosis induced by a range of stimuli. For example, elevated [K+]ext inhibits anti-CD95 antibody-induced apoptosis in Jurkat T cells (Bortner et al., 1997; Orlov et al., 1999) and both cycloheximide and anti-CD95 antibody-induced apoptosis in polymorphonuclear leukocytes (Chacon-Cruz et al., 1998). The inhibition of bacterial toxin-induced necrosis by elevated [K+]ext in monocytes has also been reported (Warny and Kelly, 1999). In the latter study, however, the classification of necrosis was questionable as the mode of cell death involved some components of the apoptotic program such as caspase activation and mitochondrial membrane depolarisation.

Elevated [K+]ext is a commonly practiced method of manipulating both transmembrane K+ flux and membrane potentials. As previously demonstrated (Chapter Three), elevated [K+]ext prevents cells from undergoing ‘spontaneous’ apoptosis in CGN cultures, possibly by inducing changes in the plasma membrane potential. In lymphocyte cell lines, however, it is hypothesised (Bortner et al., 1997; Bilney and Murray, 1998; Orlov et al., 1999) that elevated [K+]ext inhibits apoptosis by preventing efflux of intracellular K+. This is supported by Chapter Five (Figures 5.2 and 5.3) and studies such as Dallaporta et al. (1999) and Maeno et al. (2000) demonstrate that blockade of K+ channels inhibits the induction of apoptosis possibly by preventing the efflux of intracellular K+. Consistent with these observations, inhibition of apoptosis by elevated [K+]ext in lymphocyte cell lines might be achieved by the prevention of intracellular K+ efflux from the cell by significantly reducing the electrochemical driving
force of potassium ions. Maintenance of a normal cytoplasmic \([K^+]\) might thereby prevent the activation of apoptotic effector proteins, as predicted by Walev et al. (1995) and Hughes et al. (1997). As in CGN cultures, however, elevated \([K^+]_{\text{ext}}\) would also be predicted to have profound electrical effects on the plasma membrane and associated proteins. Taking into account these observations, the aims of this chapter were:

1. to characterise the inhibition of apoptosis in cell lines by elevated \([K^+]_{\text{ext}}\) and to examine the extent of this phenomenon by examining induction of apoptosis by a range of pro-apoptotic stimuli in several cell lines.
2. to elucidate what processes in the apoptotic program are inhibited by \(K^+\).
3. to investigate how specific mechanisms within the apoptotic program are inhibited by potassium ions.

6.2 Results

6.2.1 Elevated \([K^+]_{\text{ext}}\) inhibits apoptosis prior to caspase activation

Following on from the studies of Bortner et al. (1997) and Hughes et al. (1997), an investigation was designed to test whether elevated \([K^+]_{\text{ext}}\) would affect apoptosis in Jurkat T cells. Cells were exposed to anti-CD95 antibody or etoposide for 5 h in either normal (5 mM K\(^+\), 135 mM Na\(^+\)) or elevated \([K^+]_{\text{ext}}\) (135 mM K\(^+\), 5 mM Na\(^+\)). Both stimuli induced apoptosis in normal \([K^+]_{\text{ext}}\), which was almost completely inhibited in elevated \([K^+]_{\text{ext}}\) (Figure 6.1C). Control cells showed a low level of spontaneous apoptosis in both normal and elevated \([K^+]_{\text{ext}}\) (Figure 6.1C).

Activated caspases play a major role in the execution phase of apoptosis induced by many stimuli (Cohen, 1997). To investigate if elevated \([K^+]_{\text{ext}}\) inhibited apoptosis prior to caspase activation, cell pellets were analysed by Western blot for processing of caspases -3 and -8. In control cells, both caspases -3 and -8 were present entirely in their proforms (Figures 6.1, A and B, lanes 1 and 4). Induction of apoptosis by both stimuli in normal \([K^+]_{\text{ext}}\) 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 6.1A and B, lanes 2 and 3). All of the caspase-8 was processed to its p43 and p41 immunoreactive fragments (Scaffidi et al., 1997) and caspase-3 to its p20, p19 and p17 fragments (Fernandes-Alnemri et al., 1996). The inhibition by elevated \([K^+]_{\text{ext}}\) 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 6.1, A and B, lanes 5 and 6), except for a small amount of an uncharacterized caspase-3 immunoreactive product (Figure 6.1B, lane 6). This indicated that inhibition of apoptosis by elevated \([\text{K}^+]_{\text{ext}}\) was upstream of caspase activation.

---

**Figure 6.1: Elevated \([\text{K}^+]_{\text{ext}}\) inhibits apoptosis induced by anti-CD95 antibody and etoposide upstream of caspase activation.** Jurkat T cells were incubated for 5 h with vehicle (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 μM) in either normal or elevated \([\text{K}^+]_{\text{ext}}\). Processing of caspase-8 (A) and caspase-3 (B) was analysed by Western blot. Apoptosis was assessed by PS externalisation (C). Data are representative of 3 independent experiments.
6.2.2 Treatment of cells in elevated \([K^+]_{\text{ext}}\) with apoptotic stimuli does not cause a change in cell volume

The relation between apoptotic cell shrinkage and PS externalisation, plasma membrane depolarisation, efflux of intracellular K\(^+\), mitochondrial depolarisation and caspase activation was examined in Chapter Four. It was determined that apoptotic cell shrinkage occurred in synchrony with these markers of apoptosis (Figures 4.4 and 4.12), while inhibition of caspase activity prevented cell shrinkage in CD95- but not chemical-mediated apoptosis (Figure 4.12). In order to assess whether elevated \([K^+]_{\text{ext}}\) inhibited apoptosis prior to or downstream of cell shrinkage, the size of Jurkat cells in elevated \([K^+]_{\text{ext}}\) was assessed following treatment with vehicle, anti-CD95 antibody or etoposide. Increasing \([K^+]_{\text{ext}}\) induced cell shrinkage independently of apoptotic treatments, however, with the peak size of control cells decreasing and peak density increasing with increasing \([K^+]_{\text{ext}}\) (Figure 6.2, A to D). This was not due to differences in osmolarity between normal and elevated \([K^+]_{\text{ext}}\) media, as this was measured for all media of defined ionic composition (as described in section 2.6.2) and was found to be between 270 and 300 mOsm in each case. It is more likely that volume changes resulted from the depolarisation of the plasma membrane, induced by elevated \([K^+]_{\text{ext}}\), which would likely inhibit the normal operation of volume regulatory mechanisms.
Figure 6.2: Elevated $[K^+]_{\text{ext}}$ inhibits apoptotic cell volume changes induced by anti-CD95 antibody and etoposide. Jurkat T cells were incubated with vehicle (DMSO; A-D), anti-CD95 antibody (50 ng/ml; E-H) or etoposide (50 μM; I-L) for 5 h in varying $[K^+]_{\text{ext}}$ with $[Na^+]_{\text{ext}}$ correspondingly decreased to maintain a monovalent cation concentration of 140 mM. Cell size was assessed by flow cytometry, with the quadrant marker indicating the peak population size of control cells in normal $[K^+]_{\text{ext}}$ (5 mM). Data are representative of 3 independent experiments.
The size and density of cultures treated with anti-CD95 antibody or etoposide in normal $[K^+]_{\text{ext}}$ became more dispersed, as revealed by flow cytometric analysis, with an increase in shrunken cells (Figure 6.2, E and I). The profile of etoposide-treated cells became more similar to the control with increasing $[K^+]_{\text{ext}}$, with the culture in 135 mM $[K^+]_{\text{ext}}$ indistinguishable from the control (Figure 6.2, compare E and L). The size and density profiles of anti-CD95 antibody-treated cultures changed little from 5 to 95 mM $[K^+]_{\text{ext}}$ (Figure 6.2, E to G), but cells in 135 mM $[K^+]_{\text{ext}}$ were indistinguishable from control cells (Figure 6.2, compare E and H). These trends in cell shrinkage for both apoptotic stimuli were consistent with inhibition of apoptosis by increasing $[K^+]_{\text{ext}}$, as detailed in section 6.2.6. Although control cells in 135 mM $[K^+]_{\text{ext}}$ were shrunken relative to 5 mM $[K^+]_{\text{ext}}$, treatment with anti-CD95 antibody or etoposide did not result in any further change in cell volume, which suggested that elevated $[K^+]_{\text{ext}}$ inhibited apoptosis prior to apoptotic cell shrinkage. As elevated $[K^+]_{\text{ext}}$ induced cell shrinkage, however, the data did not support the hypothesis that apoptosis was inhibited primarily by prevention of cell shrinkage (Bortner et al., 1997; Bilney and Murray, 1998; Orlov et al., 1999).

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

The possibility that elevated $[K^+]_{\text{ext}}$ might block CD95-mediated apoptosis in Jurkat cells by preventing binding of anti-CD95 antibody was investigated by utilising 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 $[K^+]_{\text{ext}}$ (Figure 6.3). The point of inhibition of apoptosis by elevated $[K^+]_{\text{ext}}$ was therefore subsequent to binding of anti-CD95 antibody to the CD95 receptor.
Figure 6.3: Elevated [K⁺]_{ext} does not prevent binding of anti-CD95 antibody to Jurkat 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}. Data are representative of 2 independent experiments.

This result was confirmed by treating cells in elevated [K⁺]_{ext} with anti-CD95 antibody, then washing and re-suspending the cells in normal [K⁺]_{ext}. Treatment of cells with anti-CD95 antibody in normal [K⁺]_{ext} for 5 h induced apoptosis in 62.8 %, as assessed by PS externalisation. Incubation of cells with anti-CD95 antibody for 30 min in elevated [K⁺]_{ext} before switching to normal [K⁺]_{ext} and incubating for 5 h induced 55.7 % apoptosis (n=2). This confirmed that the elevated [K⁺]_{ext} did not inhibit the binding of the anti-CD95 antibody, and demonstrated that the ligated receptor could still activate the caspase cascade when the [K⁺]_{ext} was returned to normal. This also demonstrated that cells in elevated [K⁺]_{ext} are not ‘fixed’ and that inhibition of CD95-mediated apoptosis was reversible.
6.2.4 Elevated \([K^+]_{\text{ext}}\) inhibits apoptosis upstream of cytochrome \(c\) release and reduction of mitochondrial transmembrane potential \((\Psi_m)\)

Release of cytochrome \(c\) from the mitochondria is considered a critical step in chemical-induced apoptosis (Kluck et al., 1997; Green and Reed, 1998). 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 post-mitochondrial 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 cell stress.

As previously discussed (see section 1.5), reduction of \(\Psi_m\) is a key event in apoptosis induced by diverse stimuli (Petit et al., 1996). Putative interactions between reduction of \(\Psi_m\) and cytochrome \(c\) release are nonetheless controversial and are currently under intense investigation. In order to investigate the effect of alterations in \([K^+]_{\text{ext}}\) on cytochrome \(c\) release, cytosolic lysates were prepared from cells treated with anti-CD95 antibody and etoposide and incubated in normal and elevated \([K^+]_{\text{ext}}\) for 5 h. Control cells in both media showed no release of cytochrome \(c\) from the mitochondria (Figure 6.4A, lanes 1 and 4). Both apoptotic stimuli induced release of cytochrome \(c\) in normal \([K^+]_{\text{ext}}\), with a greater release induced by anti-CD95 antibody, consistent with its greater induction of apoptosis as assessed by PS externalisation (Figures 6.4, A and C, lanes 2 and 3). This increase in cytosolic cytochrome \(c\) was almost completely inhibited in elevated \([K^+]_{\text{ext}}\) (Figure 6.4A, 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 \([K^+]_{\text{ext}}\) (Figures 6.4, A and C, lane 5). Equal protein loading was demonstrated by Western blotting for the cytoskeletal protein \(\alpha\)-tubulin (Figure 6.4B). Treatment of cells in normal \([K^+]_{\text{ext}}\) with anti-CD95 antibody or etoposide also induced reduction of \(\Psi_m\) (Figure 6.4D, lanes 2 and 3), though with etoposide treatment in normal \([K^+]_{\text{ext}}\), fewer cells exhibited reduction of \(\Psi_m\) than PS externalisation (9 % low \(\Psi_m\) compared to 31 % with PS externalisation). In elevated \([K^+]_{\text{ext}}\), reduction of \(\Psi_m\) was almost totally inhibited (Figure 6.4D, lanes 5 and 6), indicating that apoptosis induced by both stimuli was inhibited prior to reduction of \(\Psi_m\).
Figure 6.4: Elevated $[K^+]_\text{ext}$ inhibits apoptosis induced by anti-CD95 antibody and etoposide prior to mitochondrial perturbation. Jurkat T cells were incubated for 5 h with vehicle (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 µM) in either normal or elevated $[K^+]_\text{ext}$. Western blots were performed to investigate cytochrome c release (A), and also for $\alpha$-tubulin to demonstrate equal protein loading (B). Cells were assessed by flow cytometry for PS externalisation (C) and reduction of $\Psi_m$ (D). Data are representative of 3 independent experiments.

6.2.5 Elevated $[K^+]_\text{ext}$ does not prevent anti-CD95 antibody-induced recruitment of FADD and caspase-8 to the DISC

As previously detailed (section 1.4.2 and Figure 1.4), binding of CD95 ligand or anti-CD95 antibody to the CD95 receptor leads to recruitment of FADD to the death domain of the oligomerised CD95 receptors, which in turn recruit procaspase-8 to their death effector domain, thus forming the DISC. This protein complex mediates autoactivation of procaspase-8 (Kischkel et al., 1995; Medema et al., 1997), which either activates effector caspases directly or indirectly (Scaffidi et al., 1998), possibly by cleaving Bid (Li et al., 1998; Luo et al., 1998),...
a pro-apoptotic member of the Bcl-2 family of proteins, which then triggers the release of mitochondrial cytochrome c and initiates a caspase cascade.

Data has been presented demonstrating inhibition of CD95-mediated apoptosis upstream of caspase-8 activation, cytochrome c release, mitochondrial depolarisation and PS externalisation but downstream of anti-CD95 antibody binding. It was therefore investigated whether recruitment of proteins to the DISC during CD95-mediated apoptosis was inhibited by elevated $[K^+]_{\text{ext}}$. The subcellular fraction containing the plasma membrane of untreated cells incubated in complete RPMI medium for 1 or 5 h contained a small quantity of procaspase-8 and FADD, as assessed by Western blotting (Figure 6.5A, lanes 1 and 3). Treatment of cells in complete medium with anti-CD95 antibody led to an increase in both procaspase-8 and FADD in the plasma membrane fraction (Figure 6.5, A lanes 2 and 4) compared to control cells. Anti-CD95 antibody-treated cells showed processing of caspase-8 to its p43 and p41 immunoreactive fragments after 5 h, consistent with the induction of apoptosis. No processing of caspase-8 was seen after 1 h incubation with anti-CD95 antibody.

Changes in the distribution of FADD and caspase-8 following anti-CD95 antibody treatment were consistent with recruitment of these proteins to the DISC. The relative change in the quantities of caspase-8 and FADD in the plasma membrane fractions was less striking than expected. This might be because the proteins form the DISC only transiently before caspase-8 is released in its active form into the cytoplasm. As the 1 h incubation with anti-CD95 antibody showed easily comparable levels of caspase-8 without a decrease in proform due to processing, this incubation time was selected for experiments in media of defined ionic composition. The recruitment of proteins to the plasma membrane of cells in normal $[K^+]_{\text{ext}}$ was comparable with complete medium; there was a relative increase in the quantities of both caspase-8 and FADD in the plasma membrane fraction of cultures treated with anti-CD95 antibody relative to untreated cells (Figure 6.3B, lanes 1 and 2). Intriguingly, cells in elevated $[K^+]_{\text{ext}}$ also showed an increase in the quantities of both caspase-8 and FADD in the membrane fraction of cells treated with anti-CD95 antibody compared to untreated cells in elevated $[K^+]_{\text{ext}}$ (Figure 6.5B, lanes 3 and 4). This suggested that DISC formation still occurred following anti-CD95 antibody treatment in elevated $[K^+]_{\text{ext}}$ and that inhibition of apoptosis was downstream of recruitment of procaspase-8 and FADD to the DISC.
Figure 6.5: Inhibition of anti-CD95 antibody-induced apoptosis by elevated $[\text{K}^+]_{\text{ext}}$ is downstream of FADD and procaspase-8 recruitment to the plasma membrane. Cells were treated for 1 or 5 h with vehicle (PBS) or anti-CD95 antibody (50 ng/ml) in complete medium (A) or for 1 h in medium of defined ionic composition containing normal or elevated $[\text{K}^+]_{\text{ext}}$ (B). The subcellular fraction containing the plasma membrane was probed for procaspase-8 and FADD. Data in sections A and B are representative of 2 and 3 independent experiments, respectively.
6.2.6 Properties of inhibition of CD95-mediated apoptosis by elevated $[K^+]_{\text{ext}}$

As 135 mM $[K^+]_{\text{ext}}$ almost completely inhibited apoptosis, an investigation into the concentration-dependence of this inhibition was undertaken. Jurkat cells were incubated for 5 h in media of differing $[K^+]_{\text{ext}}$, where extracellular $[Na^+]$ ($[Na^+]_{\text{ext}}$) was varied reciprocally in order to maintain osmolarity and a monovalent 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 $[Na^+]_{\text{ext}}$ and $[K^+]_{\text{ext}}$. In anti-CD95 antibody-treated cells, increasing $[K^+]_{\text{ext}}$ up to 95 mM had little effect on the induction of apoptosis, whereas $[K^+]_{\text{ext}}$ above this inhibited apoptosis in a concentration-dependent fashion (Figure 6.6A). Interestingly, apoptosis induced by etoposide was significantly inhibited by lower $[K^+]_{\text{ext}}$ than anti-CD95 antibody-induced apoptosis, and was almost completely inhibited at 120 mM $[K^+]_{\text{ext}}$.

Assessment of caspase-8 processing by Western blotting of cell lysates from selected cultures showed that cleavage was proportionate to the extent of apoptosis, as assessed by PS externalisation. Control cells in both 5 and 135 mM $[K^+]_{\text{ext}}$ showed a strong immunoreactive band for the proform of caspase-8, with no cleavage fragments (Figure 6.6B, lanes 1 and 2). Anti-CD95 antibody-treated cells showed complete processing to the p43 and p41 fragments in 5, 35, 65 and 95 mM $[K^+]_{\text{ext}}$ (lanes 3 to 6), consistent with a high induction of apoptosis, as assessed by PS externalisation (Figure 6.6A). At 115 mM $[K^+]_{\text{ext}}$, the proform and fragment bands were approximately of equal strength (lane 7), consistent with a significant reduction in the induction of apoptosis from 86.6 % at 5 mM $[K^+]_{\text{ext}}$ to 50.4 % at 115 mM $[K^+]_{\text{ext}}$. At 135 mM $[K^+]_{\text{ext}}$ caspase-8 was almost entirely in its proform (lane 8).
Figure 6.6: Treatment of cells with anti-CD95 antibody and etoposide in varying $[\text{K}^+]_{\text{ext}}$. Jurkat T cells were incubated with vehicle (DMSO; ○), anti-CD95 antibody (50 ng/ml; ■) or etoposide (50 μM; ▲) for 5 h in varying $[\text{K}^+]_{\text{ext}}$ with $[\text{Na}^+]_{\text{ext}}$ correspondingly decreased to maintain a monovalent cation concentration of 140 mM. Apoptosis was assessed by PS externalisation (A). Data displayed are mean and S.E.M. of 4 independent experiments. Processing of caspase-8 was assessed by Western blotting (B).
Etoposide-treated cultures showed a similar correlation between processing of caspase-8 and externalisation of PS. At 5, 35 and 65 mM \([K^+]_{\text{ext}}\) the immunoreactive bands for the proform and cleavage fragments were approximately of equal strength (Figure 6.5B, lanes 9 to 11), consistent with the lesser induction of apoptosis by etoposide compared to anti-CD95 antibody. At 95 mM \([K^+]_{\text{ext}}\) there was a slight decrease in the amount of caspase-8 processing (lane 12), and at 115 and 135 mM \([K^+]_{\text{ext}}\) caspase-8 was entirely in its proform (lanes 13 and 14), consistent with inhibition of apoptosis to control levels. The almost complete inhibition of etoposide-induced apoptosis at 115 mM \([K^+]_{\text{ext}}\) (8.7 % apoptotic) compared to induction of 50.4 % apoptosis by anti-CD95 antibody suggested that elevated \([K^+]_{\text{ext}}\) might act at different targets to inhibit the two apoptotic stimuli.

6.2.7 Elevated \([K^+]_{\text{ext}}\) is required from initiation of signal transduction by anti-CD95 antibody to inhibit apoptosis

It has so far been demonstrated that apoptosis induced by anti-CD95 antibody was inhibited downstream of DISC formation but prior to activation of procaspase-8 and mitochondrial perturbation. Etoposide-induced apoptosis was inhibited prior to both mitochondrial changes and caspase activation. To further elucidate the point at which elevated \([K^+]_{\text{ext}}\) inhibited apoptosis, cells were incubated in normal \([K^+]_{\text{ext}}\) and treated with vehicle, anti-CD95 antibody or etoposide for the indicated times and then media switched to elevated \([K^+]_{\text{ext}}\). Surprisingly, incubation with anti-CD95 antibody in normal \([K^+]_{\text{ext}}\) for just 15 min before switching to elevated \([K^+]_{\text{ext}}\) resulted in an increased level of apoptosis (Figure 6.7A). The extent of apoptosis induced by anti-CD95 antibody increased with time in normal \([K^+]_{\text{ext}}\), approaching maximal induction after 2 - 3 h. This implied that the inhibition of CD95-mediated apoptosis by elevated \([K^+]_{\text{ext}}\) occurred in some cells within 15 min of treatment with anti-CD95 antibody.

In contrast, etoposide-treated cells responded with similar kinetics to time-dependent induction of apoptosis in normal \([K^+]_{\text{ext}}\), with the incubation time in elevated \([K^+]_{\text{ext}}\) appearing to have no bearing on the extent of apoptosis. This suggested that switching cells from normal to elevated \([K^+]_{\text{ext}}\) immediately inhibited apoptosis. Control cells showed a consistently low level of apoptosis (<4 %), demonstrating that the centrifugation and washing did not cause a decrease in culture viability. Western blot analysis of caspase-8 cleavage was performed for
anti-CD95 antibody-stimulated cells. Cleavage of caspase-8 to its p43 and p41 fragments was commensurate with the level of apoptosis assessed by PS externalisation, with appearance of the p41 and p43 fragments observed after 30 min, increasing to complete cleavage of the proform after 2 h.
Figure 6.7: Elevated [K⁺]_{ext} is required from the outset of anti-CD95 antibody treatment to inhibit apoptosis. Jurkat T cells were incubated with vehicle (DMSO; ○), anti-CD95 antibody (50 ng/ml; ■) or etoposide (50 μM; ▲) in normal [K⁺]_{ext} for the indicated times before being washed and cultured in elevated [K⁺]_{ext} medium. Cells were treated again with vehicle, anti-CD95 antibody or etoposide and after incubation to give a total of 5 h in both media, apoptosis was quantified by PS externalisation (A), with samples taken for analysis of caspase-8 processing in the anti-CD95 antibody-treated cells by Western blot (B). Data are representative of 2 independent experiments.
The data suggested that elevated [K⁺]_{ext} inhibited CD95-mediated apoptosis at a relatively early point since a proportion of cells that were switched from normal to elevated [K⁺]_{ext} after this point still underwent apoptosis. This is in agreement with Chacon-Cruz et al., (1998), who demonstrated a significant delay in cycloheximide-induced apoptosis if cells were switched from normal to elevated [K⁺]_{ext} within 12 min of treatment, but less effectual inhibition of apoptosis after this time. In contrast with CD95, etoposide-mediated apoptosis appeared to be inhibited at a late stage since switching cells to elevated [K⁺]_{ext} prevented any further induction of apoptosis. This supported the data from 6.2.6 that elevated [K⁺]_{ext} inhibited CD95- and chemical-mediated apoptosis at different targets.

6.2.8 Inhibition of CD95-mediated apoptosis is independent of decreased extracellular [Na⁺]

In experiments investigating the effect of increasing [K⁺]_{ext} on apoptosis, [Na⁺]_{ext} had been simultaneously decreased in order to maintain osmolarity and monovalent 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} whilst keeping [K⁺]_{ext} constant were investigated. Experimental media were prepared with the cell-impermeant organic cation 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} decreased to 5 mM by replacement with NMDG (Figure 6.8). 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, though not to the same extent as elevated [K⁺]_{ext}.

The extent of caspase-8 processing, as assessed by Western blot of cell lysates, correlated with the quantitation of apoptosis by PS externalisation. Cells cultured in 5 mM K⁺/135 mM NMDG medium exhibited processing of caspase-8 to its p43 and p41 fragments following treatment with anti-CD95 antibody (Figure 6.9A), whereas the caspase-8 of cells treated with etoposide was entirely in its proform. This inhibition of etoposide-induced apoptosis by 5 mM K⁺/135 mM NMDG medium was less striking after 10 h, as cells had
decreased viability compared to control, with some processing of caspase-8 (Figure 6.9B). Protection against etoposide-induced apoptosis by 5 mM K⁺/135 mM NMDG medium was completely lost by 24 h, with culture viability and caspase-8 processing comparable to etoposide-treated cells in normal [K⁺]_{ext} (Figure 6.9C).

Taken together, these data implied that induction of apoptosis by etoposide but not anti-CD95 antibody was partially dependent upon the presence of Na⁺ in the medium, as depletion of or decreased [Na⁺]_{ext} inhibited etoposide- but not anti-CD95 antibody-induced apoptosis. Examination of this inhibition over 24 h demonstrated that culturing in decreased [Na⁺]_{ext} medium delayed rather than completely blocked the induction of apoptosis by etoposide.

Figure 6.8: 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 (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 μM) for 5 h. Apoptosis was assessed by PS externalisation. Data are mean and S.E.M. of 3 independent experiments.
6.2.9 Cell death in elevated $[K^+]_{\text{ext}}$ is independent of caspase-8 activation over 24 h

Data have been presented demonstrating the almost-complete inhibition of CD95- and chemical-mediated apoptosis by elevated $[K^+]_{\text{ext}}$ over 5 h. To investigate whether apoptosis was inhibited or delayed over a greater period of time, cells in normal and elevated $[K^+]_{\text{ext}}$ were treated with vehicle, anti-CD95 antibody or etoposide and assays performed for PS externalisation and caspase-8 activation after 5, 10 and 24 h. Both anti-CD95 antibody and etoposide induced apoptosis after 5 h with commensurate processing of caspase-8 in normal $[K^+]_{\text{ext}}$ that was completely inhibited in elevated $[K^+]_{\text{ext}}$ (Figure 6.9A), consistent with the data in Figure 6.1. After 10 h control cells showed a slight decrease in viability, and induction of apoptosis by etoposide in normal $[K^+]_{\text{ext}}$ was greater than at 5 h, with a slight increase in anti-CD95 antibody-induced apoptosis (Figure 6.9B). Cells in elevated $[K^+]_{\text{ext}}$ did not show any cleavage of caspase-8, regardless of stimulus, but culture viability was decreased compared to 5 h. After 24 h, control cells in normal $[K^+]_{\text{ext}}$ showed a further decrease in viability, though the culture was still relatively healthy (Figure 6.9C). This decrease in cell viability may have been due to the lack of vital nutrients in the normal $[K^+]_{\text{ext}}$ medium. Of the cells stimulated with anti-CD95 antibody, 98.5% were dead after 24 h, with complete processing of caspase-8 to its immunoreactive fragments. The majority (76.6%) of etoposide-treated cells were dead after 24 h, with corresponding caspase-8 cleavage.

In contrast, while culture viability of cells in elevated $[K^+]_{\text{ext}}$ did decrease over 24 h, there was little difference between vehicle-, anti-CD95 antibody- and etoposide-treated cells and procaspase-8 remained completely in its proform in all cases (Figure 6.9, A to C). This implied that the cells died as a result of being cultured in elevated $[K^+]_{\text{ext}}$, possibly due to prolonged depolarisation of the plasma membrane (i.e. over the course of 10 h). This cell death, however, was independent of caspase-8 activation. This demonstrated that $[K^+]_{\text{ext}}$ inhibited rather than delayed the apoptotic program, since there was no induction of apoptosis in elevated $[K^+]_{\text{ext}}$ at 6, 10 or 24 h by anti-CD95 antibody or etoposide.
<table>
<thead>
<tr>
<th>Media</th>
<th>5 K/135 Na</th>
<th>5 K/135 NMDG</th>
<th>135 K/5 Na</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Anti-CD95</td>
<td>Etoposide</td>
</tr>
<tr>
<td>A 5 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Viable</td>
<td>94.5</td>
<td>17.8</td>
<td>71.9</td>
</tr>
<tr>
<td>B 10 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Viable</td>
<td>89.2</td>
<td>3.9</td>
<td>31.6</td>
</tr>
<tr>
<td>C 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Viable</td>
<td>81.2</td>
<td>1.5</td>
<td>23.4</td>
</tr>
</tbody>
</table>

Figure 6.9: Cell death in elevated \([K^+]_{ext}\) is independent of caspase-8 activation over 24 h, though cytoprotection by 5K/130 NMDG medium against etoposide-induced apoptosis is lost. Jurkat cells in normal or elevated \([K^+]_{ext}\) media or 5 mM K+/135 mM NDMG medium were treated with vehicle (DMSO), anti-CD95 antibody (50 ng/ml) or etoposide (50 μM). At 5, 10 and 24 h (A, B and C, respectively) the percentage of viable cells was determined by PS externalisation and samples taken for Western blot analysis of caspase-8 processing.
6.2.10 Elevated [K+]\text{ext} inhibits apoptosis induced by a range of stimuli

An investigation was designed to investigate whether elevated [K+]\text{ext} protected against other apoptotic stimuli. Both TRAIL, another member of the tumour necrosis factor (TNF) family, and the proteasomal inhibitor lactacystin induce apoptosis in Jurkat T cells (MacFarlane et al., 2000). Treatment of Jurkat T cells in normal [K+]\text{ext} with TRAIL, staurosporine (STS), or MG132, another proteasomal inhibitor (Lee and Goldberg, 1998), induced significant levels of apoptosis as assessed by PS externalisation (Figure 6.10A). Induction of apoptosis by these three stimuli was markedly inhibited by elevated [K+]\text{ext}, though inhibition was not total for TRAIL or STS. Western blot analysis of caspases-3 and -8 processing confirmed this inhibition; treatment of cells in normal [K+]\text{ext} with TRAIL, STS or MG132 induced cleavage of procaspase-8 to its p43 and p41 fragments (Figure 6.10B, lanes 2 to 4). Both TRAIL and MG132 induced processing of caspase-3 to its p20 fragment in normal [K+]\text{ext} (Figure 6.10B, lanes 2 and 4), but interestingly STS did not (Figure 6.10C, lane 3). Cells in elevated [K+]\text{ext} did not exhibit caspase-8 or caspase-3 processing following treatment with TRAIL, STS or MG132 (Figures 6.10, B and C, lanes 6 to 8). Control cells in both normal and elevated [K+]\text{ext} exhibited no processing of either caspase-3 or -8 (Figure 6.10, B and C, lanes 1 and 5).
Figure 6.10: Elevated [K+]_{ext} inhibits apoptosis induced by TRAIL, STS and MG132 upstream of caspase activation. Jurkat T cells were incubated for 5 h with vehicle (DMSO), TRAIL (1 μg/ml), STS (1 μM) or MG132 (1 μM) in either normal (open bars) or elevated [K+]_{ext} (filled bars). Apoptosis was assessed by PS externalisation (A), with the mean and S.E.M. of 3 independent experiments displayed. Processing of caspase-8 (B) and caspase-3 (C) was analysed by Western blot.
6.2.11 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. Etoposide and MG132 induced apoptosis in normal $[K^+]_{ext}$, as assessed by PS externalisation (Figure 6.11A), with processing of caspase-3 and -8 to their respective immunoreactive bands that was commensurate with induction of apoptosis (Figure 6.11B and 6.11C, lanes 2 and 4). STS induced apoptosis to an extent comparable with MG132, but only induced a small amount of caspase-8 processing and almost no processing of caspase-3. Cells in elevated $[K^+]_{ext}$ did not undergo apoptosis upon treatment with etoposide or MG132. Both caspase-3 and -8 remained entirely in their proforms with etoposide treatment (Figure 6.11, B and C, lane 6). MG132 did not induce processing of caspase-8 in elevated $[K^+]_{ext}$ (Figure 6.11B, lane 8), and only a very small amount of caspase-3 was processed to its p20 subunit (Figure 6.11C, lane 8). In contrast, STS induced apoptosis in elevated $[K^+]_{ext}$ that was comparable with induction in normal $[K^+]_{ext}$ (Figure 6.11A), though unlike cells in normal $[K^+]_{ext}$, both caspase-3 and -8 remained entirely in their proforms (Figure 6.11, B and C, lane 7). Control cells in both normal and elevated $[K^+]_{ext}$ exhibited little apoptosis (Figure 6.11A) and there was no processing of either caspase-3 or -8 (Figures 6.11, B and C, lanes 1 and 5).

The incomplete inhibition by elevated $[K^+]_{ext}$ of STS-induced apoptosis in Jurkat cells (Figure 6.10A) and total lack of inhibition in THP.1 cells (Figure 6.11A), combined with the caspase-processing data was of great interest. STS-induced apoptosis in Jurkat cells appeared to be mediated by caspase activation to a greater extent than in THP.1 cells, though there was a greater induction of apoptosis in Jurkat cells (43.9%) compared to THP.1 cells (24.8%). A possible hypothesis is the existence of two pathways by which STS induced apoptosis, one inhibited by elevated $[K^+]_{ext}$ and the other not. The pathway inhibited by elevated $[K^+]_{ext}$ may be mediated by caspase activation, since STS-treated Jurkat cells in elevated $[K^+]_{ext}$ showed a decrease in apoptosis along with the complete inhibition of caspase processing compared to cells in normal $[K^+]_{ext}$. In THP.1 cells there was little caspase activation in normal $[K^+]_{ext}$, suggesting that induction of apoptosis by STS was almost completely caspase-independent. The complete lack of inhibition in elevated $[K^+]_{ext}$ of apoptosis in STS-treated THP.1 cells suggested that elevated $[K^+]_{ext}$ only inhibited caspase-dependent apoptotic pathways. Since caspase-9 is the apical caspase in the extrinsic cell death pathway (Sun et al., 1999), however,
it would be preferable to examine caspase-9 processing during chemical-induced apoptosis. Taking these data together, elevated $[K^+]_{\text{ext}}$ inhibited a range of stimuli in two cell lines, which suggested that either it inhibited a fundamental process common to most stimuli or that it inhibited apoptosis at multiple loci.
Figure 6.11: Elevated $[K^+]_{ext}$ inhibits etoposide- and MG132- but not STS-induced apoptosis upstream of caspase activation in THP.1 cells. THP.1 cells were incubated for 8 h with vehicle (DMSO), etoposide (50 μM), STS (1 μM) or MG132 (1 μM) in either normal or elevated $[K^+]_{ext}$. Apoptosis was assessed by PS externalisation (A), with the mean and S.E.M. of 3 independent experiments displayed. Processing of caspase-8 (B) and caspase-3 (C) was analysed by Western blot.
6.2.12 Addition of K⁺ or Na⁺ to cytosolic lysates inhibits caspase activation and formation of the ~700 kDa apoptosome complex

Elevated [K⁺]_{ext} inhibits both anti-CD95 antibody- and etoposide-induced apoptosis in cultured Jurkat T cells upstream of mitochondrial cytochrome c release. It was decided, however, to also 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. The influence of varying ionic concentration on post-mitochondrial activation of caspases in THP.1 lysates was investigated by addition of KCl or NaCl prior to activation with dATP. The caspase activation of the lysates was then assessed using the fluorogenic substrate z-DEVD.AFC. Unactivated lysates showed a very low level of DEVDase activity compared to the dATP-activated lysates (Figure 6.12). Addition of either KCl or NaCl decreased activity in a concentration-dependent fashion, with 50 mM inhibiting back to basal levels of activity. Inhibition of caspase activation was not cation specific as either NaCl or KCl was equally effective, suggesting the inhibition may be charge- rather than ion-specific in cell free extracts.

The formation of the apoptosome can be modelled *in vitro* by adding dATP and cytochrome c to cell lysates (Cain et al., 1999; Cain et al., 2000). Since caspase activity was almost completely inhibited by 50 mM KCl and NaCl (Figure 6.12) the effect of this ionic concentration on apoptosome formation was investigated using gel filtration chromatography. Cytosolic lysates from Jurkat cells were separated on gel filtration columns and the individual fractions immunoblotted for Apaf-1. In unactivated lysates, Apaf-1 eluted in its monomeric form, with an estimated Mr ≤158,000 (Figure 6.13A). After control dATP activation, three different peaks of Apaf-1 were seen at ~1.4 MDa, ~700 kDa and 130 kDa (Figure 6.13A), corresponding to the previously described apoptosome complexes, and the monomeric form of Apaf-1 (Cain et al., 2000). Addition of 50 mM KCl prior to dATP activation of the lysates completely suppressed caspase activation and Apaf-1 only eluted in the ~1.4 MDa and 130 kDa fractions (Figure 6.13A). This strongly suggested that the oligomerisation of Apaf-1 to form the biologically active ~700 kDa apoptosome complex was inhibited by 50 mM KCl.
Figure 6.12: Pre-treatment of THP.1 lysates with KCl or NaCl inhibits caspase activation. THP.1 lysates (10 mg/ml) were incubated with the indicated concentrations of NaCl or KCl for 30 min at 4°C and then activated with dATP/MgCl₂ at 37°C for 30 min. Controls were treated with vehicle alone and not activated. Caspase activation was measured by assaying for DEVDase activity.

The caspase processing activity of these two apoptosome complexes was assessed using z-DEVD.AFC. Fractions corresponding to the ~700 kDa complex (Figure 6.13B, 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 6.13A). In the presence of 50 mM NaCl or 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.
Figure 6.13: 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°C without dATP (○), with dATP (●) and with dATP, having been pre-treated with 50 mM KCl at 4°C for 30 min (▲). Lysates (~2.5 mg 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 weight standards are shown in the centre of the figure.
In order to confirm that inhibition of apoptosome formation did not relate only to Jurkat T cell lysates, the experiment was repeated with lysates from THP.1 cells, in which the formation of the apoptosome complex has been well characterised (Cain et al., 1999; Cain et al., 2000). Activation with dATP resulted in the formation of both ~700 kDa and ~1.4 MDa apoptosome complexes (Figure 6.14). However, after pre-treatment of the lysates with either 50 mM KCl or NaCl, little or no Apaf-1 eluted in the ~700 kDa fractions. The inhibition of apoptosome formation and function by relatively low concentrations K⁺ and Na⁺ suggests that normal [K⁺], may prevent inappropriate formation of the apoptosome complex and activation of the death pathway. This may, as suggested previously (Hughes et al., 1997), 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.
Figure 6.14: Both NaCl and KCl inhibit ~700 kDa apoptosome complex formation in THP.1 lysates. Control, dATP-activated and dATP-activated lysates pre-treated with 50 mM NaCl or 50 mM KCl at 4°C for 30 min were separated by Superose 6 gel filtration. Equal volumes of the fractions were immunoblotted for Apaf-1. Fraction numbers and the position of the high molecular weight standards are shown at the top of the figure.
6.3 DISCUSSION

Elevated $[K^+]_{\text{ext}}$ inhibited both death receptor- and chemical-mediated apoptosis at a very early stage, as PS externalisation, caspase activation (Figure 6.1), cytochrome $c$ release and reduction of $\Psi_m$ (Figure 6.4) were almost completely inhibited. Observation of the direct interaction between elevated $[K^+]_{\text{ext}}$ and apoptotic cell shrinkage was difficult as elevated $[K^+]_{\text{ext}}$ induced shrinkage in all cells (Figure 6.2), possibly due to sustained depolarisation of the plasma membrane disrupting regulatory volume decrease mechanisms. There was no change in cell volume for cells in elevated $[K^+]_{\text{ext}}$ when apoptotic stimuli were added, however, suggesting that elevated $[K^+]_{\text{ext}}$ inhibited apoptosis prior to cell shrinkage.

It was not possible to investigate whether elevated $[K^+]_{\text{ext}}$ inhibited depolarisation of the plasma membrane using DiBAC$_4$(3) (as described in Chapter Four, Figures 4.1 to 4.6) as elevated $[K^+]_{\text{ext}}$ would have almost completely dissipated the plasma membrane potential. The flux of intracellular $K^+$ using $^{86}\text{Rb}^+$ was investigated in elevated $[K^+]_{\text{ext}}$ since two possible mechanisms by which apoptosis might be inhibited were the elevation of $[K^+]_i$ or by preventing the efflux of intracellular $K^+$. Intracellular $[^{86}\text{Rb}^+]$ decreased very quickly in elevated $[K^+]_{\text{ext}}$. This was not necessarily due to shrinkage of cells but because depolarisation of the plasma membrane by elevated $[K^+]_{\text{ext}}$ would have opened $K^+$ ion channels in the plasma membrane (such as voltage gated or inward rectifying channels), leading to a rapid equilibration of $^{86}\text{Rb}^+$ between the cytoplasm and culture medium. Consequently it was not possible to ascertain whether culturing cells in elevated $[K^+]_{\text{ext}}$ led to an increase in $[K^+]_i$ or prevented efflux of intracellular $K^+$ during apoptosis.

Since elevated $[K^+]_{\text{ext}}$ did not prevent binding of anti-CD95 antibody to cells (Figure 6.3) but no processing of caspase-8 was observed, inhibition of apoptosis occurred between binding of the antibody to the cell and activation of capase-8. This led to investigation of formation of the DISC. Analysis of recruitment of caspase-8 and FADD to the plasma membrane demonstrated that elevated $[K^+]_{\text{ext}}$ inhibited apoptosis downstream of these events (Figure 6.5). It was therefore concluded that CD95-mediated apoptosis was inhibited at the level of the plasma membrane, by preventing the activity of the DISC. Although differences in protein redistribution in the plasma membrane were clear upon anti-CD95 antibody stimulation, the use of a type I cells line such as SKW6.4 B lymphocytes might have yielded
better results. As detailed in Chapter One (section 1.4.2) Jurkat T cells are type II cells, with death receptor-induced apoptosis being mediated by amplification of the apoptotic signal via the mitochondria, since relatively few DISC complexes are formed in type II cells, with a correspondingly low amount of caspase-8 being activated prior to mitochondrial involvement (Figure 1.4). Type I cells form a greater quantity of DISC complexes upon death receptor ligation and are capable of activating sufficient caspase-8 to directly activate effector caspases independent of the mitochondria. Therefore analysis of DISC formation in a type I cell line might give clearer results due to the greater quantity of DISC complexes formed in the plasma membrane.

In contrast with CD95-mediated apoptosis, the plasma membrane is not known to be involved in initiating etoposide-induced apoptosis, which strongly suggested that elevated $[K^+]_{\text{ext}}$ had different loci of action for the two apoptotic stimuli. Although etoposide induced processing of caspase-8, it has been demonstrated that this activation is independent of the CD95 receptor system (Boesen-de Cock et al., 1998; Ferrari et al., 1998). Processing of caspase-8 was likely mediated by death receptor-independent intracellular signalling, and therefore inhibition of etoposide-induced apoptosis by elevated $[K^+]_{\text{ext}}$ was unlikely to have been at the level of caspase-8 activation. Release of cytochrome c in etoposide-mediated apoptosis can occur independently of cleaved Bid, whereas such cleavage is required in CD95-mediated apoptosis (Sun et al., 1999). Etoposide-induced cytochrome c release may be mediated via modulation of the stress-activated protein kinase cascade (Kauffmann, 1998), release of nuclear factors or direct action on the mitochondria (Robertson et al., 2000). Our data suggest that elevated $[K^+]_{\text{ext}}$ inhibited etoposide-induced apoptosis by interfering with the intracellular signalling 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 non-selective K$^+$ channel blocker tetrapentyl ammonium (Dallaporta et al., 1999).

Previous studies have proposed that elevated $[K^+]_{\text{ext}}$ inhibits apoptosis by preventing efflux of intracellular K$^+$, thereby preventing activation of caspases (Bortner et al., 1997; Bilney and Murray, 1998; Orlov et al., 1999). 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\(^+\)]\(_{\text{ext}}\) (Chapter Four, Figure 4.12), consistent with activation of caspases in low [K\(^+\)]. However, elevated [K\(^+\)]\(_{\text{ext}}\) inhibited cytochrome c release (Figure 6.4), thus preventing oligomerisation of Apaf-1 and formation of the caspase-activating apoptosome complex. It was therefore concluded that elevated [K\(^+\)]\(_{\text{ext}}\) inhibited etoposide-induced apoptosis prior to any involvement of caspases.

Although the inhibition of apoptosis prior to apoptosome complex formation was demonstrated, it was decided to further investigate previous findings that show inhibition by increased [K\(^+\)] of caspase-3-like activation but not caspase activity in cell extracts (Bortner et al., 1997; Hughes et al., 1997). In addition, the cell free system allowed us to examine actions of K\(^+\) on cytoplasmic proteins without changes in membrane properties, since elevated [K\(^+\)]\(_{\text{ext}}\) would depolarise the plasma membrane of cultured cells and also dissipate the electrochemical gradient for the flow of K\(^+\) out of the cell. These 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 (Figures 6.13 and 6.14). This explains why activation of caspases but not their activity per se is inhibited by increased [K\(^+\)]. Although inhibition was not specific for K\(^+\) or Na\(^+\), inhibition by 50 mM Na\(^+\) was not physiologically relevant as the intracellular [Na\(^+\)] of cells is unlikely to fluctuate much from the normal value of ~12 mM, due to the impermeability of the plasma membrane to Na\(^+\). This demonstrates that, independent of the effects of elevated [K\(^+\)]\(_{\text{ext}}\) on the plasma membrane potential and electrochemical gradients, maintained cytoplasmic [K\(^+\)] will inhibit the initiation of the post-mitochondrial caspase cascade. As caspases are activated during the execution phase of apoptosis, other cellular factors, possibly not present in the cell-free system used, must be present to alleviate this inhibition of caspase activation by cytoplasmic [K\(^+\)].

The point at which elevated [K\(^+\)]\(_{\text{ext}}\) blocked the apoptotic program has been established, but the mechanisms by which apoptosis was inhibited were more difficult to determine. Varying [Na\(^+\)]\(_{\text{ext}}\) against [K\(^+\)]\(_{\text{ext}}\) revealed a sharp increase in the inhibition of CD95-mediated apoptosis at approximately 95 mM K\(^+\) (Figure 6.6). This is consistent with previous findings (Walev et al., 1995), where caspase-1-mediated processing of IL-1\(\beta\) was inhibited by 150 but not 120 mM potassium. Inhibition of apoptosis may have been mediated by a reversal
in ionic driving force preventing $K^+$ efflux or even increasing $[K^+]_i$. Alternatively, depolarisation of the plasma membrane by elevated $[K^+]_{\text{ext}}$ might have disrupted apoptotic signalling events by changing the state of transmembrane ion channels or by affecting the movement of other voltage-sensitive proteins embedded within the membrane. There is some evidence for an early depolarisation of the plasma membrane prior to reduction of $\Psi_m$ (Ferlini et al., 1999; Chapter Four), and therefore, as suggested previously (Chacon-Cruz et al., 1998), elevated $[K^+]_{\text{ext}}$ might inhibit CD95-mediated apoptosis by depolarising the plasma membrane rather than buffering movement of $K^+$ ions.

As previously stated, initiation of etoposide-induced apoptosis has no known involvement at the plasma membrane, leading to the suggestion that $[K^+]_{\text{ext}}$ affected $[K^+]_i$ by altering driving forces of $K^+$ across the plasma membrane. The inhibition of etoposide-induced apoptosis by depletion of $[Na^+]_{\text{ext}}$ (Figure 6.8), however, indicated a possible involvement of ion flux across the plasma membrane. This result was consistent with Tsao et al. (1996), who showed that depletion of $Na^+$ from medium delays or completely inhibits spontaneous apoptosis in thymocytes. In following with this, Kawazoe et al. (1999) showed inhibition of bufalin-induced apoptosis in HL60 cells by $Na^+$-deficient medium. While it was not ruled out that NMDG was responsible for the inhibition of apoptosis, inhibition by the depletion of $[Na^+]_{\text{ext}}$ appears to support data from Chapter Four demonstrating the depolarisation of the plasma membrane during apoptosis. One way that plasma membrane depolarisation might occur is the influx of a cation such as $Na^+$, and thus the removal of extracellular $Na^+$ might inhibit apoptosis by preventing this influx. Depletion of $[Na^+]_{\text{ext}}$ might have inhibited etoposide-induced apoptosis by a different mechanism from elevated $[K^+]_{\text{ext}}$ as depletion of $[Na^+]_{\text{ext}}$ only delayed induction of apoptosis and still permitted activation of caspases after 10 h (Figure 6.9). Although anti-CD95 antibody- and etoposide-treated cells in elevated $[K^+]_{\text{ext}}$ did eventually die, caspase activation was completely inhibited up to 24 h. An interesting further experiment would have to assess the inhibition of apoptosis while titrating of $[Na^+]$ against $[\text{NMDG}]$ in a similar fashion to the titration of $[Na^+]$ against $[K^+]$ in section 6.2.6.

These data do not support the hypothesis that elevated $[K^+]_{\text{ext}}$ inhibits apoptosis by preventing cell shrinkage or efflux of intracellular $K^+$. While the data suggested that both CD95- and chemical- mediated apoptosis were inhibited upstream of apoptotic cell shrinkage.
by elevated $[K^+]_{\text{ext}}$ (Figure 6.2), it was demonstrated that caspase-8 activation in CD95-mediated apoptosis was prior to cell shrinkage (Figure 4.12) and that etoposide-induced cell shrinkage was concomitant with but did not precede reduction of $\Psi_m$ or PS externalisation (Figure 4.12). Since no caspase activation, reduction of $\Psi_m$ or PS externalisation was seen in either anti-CD95 antibody- or etoposide-treated cells, it is concluded that elevated $[K^+]_{\text{ext}}$ inhibited apoptosis prior to cell shrinkage but the inhibition of apoptosis was not mediated primarily by prevention of this shrinkage.

The failure of elevated $[K^+]_{\text{ext}}$ to inhibit STS-induced apoptosis in THP.1 cells and only partial inhibition in Jurkat cells was interesting. The differences in the apoptotic mechanisms between STS and other apoptotic stimuli may indicate the point of inhibition of apoptosis. The minimal processing of caspase-3 and -8 in STS-treated THP.1 cells indicated that perhaps elevated $[K^+]_{\text{ext}}$ inhibited caspase-dependent mechanisms, while STS-induced apoptosis proceeded by a caspase-independent mechanism. This would be consistent with Déas et al. (1998), who showed that STS induces caspase-independent apoptosis in T cells. The investigation of inhibition by elevated $[K^+]_{\text{ext}}$ of apoptotic stimuli with mechanisms of action similar to STS might show whether the STS result was anomalous or whether only stimuli dependent on certain processes such as caspase activation are inhibited. Alternatively, STS-treatment of THP.1 and Jurkat cells may have induced a combination of apoptosis and necrosis. While induction of apoptosis in Jurkat cells might have been inhibited by elevated $[K^+]_{\text{ext}}$ to give partial protection from STS, the necrotic cell death might not have been inhibited, particularly in the THP.1 cells, where no decrease in induction of cell death in elevated $[K^+]_{\text{ext}}$ was observed.

Manipulation of $[K^+]_{\text{ext}}$ greatly affects the execution of apoptosis, possibly acting at several loci and by several mechanisms to inhibit induction of apoptosis by different stimuli. Elevated $[K^+]_{\text{ext}}$ acted at the plasma membrane, inhibiting CD95-mediated apoptosis by preventing activation of caspase-8 at the DISC, and also appeared to act at an intracellular level, blocking signalling upstream of mitochondrial perturbation in chemical-mediated apoptosis, and inhibiting caspase activation by preventing formation of the biologically active ~700 kDa apoptosome complex. While 135 mM $[K^+]_{\text{ext}}$ is a supra-physiological concentration, its putative effect in reversing driving force potassium ions across the semi-permeable
membrane, increasing cytoplasmic \([K^+]\), may yield a physiological response in its inhibition of apoptosis.

There are still several interesting experiments that might be performed for a fuller understanding of the effects of potassium on this signal transduction pathway. Caspase-8 cleavage was often used as a marker for caspase activation but is not necessarily appropriate for chemical-mediated apoptosis. It might have been more relevant to investigate caspase-9 activation in elevated \([K^+]_{\text{ext}}\) as this is the apical caspase in chemical-mediated apoptosis (as detailed in 1.4.3). The antibody for caspase-9 gave poor results in these studies, however, whereas immunoblotting for caspase-8 gave unambiguous indication of caspase activation. Western blots for Bid cleavage were also performed but again yielded poor results. The demonstration of induction of apoptotic markers upstream of inhibition by elevated \([K^+]_{\text{ext}}\) in etoposide-treated cells such as DNA damage would have been useful in narrowing down the point of inhibition. As described in Chapter Three, a conventional explanation for the mechanism by which 25 mM medium \([K^+]\) might protect CGN cultures from spontaneous apoptosis is by opening voltage-gated ion channels in the plasma membrane and causing an influx of \(Ca^{2+}\). By adding the ion chelator EDTA to the elevated \([K^+]_{\text{ext}}\) medium, thus removing all free \(Ca^{2+}\) and thus preventing any influx of \(Ca^{2+}\), it could have been tested whether inhibition of CD95- or chemical-mediated apoptosis by elevated \([K^+]_{\text{ext}}\) was mediated by an influx of \(Ca^{2+}\).

6.4 SUMMARY

- Elevated \(K^+\) inhibited CD95-mediated apoptosis by preventing the activation of caspase-8 by the DISC.
- Etoposide-induced apoptosis was inhibited prior to cytochrome c release, possibly by interference with the intracellular signalling of DNA damage.
- Formation of the functional \(~700\) kDa apoptosome formation was prevented by 50mM \([K^+]_{\text{ext}}\). This offers an explanation for previous literature (Hughes et al., 1997), which showed a lack of caspase activation in cell lysates with elevated \([K^+]\).
- From present experiments, the mechanism by which elevated \([K^+]_{\text{ext}}\) inhibited apoptosis was complex. Inhibition of apoptotic stimuli with diverse modes of action
suggested that inhibition was at several loci or by several mechanisms. Two mechanisms that seem more plausible are depolarisation of the plasma membrane and maintenance of normal \([K^+]_i\).

**ACKNOWLEDGEMENTS**

Gel filtration, cell lysate activation and Western blotting for Apaf-1 were performed by Claudia Langlais.

Parts of this Chapter have been published in:


This publication is included in the Appendix.
Chapter Seven. Mechanisms of N-Acetylleucine chloromethylketone-induced apoptosis.

7.1 INTRODUCTION

Work by Yamaguchi et al. (1999) has shown that N-acetylleucine chloromethylketone (ALCK), a proposed specific inhibitor of acylpeptide hydrolase (ACPH) (Krishna and Wold, 1992), induces apoptosis in U937 cells. The inference from these data was that ACPH activity is essential for cell survival and its inhibition leads to induction of apoptosis. As described in Chapter Six, apoptotic stimuli such as etoposide that induce chemical stress are mediated by as yet unknown signalling pathways prior to mitochondrial perturbation. Induction of apoptosis by inhibition of ACPH suggests that this enzyme may have a functional role in intrinsic apoptotic signalling pathways. ACPH is a serine peptidase that cleaves N-terminal acylated amino acids from short peptides to produce an acylated amino acid and a peptide with a free NH$_2$-terminus (Krishna and Wold, 1992). ACPH belongs to a novel family of serine peptidases, the prolyl oligopeptidase family, that is unrelated to the trypsin and subtilisin family of serine hydrolases and has evolved an Asp-His-Ser catalytic triad by convergent evolution (Rawlings et al., 1991). Although the catalytic activity of ACPH has been characterised, the exact biological function of the enzyme remains unknown.

Recent biochemical evidence indicates that inhibition of ACPH activity is correlated with improved learning and memory in a rat behavioural model (van der Staay et al., 1996; Richards et al., 2000). This has led to the hypothesis that acylpeptide hydrolase may play a role in cognition, possibly through the hydrolysis of N-acetylated neuropeptides (Richards et al., 2000). The enzyme is therefore of potential therapeutic value as a target for cognition-enhancing drugs. Although the mechanism through which this effect is mediated is unknown, it seems plausible that inhibition of the hydrolysis or processing of N-acetylated neuropeptides is the primary event. Thus inhibition of ACPH activity may prove to be a therapeutic target for the development of new drugs for the treatment of cognitive disorders such as in Alzheimer’s disease. The induction of apoptosis by inhibitors of ACPH, however, would prevent the therapeutic use of these agents in the
treatment of cognitive disorders such as Alzheimer's disease, but may instead lead to the development of novel cancer therapeutics.

Several organophosphate (OP) compounds that are potent inhibitors of ACPH activity (Figure 7.1) were employed in three cell lines to explore the link between ACPH inhibition and apoptosis. The aims of this chapter were:

i) to confirm the results of Yamaguchi et al. (1999) that ALCK induces apoptosis.

ii) to examine, if ALCK does induce apoptosis, whether this is achieved by inhibition of ACPH.

iii) to explore the hypothesis that ACPH activity is essential to cell survival and whether its inhibition is centrally involved in the induction of apoptosis.
Figure 7.1. Structure of organophosphate ACPH inhibitors. IC₅₀ values for the inhibition of ACPH are given for a 20 min incubation at 37 °C in 200 mM Tris/HCl, pH 7.4.


7.2 Results

7.2.1 Concentration-dependent inhibition of ACPH activity and induction of apoptosis by ALCK.

It has previously been established that ALCK induces apoptosis in U937 cells (Yamaguchi et al., 1999). The induction of apoptosis by ALCK treatment was investigated in Jurkat cells, a cell line that has previously been demonstrated to be sensitive to a number of apoptotic stimuli (Sun et al., 1999; Chapters Four and Six). The potential of ALCK to inhibit ACPH and induce cell death was tested over a range of concentrations. Cells were incubated for 12 h with various concentrations of ALCK or the DNA topoisomerase II inhibitor etoposide (Chen et al., 1984), as a positive control for induction of apoptosis. Cell lysates were then analysed for ACPH activity and cell cultures for externalisation of PS, a plasma membrane phospholipid that is flipped to the cell surface as a phagocytotic marker and is a signatory event in many forms of apoptosis (Martin et al., 1995). ALCK caused a concentration dependent induction of apoptosis in Jurkat cells with a 50 % effect level at a concentration of 60.0 ± 7.1 μM (Figure 7.2). In contrast, the IC₅₀ for the inhibition of ACPH activity by ALCK was 3.6 ± 0.7 μM. At an ALCK concentration of 40 μM the level of ACPH inhibition was 96.9 ± 1.6 %, showing nearly complete inhibition of the enzyme activity at a concentration below that which induced 50 % cell death. These data suggested that cell death induced by ALCK was not directly mediated through inhibition of ACPH activity, as ACPH inhibition was much more sensitive than the induction of cell death.

Induction of apoptosis was confirmed by assessing cleavage of PARP, which is cleaved by caspases -3 and -7 at a DEVD-G motif to give a characteristic 85 kDa signature fragment (Lazebnik et al., 1994; Cohen, 1997). The PARP of control cells was entirely in its proform (Figure 7.3A, lane 1). The appearance of the 85 kDa signature fragment along with a corresponding disappearance in the proform was observed in etoposide-treated Jurkat cells (Figure 7.3A, lane 5), consistent with the 80.1 % of cells quantified as apoptotic by PS externalisation (Figure 7.3C). A decrease in caspase-3 proform relative to the control was also seen in etoposide-treated cells (Figure 7.3B, compare lanes 1 and 5), consistent with processing to its active form. ALCK treatment almost completely inhibited ACPH activity (94.8 % inhibition of control; Figure 7.3D, lane 4) with associated induction of apoptosis (39.5 %; Figure 7.3C, lane 4).

Although no cleaved fragments or change in proform of caspase-3 were observed (Figure 7.3B), ALCK induced cleavage of PARP (Figure 7.3A, lane 4), confirming that
cell death was principally by apoptosis. Changes in cell morphology were assessed by light microscopy as an additional marker of apoptosis. Cell shrinkage, membrane blebbing and nuclear fragmentation were seen in cell cultures exhibiting externalisation of PS and cleavage of PARP, indicating that apoptosis rather than necrosis was the principal mode of cell death induced by ALCK in Jurkat cells.

Figure 7.2: ALCK inhibits ACPH and induces apoptosis in Jurkat cells. The percentage of apoptotic cells (○) or ACPH inhibition relative to control cells (●) was recorded 12 h after the addition of various concentrations of ALCK, as assessed by PS externalisation and AANA cleavage, respectively. The control level of ACPH activity in Jurkat cells was 7 ± 1 nmol AANA hydrolysed/min /10^6 cells. Data are the mean and S.E.M. of 3 independent experiments.
Figure 7.3: ALCK induces PARP cleavage and caspase-3 processing in Jurkat, SKW6.4 and U937 cells. Cells were treated with vehicle (DMSO), dichlorvos (1 μM), mipafox (1 μM), ALCK (40 μM) or etoposide (50 μM) for 12 h before analysis of PARP cleavage (A) and caspase-3 processing (B) by Western blotting, quantification of apoptosis by PS externalisation (C) and inhibition of ACPH activity as assessed by AANA cleavage (D). The control levels of ACPH activity in various cell lines (nmol AANA hydrolysed/min /10^6 cells) were Jurkat, 6 ± 2; SKW6.4, 9 ±3; U937, 12 ± 4. Data are representative of 3 independent experiments.
7.2.2 Dissociation between the inhibition of ACPH and induction of apoptosis using dichlorvos in Jurkat cells.

To further explore the structure-activity relationship between ACPH inhibition and apoptosis, two OP esters that have been shown to be potent inhibitors of ACPH activity (Richards et al., 2000) were used, namely dichlorvos and mipafox (Figure 7.1). Dichlorvos was found to inhibit ACPH activity in Jurkat cells at concentrations as low as 100 nM where 83.5 ± 6.3 % ACPH inhibition was detected (Figure 7.4). At concentrations of 1 μM dichlorvos and above, >95 % inhibition of ACPH was achieved. In contrast to the high levels of ACPH inhibition, apoptosis was apparent only at dichlorvos concentrations of 100 μM and above and did not appear to be correlated to the inhibition of ACPH. Neither PARP nor caspase-3 cleavage was observed at 1 μM dichlorvos (Figure 7.3, A and B, lane 2), a concentration that inhibited 95.6 % of ACPH activity (Figure 7.3D, lane 2).

Figure 7.4. Response of Jurkat cells to dichlorvos. The percentage of apoptotic cells (○) and ACPH inhibition relative to control cells (●), as assessed by PS externalisation and AANA cleavage, respectively, were recorded 12 h after the addition of various concentrations of dichlorvos. The control level of ACPH activity in Jurkat cells was 6 ± 3 nmol AANA hydrolysed/min /10⁶ cells. Data are the mean and S.E.M. of 3 independent experiments.
A concentration of 1 µM mipafox induced 97% inhibition of ACPH activity (Figure 7.3D, lane 3) without cleavage of PARP, caspase-3 cleavage or induction of apoptosis (Figure 7.3, A, B and C respectively). Thus using dichlorvos or mipafox, almost complete inhibition of ACPH could be observed with no induction of apoptosis.

7.2.3 Response of SKW6.4 and U937 cell lines to ALCK and dichlorvos: effects on cell viability and ACPH activity.

As inhibition of ACPH could be observed in the absence of apoptosis in Jurkat cells, the study was extended to include other cell lines, namely SKW6.4 B lymphocytic and monocytic U937 cell lines, to verify that the results hitherto obtained were not cell line specific. In agreement with the observation of Yamaguchi et al. (1999), ALCK induced apoptosis and inhibited ACPH activity in U937 cells (Table 7.1). It is clear that although ALCK at 40 µM inhibited ACPH activity, dichlorvos (1 µM) produced similar levels of ACPH inhibition without induction of apoptosis above control levels (< 5%). ACPH activity was also decreased in samples where there had been extensive cell death. This decrease would be expected, as ACPH is a cytoplasmic enzyme that would be released from cells after loss of membrane integrity. The extent of apoptosis induced by ALCK was cell-type specific, with Jurkat cells being the most responsive and both SKW 6.4 and U937s showing a relatively slight response. Again the PARP cleavage data (Figure 7.3A) was consistent with ALCK inducing apoptosis, whereas other ACPH inhibitors had no apparent effect on cell survival.


<table>
<thead>
<tr>
<th></th>
<th>Jurkat</th>
<th>SKW 6.4</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loss of</td>
<td>Loss of</td>
<td>Loss of</td>
</tr>
<tr>
<td></td>
<td>ACPH activity (%)</td>
<td>ACPH activity (%)</td>
<td>ACPH activity (%)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.8 ± 0.4</td>
<td>4.9 ± 0.9</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>ALCK</td>
<td>95 ± 0.6</td>
<td>96 ± 1.2</td>
<td>96 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>43 ± 1.9</td>
<td>20 ± 1.7</td>
<td>14.3 ± 2.2</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>96 ± 0.6</td>
<td>96 ± 0.1</td>
<td>96 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>4 ± 0.6</td>
<td>4 ± 1.2</td>
<td>3 ± 1.2</td>
</tr>
<tr>
<td>Etoposide</td>
<td>71 ± 13.9</td>
<td>43 ± 7.5</td>
<td>42 ± 4</td>
</tr>
<tr>
<td></td>
<td>71 ± 6.4</td>
<td>9 ± 1.7</td>
<td>52 ± 8.7</td>
</tr>
</tbody>
</table>

Table 7.1. Effect of ACPH inhibitors and etoposide on culture viability and ACPH activity. Percentage of apoptotic cells or percentage ACPH inhibition relative to control samples were determined in Jurkat, SKW6.4 and U937 cell lines, 12 h after addition of ALCK (40 μM), dichlorvos (1 μM) or etoposide (50 μM). Control levels of ACPH activity in various cell lines (nmol AANA hydrolysed/min /10^6 cells) were Jurkat, 6 ± 1.2; SKW6.4, 9 ± 1.7; U937, 12 ± 2.3. Data are mean and S.E.M. of 3 independent experiments.

7.2.4 Dissociation between the inhibition of ACPH and induction of apoptosis in Jurkat, U937 and SKW6.4 cells using the ACPH inhibitors mipafox and chlorpyrifos methyl oxon.

Two further OP ester inhibitors of ACPH, mipafox and chlorpyrifos methyl oxon (Figure 7.1) were included in this study to explore the relationship between ACPH inhibition and cell survival. Both of these compounds have been used for in vitro neuropathy screening using a number of neuronal cell lines (Schmuck and Ahr, 1997). The OP esters were added at four different concentrations (1 mM, 100 μM, 10 μM and 1 μM) to Jurkat, U937 and SKW6.4 cell cultures and effects on ACPH inhibition and culture viability studied. ACPH activity was inhibited to >90 % compared to controls for all combinations except for 1 μM chlorpyrifos methyl oxon against Jurkat cells where only 75.8 % inhibition of ACPH activity was observed. ACPH in the presence of 1 μM mipafox was inhibited to between 92.2 and 99.5 % of control activity. Yet, despite this high level of inhibition, no cell death or PARP cleavage was observed (Figure 7.3A). Chlorpyrifos methyl oxon induced a notable degree of apoptosis in all cell lines at 1 mM, and in Jurkat cells at 10 and 100 μM. However, as ACPH activity was inhibited to >90 % at a
concentration of 1 μM, this effect appeared to be unrelated to ACPH inhibition. Analysis of cells after treatment for 3 h showed >90% inhibition of ACPH with all of the above compounds, demonstrating that ACPH activity was rapidly and persistently inhibited for at least 9 h with the above inhibitors.

7.2.5 Investigation into the specificity of the reaction of ALCK with serine hydrolases.

The data presented in this chapter demonstrate that prolonged inhibition of ACPH did not induce apoptosis. Thus to investigate the mechanism by which ALCK induces apoptosis, tritiated diisopropylfluorophosphate (DFP) was used as a selective reagent to label serine hydrolases in cell lysates. If ALCK reacts covalently in a non-specific fashion with serine hydrolases, then one would predict widespread inhibition of DFP-labelling in cells pre-treated with ALCK. In the concentration range of ALCK that induced apoptosis (20 to 100 μM), a broad inhibition of DFP-labelling of cellular proteins was observed (Figure 7.5). This suggested that ALCK covalently bound to many targets, any one of which may be involved in the apoptotic program. Taken together with the results from the inhibition of ACPH by OP compounds, these data revealed a complete dissociation between apoptosis and the inhibition of ACPH activity and showed that ALCK is not a specific reagent for the inhibition of ACPH.
Figure 7.5: Inhibition of [H\(^3\)]-DFP labelling by ACLK. Profile of [H\(^3\)]-DFP labelling after cells were treated with various concentrations of ALCK for 12 h. The labelled proteins were detected using a microchannel plate detector after blotting onto a membrane. A decrease in labelling shows covalent reaction with serine hydrolases in the lysate. ACPH is a homotetrameric protein with a subunit mass of 82 kDa.

7.2.6 Potentiation of apoptosis by ALCK.

Although direct inhibition of ACPH activity did not induce apoptosis in any of the cell lines tested, potentiation of apoptosis by inhibition of ACPH was investigated. Cells were treated with ALCK in conjunction with the apoptotic inducers etoposide or staurosporine (STS; a protein kinase inhibitor). The concentration of ALCK chosen (10 \(\mu\)M) was below the threshold for inducing a discernable level of apoptosis in Jurkat cells but still inhibited ACPH to a significant degree (Figure 7.2).

Treatment of Jurkat, SKW 6.4 and U937 cells with dichlorvos did not induce apoptosis above control levels (<2.5 %, as assessed by PS externalisation; Figure 7.6). Treatment with ALCK alone induced very low levels of apoptosis (~5 %). Etoposide treatment induced apoptosis to varying degrees in the three cell lines, which was unaffected by dichlorvos treatment in all three cell lines. In Jurkat and SKW6.4 cells,
ALCK had no discernable effect on etoposide-induced apoptosis. Etoposide-induced apoptosis in U937 cells, however, was strongly inhibited by ALCK (19.5 vs. 61.3 % apoptosis). In contrast, while STS-induced apoptosis in Jurkat and U937 cells was unaffected by ALCK treatment, co-treatment of SKW6.4 cells with both STS and ALCK appeared to potentiate apoptosis (23.2 vs. 12.4 %). Dichlorvos had no discernable effect on STS induced apoptosis in all three cell lines.

The results demonstrate the non-specific nature of the action of ALCK, potentiating STS-induced apoptosis in SKW6.4 cells and inhibiting etoposide-induced apoptosis in U937 cells. Dichlorvos did not affect the induction of apoptosis in any of the three cell lines, demonstrating that specific inhibition of ACPH activity had no effect on the apoptotic pathways initiated by etoposide and STS. These data present further evidence that ALCK influences cell survival by a mechanism other than inhibition of ACPH activity.
Figure 7.6: Potentiation of apoptosis by ALCK but not dichlorvos. Jurkat, SKW6.4 and U937 cells were incubated for 12 h with vehicle (DMSO), etoposide (50 μM) or STS (200 nM) in the presence of vehicle (DMSO), ALCK (10 μM) or dichlorvos (1 μM). Apoptosis was assessed by PS externalisation. Data are the mean and S.E.M. of three independent experiments.
7.3 Discussion

*ACPH inhibition alone does not induce apoptosis.* The clear conclusion from the data presented in this chapter is that ACPH activity can undergo virtually complete and sustained inhibition without inducing apoptosis in Jurkat, U937 or SKW6.4 cells. The *in vitro* data presented strongly support previous observations that ACPH activity can be inhibited *in vivo* with no obvious detrimental effect on animal health (Richards et al., 2000). In this case, rats were treated with a single dose of dichlorvos (4 mg/kg/i.p.) that produced an average of 93% inhibition of ACPH activity. Activity recovered to only 50% after 5 days, yet no detrimental effects on health or body weight were observed over this time period.

The data demonstrate that ACPH is not directly involved in apoptosis. The role of ACPH in cellular function, however, is unknown. There have been a number of suggestions regarding the role of ACPH in controlling the hydrolysis of N-acylated neuropeptides and growth factors (Jones et al., 1986). A region of chromosome 3 containing the gene for ACPH is deleted in some forms of kidney and small cell lung carcinomas, leading to the suggestion that it may catabolise growth factors and that gene deletion causes accumulation of these substances (Erlandsson et al., 1991). More recent suggestions have included a role for ACPH in the hydrolysis of oxidised proteins (Fujino et al., 1998; Fujino et al., 2000) and a possible role in learning and memory formation (Richards et al., 2000). If ACPH plays a role in the cognitive process and inhibitors of the enzyme have therapeutic potential, dissociation of ACPH inhibition from apoptosis is an important finding for the design of therapeutic inhibitors.

*ALCK induces apoptosis by non-specific inhibition of serine hydrolases.* While the cellular target of ALCK for the identification of apoptosis was not identified, analysis of inhibition of DFP labelling sites showed that, at concentrations of ALCK required for induction of apoptosis, the compound reacted with a number of serine hydrolases (Figure 7.5). This extensive inhibition of serine hydrolases would be likely to cause accumulation of substrates in the cell. The induction of apoptosis by ALCK therefore most likely results from cell stress mechanisms. The potentiation of STS-induced apoptosis in SKW6.4 cells and inhibition of etoposide-induced apoptosis in U937 cells (Figure 7.6) further illustrates the non-specific action of ALCK on cellular processes.
7.4 SUMMARY

- Using alternative and more potent inhibitors of ACPH, prolonged and virtually complete inhibition of this enzyme could be established in Jurkat, U937 and SKW 6.4 cell lines without induction of apoptosis.
- As direct inhibition of ACPH did not induce apoptosis, it remains a valid target for development of cognition enhancing drugs.
- ALCK did not specifically inhibit ACPH at concentrations that induce apoptosis. Rather it had a broad reactivity with serine hydrolases at concentrations that induced apoptosis.
- Non-specific inhibition of a range of serine hydrolases in the cell appeared to induce apoptosis by cellular stress mechanisms.

ACKNOWLEDGEMENTS

The ACPH assay and tritiated-DFP labelling were performed by Paul Richards (M.R.C., Leicester).
Chapter Eight. General discussion

8.1 Flux of intracellular \( K^+ \) during apoptosis

8.1.1 Efflux of intracellular \( K^+ \)

Overall, this study demonstrated that induction of both death receptor- and chemical-mediated apoptosis in Jurkat T cells was associated with an efflux of intracellular \( K^+ \) that was concomitant with cell shrinkage, reduction of \( \Psi_m \) and PS externalisation (sections 4.2.3 and 4.2.5). The data did not support the hypothesis that efflux of \( K^+ \) is a relatively early event, as proposed by Bortner and Cidlowski (1996) and Hughes et al. (1997). Neither was it a late-stage secondary event, but data did indicate that efflux of \( K^+ \) contributes to the apoptotic phenotype. The close association of \( K^+ \) efflux and cell shrinkage suggests that they are related, with water leaving the cell as an osmotic consequence of an efflux of \( K^+ \) ions.

8.1.2 \( K^+ \) flux across the inner mitochondrial membrane

The involvement of \( K^+ \) flux in the reduction of \( \Psi_m \) during apoptosis was investigated by comparison of mitochondrial \( K^+ \) concentration in apoptotic and normal CEM C7H2 cells (section 5.2.3). Cells treated with anti-CD95 antibody showed an apparent increase in mitochondrial matrix \( K^+ \) concentration. These data suggest that mitochondrial changes during apoptosis are associated with gating of inner mitochondrial membrane channels, leading to an influx of \( K^+ \) and water molecules. This would lead to the observed reduction of \( \Psi_m \) with increased matrix volume resulting in rupture of the outer mitochondrial membrane and release of pro-apoptotic intermembrane proteins.

8.1.3 Depolarisation of the plasma membrane

Changes in the plasma membrane potential during apoptosis were investigated in Jurkat T cells. In both death receptor- and chemical-mediated apoptosis, depolarisation of the plasma membrane occurred synchronously with PS externalisation and cell shrinkage (section 4.2.1). This result has recently been confirmed by Bortner et al. (2001). It was not clear what mediates this plasma membrane depolarisation, but data from section 6.2.8, demonstrating inhibition of etoposide-induced apoptosis by removal of extracellular \( Na^+ \), suggests that this depolarisation might be mediated by an influx of \( Na^+ \). The lack of association between cell
shrinkage and plasma membrane depolarisation in cells undergoing apoptosis (section 4.2.1) indicates that these events are not closely related. Consistent with the observations in this study, Bortner et al. (2001) have recently reported that following apoptotic stimulation of Jurkat cells, a rapid increase in intracellular [Na+] and accompanying plasma membrane depolarisation are observed.

8.1.4 Caspase inhibition of CD95-mediated apoptosis

Using the broad-spectrum caspase inhibitor z-VAD.fmк, it was demonstrated that both plasma membrane depolarisation and efflux of intracellular K\(^+\) were dependent upon caspase-activation in CD95-mediated apoptosis (sections 4.2.2, 4.2.4 and 4.2.6). Sun et al. (1999) demonstrated that z-VAD.fmк prevents CD95-mediated caspase-8 activation, Bid cleavage, cytochrome c release and reduction of \(\Psi_m\). Combining the current mechanistic scheme for CD95-mediated apoptosis (Figure 1.4) with the data from Chapter Four, it is hypothesised that PS externalisation, cell shrinkage, efflux of intracellular K\(^+\) and plasma membrane depolarisation are mediated by intracellular signalling pathways that are dependent upon the activation of initiator caspases (Figure 8.1). It cannot be deduced from this data whether these pathways are directly activated by caspase-8 or by other effectors of apoptosis. Neither can it be discerned from this data whether PS externalisation, cell shrinkage, efflux of intracellular K\(^+\) and plasma membrane depolarisation are mediated by a common mechanism or multiple pathways. Cell shrinkage is represented in Figure 8.1 as efflux of water accompanied by K\(^+\) and Cl\(^-\) ions. Plasma membrane depolarisation is represented as the influx of cations and efflux of anions, though it is not clear which of these two possible events mediate this depolarisation.
Figure 8.1: Inhibition of CD95-mediated apoptosis by z-VAD.fmk. Inhibition is prior to shrinkage, efflux of K\(^+\), plasma membrane depolarisation and PS externalisation.
8.1.5 Caspase inhibition of etoposide-induced apoptosis

Investigations into caspase inhibition of etoposide-induced apoptosis demonstrated that cell shrinkage, efflux of intracellular $K^+$ and plasma membrane depolarisation but not PS externalisation were independent of caspase activation (sections 4.2.2 and 4.2.6). Combining the current mechanistic scheme for etoposide-induced apoptosis (Figure 1.5) with data from Chapter Four, it is hypothesised that as yet unknown caspase-independent apoptotic signalling pathways mediate cell shrinkage, efflux of intracellular $K^+$, plasma membrane depolarisation, reduction of $\Psi_m$ and cytochrome c release (Figure 8.2). From the data presented it can not be deduced whether a single or multiple pathways mediate these changes. The prevention of PS externalisation by caspase inhibition suggests that this is mediated by a different signal pathway to cell shrinkage and plasma membrane depolarisation. Although formation of the apoptosome has not been demonstrated in vivo, data from cell lysate studies in vitro indicates that release of cytochrome c from the mitochondria would stimulate apoptosome formation, but the caspase-activating activity of this complex would be blocked. Comparing the inhibition of CD95- and chemical-mediated apoptosis by z-VAD.fmk, there appear to be a caspase-dependent signalling pathway for PS externalisation and a separate pathway for cell shrinkage, efflux of intracellular $K^+$ and plasma membrane depolarisation that is dependent on initiator caspase activation in CD95- but not chemical-mediated apoptosis.
Figure 8.2: Inhibition of etoposide-induced apoptosis by z-VAD.fmk. Inhibition is prior to caspase activation and PS externalisation but downstream or independent of cell shrinkage, efflux of K+, plasma membrane depolarisation or cytochrome c release.
8.2 MECHANISM OF INTRACELLULAR K⁺ EFFLUX DURING APOPTOSIS

Several approaches were taken to investigate how the efflux of intracellular K⁺ during apoptosis is mediated. Using a range of K⁺-selective channel blockers, a role for K⁺ channels and the Na⁺,K⁺-ATPase antiporter in apoptosis was demonstrated but no further conclusions using these approaches could be made as to the identity of K⁺ channels mediating this apoptotic efflux of K⁺ (section 5.2.1). The close association between cell shrinkage and efflux of intracellular K⁺ during apoptosis suggested a possible role for Kv1.3, an important ion channel in regulatory volume decrease, but surprisingly a specific peptide blocker of Kv1.3, rMgTX, had no effect on the induction of CD95- or chemical-mediated apoptosis. The caspase cleavage of Kᵢᵣ6.1 and Kᵢᵣ6.2, two candidate ion channels for a role in apoptotic K⁺ efflux, was investigated as a possible mechanism for mediating efflux (section 5.2.2). Neither channel was cleaved by caspase-3 or -8 at the predicted sequences, suggesting that efflux of intracellular K⁺ was not mediated by caspase cleavage of these channels. This data is supported by section 4.2.6, which demonstrated that efflux of intracellular K⁺ in etoposide-induced apoptosis is independent of caspase activation. The involvement of the mitochondrial K⁺ channel mitoK⁺ATP in apoptosis was investigated by constructing constitutively open K⁺ channels that were targeted to the mitochondria (section 5.2.4). While insertion of constitutively open K⁺ channels into the inner mitochondrial membrane may have been lethal to transfected cells, no firm conclusions were possible from this work regarding the role of these channels in apoptosis.

The significance of plasma membrane depolarisation for initiation or execution of apoptosis is not clear from this study. Depolarisation of the plasma membrane might affect the state of voltage-gated ion channels or may affect localisation of voltage-sensitive proteins embedded in the plasma membrane, thus activating signalling cascades. Glyburide may have inhibited etoposide-induced apoptosis by preventing plasma membrane depolarisation, but this cannot be concluded for certain since this channel blocker would have had other effects on K⁺- and Cl⁻-selective ion channels. Elevated [K⁺]ₑₓ decreased plasma membrane potential and may have inhibited CD95- and etoposide-mediated apoptosis by this mechanism, though elevated [K⁺]ₑₓ would also have reduced driving force of K⁺. Alternatively, depolarisation of the plasma membrane potential during apoptosis might be a secondary event, reflecting other ionic changes.
8.3 CYTOPROTECTION BY EXTRACELLULAR K⁺

8.3.1 Cerebellar Granule Neurons

A model of neuronal apoptosis was set up with *ex vivo* CGN cultures. The inhibition of both spontaneous and serum-withdrawal induced apoptosis by raised [K⁺]_{ext} (25 mM) was assessed by staining under UV microscopy (section 3.2.2). Reduction of [K⁺]_{ext} from 25 to 5 mM induced apoptosis in CGN cultures, as characterised by nuclear condensation and fragmentation. Primary CGN cultures are a good approximation of *in vivo* conditions and the concentration of extracellular K⁺ required to inhibit apoptosis (25 mM) is close to physiological conditions (~5 mM), suggesting a role for K⁺ under physiological conditions. While the mechanisms of cytoprotection by 25 mM [K⁺]_{ext} were difficult to discern, the conventional hypothesis is that cytoprotection of CGN cells is achieved by the depolarisation of the plasma membrane, thus opening voltage-gated Ca²⁺ channels, increasing intracellular [Ca²⁺] and stimulating activity of pro-survival Ca²⁺-dependent enzymes (Courtney et al., 1990).

8.3.2 Inhibition of CD95-mediated apoptosis by elevated [K⁺]_{ext}

An extracellular concentration of >130 mM K⁺ was required to inhibit CD95-mediated apoptosis in Jurkat T cells (section 6.2.6). Assessment of different aspects of the apoptotic phenotype suggests that elevated [K⁺]_{ext} (135 mM) inhibited CD95-mediated apoptosis by preventing activation of caspase-8 at the DISC (Figure 8.3). While binding of anti-CD95 antibody (section 6.2.3) and recruitment of FADD and caspase-8 to the plasma membrane (section 6.2.5) were not prevented, apoptotic events downstream of DISC formation were inhibited. No caspase-8 activation was observed in elevated [K⁺]_{ext} following treatment with anti-CD95 antibody (section 6.2.1). Consistent with the current model of CD95-mediated apoptosis (Figure 1.4), inhibition of caspase-8 activation prevented reduction of Ψₘ and cytochrome c release (section 6.2.4), cell shrinkage (section 6.2.2), caspase-3 activation and PS externalisation (section 6.2.1). Inhibition of Bid cleavage was not demonstrated but without activation of caspase-8 this would not occur in CD95-mediated apoptosis. Apoptosome formation would not be predicted to occur in elevated [K⁺]_{ext} as release of mitochondrial cytochrome c was prevented. This inhibition of CD95-mediated apoptosis very early in the signalling cascade offers an explanation for the requirement of elevated [K⁺]_{ext}
from the outset of treatment with anti-CD95 antibody (section 6.2.7) to inhibit apoptosis. Formation of the DISC is an almost instantaneous process that can be detected within seconds of receptor cross-linking (Kischkel et al., 1995; Scaffidi et al., 1997). Without the inhibitor present from the outset of treatment with anti-CD95 antibody, caspase-8 would be activated at the DISC and, even if the cell is subsequently resuspended in elevated [K$^+$]$_{ext}$, the cell would undergo apoptosis (although apoptosis might still be inhibited at other points distal to caspase-8 activation). The similarity in cell phenotypes seen in Figures 8.1 and 8.3 further supports the hypothesis that that caspase inhibition by z-VAD.fmk and elevated [K$^+$]$_{ext}$ inhibit apoptosis at the same point, preventing activation of caspase-8 at the DISC.
CD95 antibody —► Anti-CD95 antibody binds to receptor (Fig 6.3)

CD95 receptor —► Procaspase-8 and FADD are recruited to the DISC (Fig. 6.5)

FADD —► Cell shrinkage is inhibited (Fig. 6.2)

Caspase-8 —► Capase-3 is not activated (Fig. 6.1)

PS externalisation is inhibited (Fig. 6.1)

Apoptosome formation and activity inhibited (Figs. 6.12-14)

Caspase-9 —► Cytochrome c release inhibited (Fig. 6.4)

Volts —► Reduction of $\Psi_m$ does not occur (Fig. 6.4)

Figure 8.3: Elevated $[K^+]_{ext}$ inhibits CD95-mediated apoptosis by preventing caspase-8 activation at the DISC. Application of experimental data to the current model of death receptor-mediated apoptosis suggests that elevated $[K^+]_{ext}$ inhibited apoptosis by preventing activation of caspase-8 at the DISC.
8.3.3 Inhibition of etoposide-induced apoptosis by elevated $[K^+]_{\text{ext}}$

An extracellular concentration of $>115$ mM $K^+$ was required to inhibit etoposide-induced apoptosis in Jurkat T cells (section 6.2.6). Analysis of different aspects of the apoptotic phenotype suggested that elevated $[K^+]_{\text{ext}}$ (135 mM) inhibited etoposide-induced apoptosis prior to mitochondrial perturbation, possibly by interfering with intracellular signalling pathways that are activated following DNA damage (Figure 8.4). In contrast with the inhibition of CD95-mediated apoptosis by prevention of caspase-8 activation, elevated $[K^+]_{\text{ext}}$ did not inhibit etoposide-induced apoptosis by the prevention of caspase activation alone. Comparisons of the inhibition of etoposide-induced apoptosis by z-VAD.fmk (Figure 8.2) and elevated $[K^+]_{\text{ext}}$ (Figure 8.4) showed that the caspase-independent cell shrinkage and mitochondrial perturbation in etoposide-induced apoptosis were inhibited by elevated $[K^+]_{\text{ext}}$ (sections 6.2.2 and 6.2.4, respectively). As in CD95-mediated apoptosis, activation of both caspase-3 and -8 and PS externalisation were inhibited by elevated $[K^+]_{\text{ext}}$ (section 6.2.1). Elevated $[K^+]_{\text{ext}}$ was not required from the outset of etoposide treatment to inhibit apoptosis (section 6.2.7) possibly because etoposide would be predicted to take time to generate DNA damage and so signalling of DNA damage would not immediately follow treatment of cells with etoposide. Elevation of $[K^+]_{\text{ext}}$ subsequent to treatment with etoposide was therefore able to inhibit apoptosis.

From present experiments, the mechanism by which elevated $[K^+]_{\text{ext}}$ inhibited apoptosis is complex. Inhibition of anti-CD95 antibody- and etoposide-induced apoptosis may have been by different mechanisms (Figures 8.3 and 8.4). This is further supported by the inhibition of etoposide- but not anti-CD95 antibody-induced apoptosis to removal of $[Na^+]_{\text{ext}}$ (section 6.2.8) and also the different sensitivity to $[K^+]_{\text{ext}}$ (section 6.2.6). In addition to anti-CD95 antibody and etoposide, elevated $[K^+]_{\text{ext}}$ inhibited apoptosis induced by the death receptor ligand TRAIL, the proteasome inhibitor MG132 and the protein kinase inhibitor STS. Inhibition of apoptotic stimuli with diverse modes of action suggested that inhibition was at several loci or by several mechanisms. Two possible mechanisms are depolarisation of the plasma membrane and maintenance of normal $[K^+]_i$. Induction of apoptosis by etoposide, MG132 or STS has no known involvement at the plasma membrane, suggesting elevated $[K^+]_{\text{ext}}$ inhibits the activity of a number of intracellular enzymes involved in the transduction of apoptotic signalling.
While experiments in 135 mM elevated $[K^+]_{\text{ext}}$ cannot be compared to physiological situations, this supraphysiological concentration may have been required to alter intracellular conditions to inhibit apoptosis. These intracellular changes were caused either by the reversal of $K^+$ driving forces or by depolarisation of the plasma membrane. While these extracellular ionic conditions are highly unlikely to occur \textit{in vivo}, the inhibition of apoptosis by elevated $[K^+]_{\text{ext}}$ suggests that there are electrically or ionically sensitive agents in the cells that might be targeted to inhibit apoptosis if an appropriate blocker can be found.
Figure 8.4: Elevated $[K^+]_{ext}$ inhibits etoposide-induced apoptosis upstream of cytochrome c release and reduction of $\Psi_m$. Application of experimental data to the current model of chemical-mediated apoptosis suggests that elevated $[K^+]_{ext}$ inhibited apoptosis upstream of mitochondrial perturbation, possibly inhibiting intracellular signalling pathways.
8.4 ROLE OF INTRACELLULAR K⁺ EFFLUX IN APOPTOSIS

The conventional hypothesis states that efflux of intracellular K⁺ during apoptosis is required to decrease [K⁺]ᵢ, thus allowing for activation of enzymes involved in the transduction of apoptotic signalling, such as caspases and nucleases, that would otherwise be inhibited by normal [K⁺]ᵢ (Bortner et al., 1997; Hughes et al., 1997; Bilney and Murray, 1998; Orlov et al., 1999). In support, this study demonstrates that formation of the caspase-activating ~700 kDa apoptosome formation was prevented by 50 mM K⁺. This offers an explanation for previous literature (Hughes et al., 1997), which shows inhibition by elevated [K⁺]ₑₓₜ of caspase activation in cell lysates. This implies that, for a cell to undergo apoptosis, [K⁺]ᵢ must be reduced sufficiently to allow for apoptosome formation. By increasing [K⁺]ₑₓₜ, the driving force of K⁺ out of the cell is decreased, thus preventing efflux of intracellular K⁺ and preventing the activation of caspases. This hypothesis also offers an explanation for the inhibition of chemical-mediated apoptosis by glyburide, with pharmacological block of K⁺-selective channels preventing efflux of K⁺ and maintaining an [K⁺]ᵢ that inhibits apoptotic effector proteins.

The inhibition of etoposide-induced apoptosis by K⁺-selective channel blockers may have been mediated by i) depolarisation of the plasma membrane ii) prevention of efflux of intracellular K⁺ iii) prevention of cell shrinkage. The inconsistency between Figures 5.3 and 5.4 suggests that inhibition of apoptosis was not mediated by preventing cell shrinkage, however, as there was little correlation between inhibition of apoptosis and prevention of cell shrinkage. As detailed above, the prevention of intracellular K⁺ efflux did not appear to be the main mechanism by which elevated [K⁺]ₑₓₜ inhibited apoptosis, which suggests that inhibition of apoptosis by K⁺-selective pharmacological block may have been by depolarisation of the plasma membrane potential.

The KCOs diazoxide, RP49356 and P1060 did not induce apoptosis in CGN cultures at concentrations that have previously been shown to cause K⁺ channel opening (section 3.2.4). This is consistent with the hypothesis that efflux of intracellular K⁺ is an event in the apoptotic phenotype that allows the activation of effector enzymes but does not play a role itself in the initiation of apoptosis.
The data in this study reveal several inconsistencies in the hypothesis that elevated \([K^+]_{\text{ext}}\) inhibits apoptosis by preventing efflux of intracellular \(K^+\), thus preventing caspase activation:

i) Efflux of intracellular \(K^+\) in CD95-mediated apoptosis was caspase-dependent (section 4.2.6), which strongly implies that activation of caspase-8 at the DISC must occur at normal \([K^+]_i\) (~150 mM). While activation might occur in a microenvironment within the cell at a lowered \([K^+]\) or caspase-8 might be less susceptible to inhibition by \(K^+\), this is not consistent with the inhibition of CD95-mediated apoptosis by elevated \([K^+]_{\text{ext}}\). Possible explanations for this inhibition might be that elevated \([K^+]_{\text{ext}}\) actually increases \([K^+]_i\) above physiological levels, thereby preventing caspase activation. Alternatively, while elevated \([K^+]_{\text{ext}}\) did not prevent recruitment of FADD and caspase-8 to the plasma membrane, it might prevent functional formation of the DISC in a similar fashion to preventing formation of the apoptosome.

ii) A second inconsistency is that elevated \([K^+]_{\text{ext}}\) inhibited several events during etoposide-induced apoptosis that were demonstrated to be independent of caspase activation, including reduction of \(\Psi_m\) and cytochrome c release. A possible explanation for this is that, in addition to caspases, \(K^+\) inhibits the intracellular enzymes that mediate these caspase-independent changes in etoposide-induced apoptosis.

iii) The time course of CD95- and etoposide-mediated apoptosis demonstrated that efflux of intracellular \(K^+\) accompanied but did not precede reduction of \(\Psi_m\), cell shrinkage and PS externalisation (section 4.2.5). A possible explanation is that resolution between events in the apoptotic phenotype was lost due to heterogeneity of the cell culture, thus obscuring the ordering of events. Alternatively, once a cell undergoing apoptosis has lost intracellular \(K^+\), events such as reduction of \(\Psi_m\), caspase activation and PS externalisation quickly follow.

8.5 INTRACELLULAR APOPTOTIC SIGNALLING MECHANISMS

Data has been presented demonstrating that cell shrinkage, plasma membrane depolarisation and reduction of \(\Psi_m\) in etoposide-induced apoptosis were independent of caspase activation, which suggests the existence of alternative signalling pathways. The mechanism by which elevated \([K^+]_{\text{ext}}\) inhibits etoposide-induced apoptosis also appears to be
independent of caspases. In addition, STS-induced apoptosis in THP.1 cells was not inhibited by elevated $[K^+]_{\text{ext}}$, implying that this is mediated by an alternative apoptotic pathway.

The hypothesis that ACPH activity is essential to cell survival and that its inhibition is centrally involved in the induction of apoptosis was explored. Although the ACPH-inhibitor ALCK did induce apoptosis in Jurkat, U937 and SKW 6.4 cell lines, apoptosis was induced by cell stress mechanisms resulting from non-specific inhibition of a range of serine hydrolases in the cell. Using alternative inhibitors, prolonged and virtually complete inhibition of ACPH could be established without induction of apoptosis, demonstrating that ACPH does not have central role in the transduction of apoptotic signalling.

### 8.6 Future Directions

The results of this thesis suggest a number of further experiments that could be performed to further elucidate the role of $K^+$ in apoptosis.

- Although inhibition of CD95-mediated apoptosis was reversible if cells were returned to normal $[K^+]_{\text{ext}}$ (section 6.2.3) and STS-induced apoptosis was not inhibited by elevated $[K^+]_{\text{ext}}$ (section 6.2.11), it was not demonstrated that elevated $[K^+]_{\text{ext}}$ inhibited apoptosis by 'fixing' cells, preventing all cellular processes. Experiments could be performed to monitor ATP generation in cells in both normal and elevated $[K^+]_{\text{ext}}$ to investigate whether this process still proceeded while the cells were in elevated $[K^+]_{\text{ext}}$.

- Regarding the failure of elevated $[K^+]_{\text{ext}}$ to completely inhibit STS-induced apoptosis in Jurkat and THP.1 cells (section 6.2.10 and 6.2.11), this study could be extended to further apoptotic stimuli and cell lines. Instances where elevated $[K^+]_{\text{ext}}$ failed to inhibit apoptosis might indicate both the mechanism and the point of inhibition.

- Real-time analysis of plasma membrane depolarisation during apoptosis would indicate the time course of this event, demonstrating whether it was an important early event or a secondary event late in apoptosis. This experiment was attempted using the Wallac spectrophotometer but was not successful due to dye interference and excessive extracellular signal, despite use of a quenching technique.

- The possibility of $Na^+$ influx, as suggested by data in this study (section 6.2.8), could be investigated using the radioisotope $^{22}Na^+$, albeit this would be a hazardous procedure.
References


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


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

Gwilym J. THOMPSON, Claudia LANGLAIS, Kelvin CAIN, Edward C. CONLEY1 and Gerald M. COHEN2

MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester LE1 9HN, U.K.

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 86Rb+, 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 a 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 apoptosis 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 apoptotic and associated proteins.

Protection of neuronal cultures [11] and lymphocyte cell lines by elevated extracellular [K+] ([K+]e) when exposed to pro-apoptotic stimuli provides further support for a role for K+ in apoptosis. Elevated [K+]e inhibits anti-CD95-antibody-induced apoptosis in Jurkat T cells [2,12], and both cycloheximide- 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+]e 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 Bel-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
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 \( \approx 1.4 \) M DA 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 postmitochondrial caspase cascade [24]. We have also demonstrated that, following dATP activation, two complexes were formed: a biologically active \( \approx 700 \) kDa complex which could activate effector caspases, and a biologically inactive \( \approx 1.4 \) 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\(^+\)]\(_{\text{int}}\) potently 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 \( \approx 700 \) 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-\( \alpha \)-tubulin antibody were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). Benzylxoycarbonyl-Val-Ala-Asp-O-methyl ester fluoromethylketone (Z-VAD.fmk) and benzylxoycarbonyl-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) were 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 30 \( \mu \)M 2-mercaptoethanol. Cells used in experiments were in the exponential growth phase.

**Ionomically and osmotically defined media**

Experimental media were made up of solution A (500 \( \mu \)M CaCl\(_2\)/500 \( \mu \)M 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 osmolality 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] \( \times 10^6\)/ml) were incubated in RPMI 1640 medium with vehicle (DMSO), 50 ng/ml anti-CD95 antibody, 1 \( \mu \)g/ml TRAIL, 50 \( \mu \)M etoposide or 1 \( \mu \)M MG132. For experiments involving elevated or normal [K\(^+\)] media, cells were washed once in experimental medium and then re-suspended to \((5-8) \times 10^6\) cells/ml. Apoptotic stimuli were added 10 min after resuspension. Where appropriate, Z-VAD.fmk (20 \( \mu \)M) 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 (\( \Psi_m \)), 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 \( \Psi_m \) 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 was assayed [29]. The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone was used to verify TMRE measured changes in \( \Psi_m \) and that the signal was not influenced by changes in plasma-membrane potential.

**\( ^{86}\)Rb\(^+\) efflux assay**

Jurkat T cells were loaded for 6 h at 2.5 \( \times 10^6\) cells/ml in complete RPMI medium containing 2 \( \mu \)Ci/ml \( ^{86}\)RbCl. Cells were washed twice in PBS, resuspended at \( 8 \times 10^6\) 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 \( K_n \). The background count was negligible and was discounted.

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

Jurkat lysates (100000 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 (DEVase) 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 (≈ 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 activity of the ≈ 700 kDa and ≈ 1.4 M Da apoptosome complexes to process and activate effector procaspases was measured 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 μg of protein) obtained following fractionation of control lysates, before being assayed for DEVase 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 ψm 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 ψm and decrease in forward light scatter as a measure of cell shrinkage. In control cells, spontaneous apoptosis was ≈ 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 ²⁹Rb⁺ and, at the indicated times following treatment with the different apoptotic stimuli, [K⁺]ᵢ was determined indirectly by measuring the retention of ²⁹Rb⁺ in the cell pellet. The change in [K⁺]ᵢ was represented by the ratio of experimental against control cell-pellet [²⁹Rb⁺]ᵢ, a parameter termed Kₑ (see the Experimental section). Induction of apoptosis led to a decrease in Kₑ (Figures 1B and 1C), which indicated a decrease in [K⁺]ᵢ. This K⁺ efflux accompanied PtdSer externalization, cell shrinkage and loss of ψm (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 Kₑ 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 μM) 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 ψm 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 ψm 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 blotting 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). Induction of apoptosis induced by many stimuli [9], 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). Etoposide 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]. Etoposide 8 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 inhibition of processing of both caspases 3 and 8, which remained entirely as their intact zymogens (Figures 2A and 2B). Etoposide 8, except for a small amount of an uncharacterized caspase-3-immunoreactive product (Figure 2B, lane 6), was not detected 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
G. J. Thompson and others

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

Jurkat T cells, either loaded with $^{86}$Rb+ or sham-loaded, were incubated with vehicle (A and D), anti-CD95 antibody (50 ng/ml; B and E) or etoposide (50 $\mu$M; C and F) for 5 h in standard RPMI medium. At the times shown, assays were performed for $^{86}$Rb+ efflux (●), PtdSer externalization (■), cell shrinkage (□) and loss of $\Psi_m$ (○). Experiments were also performed in the presence of Z-VAD.fmk (20 $\mu$M; D–F). The right-hand axis displays cell-pellet $^{86}$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 $\mu$M) 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.

© 2001 Biochemical Society
Elevated [K+]ext inhibits apoptosis by multiple mechanisms

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 3D), though with etoposide treatment in normal [K+]ext, fewer cells showed FITC fluorescence of cells treated with anti-CD95 antibody in normal and elevated [K+]ext.

Elevated [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+], where [Na+] 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 receptor could still activate the caspase cascade when the [K+]ext was returned to normal (results not shown).

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 μM) 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 ψm (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).

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 analyzed 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 μM etoposide (▲) for 5 h in [K+]ext at various values, with [Na+] correspondingly decreased to maintain a univalent-cation concentration of 140 mM. Apoptosis was assessed by PI/PI externalization. Results are means ± S.E.M. for four independent experiments.

© 2001 Biochemical Society
apoptosis, whereas $[K^+]_{\text{ext}}$ above this inhibited apoptosis in a concentration-dependent fashion (Figure 5). Interestingly, apoptosis induced by etoposide was significantly inhibited by lower $[K^+]_{\text{ext}}$ than anti-CD95-antibody-induced apoptosis, and was almost completely inhibited at 120 mM $[K^+]_{\text{ext}}$. These results imply that elevated $[K^+]_{\text{ext}}$ may act at different targets to inhibit the two apoptotic stimuli.

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

In experiments investigating the effect of increasing $[K^+]_{\text{ext}}$ on apoptosis, extracellular $[Na^+]$($[Na^+]_{\text{ext}}$) 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 a decrease in $[K^+]_{\text{ext}}$, it was possible that a decrease in $[Na^+]_{\text{ext}}$ was responsible. In order to ascertain the basis of the effects observed, the consequences of decreasing $[Na^+]_{\text{ext}}$ while keeping $[K^+]_{\text{ext}}$ 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 $[K^+]_{\text{ext}}$ was maintained at 5 mM and $[Na^+]_{\text{ext}}$ was decreased to 5 mM by replacement with NMDG (Figure 6). This demonstrated that protection against induction of apoptosis by elevated $[K^+]_{\text{ext}}$ was due to the increase in $[K^+]_{\text{ext}}$ and not decreased $[Na^+]_{\text{ext}}$. 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 $[K^+]_{\text{ext}}$. 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 $[K^+]_{\text{ext}}$ inhibits apoptosis induced by a range of stimuli

We wished to investigate whether elevated $[K^+]_{\text{ext}}$ 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 $[K^+]_{\text{ext}}$ with TRAIL or MG132, another proteasomal inhibitor, induced significant levels of apoptosis as assessed by PtdSer externalization (Table 1). Induction of apoptosis by both these stimuli was markedly inhibited by elevated $[K^+]_{\text{ext}}$.

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

We have shown that elevated $[K^+]_{\text{ext}}$ 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 effect 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
lysates, Apaf-1 was eluted in its monomeric form, with an estimated $M_r$ of $\approx 158,000$ (Figure 7A). After dATP activation, three different peaks of Apaf-1 were seen at $\approx 1.4$ MDa, $\approx 700$ kDa and $\approx 130$ 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 $\approx 1.4$ MDa and $\approx 130$ kDa fractions (Figure 7A). This strongly suggested that the oligomerization of Apaf-1 to form the biologically active $\approx 700$ 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 $\approx 700$ kDa complex (Figure 7B, fractions 9–16) exhibited increased caspase-processing activity in lysates treated with dATP alone. The $\approx 1.4$ 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 $\approx 700$ kDa fractions [9–16], consistent with formation of the caspase-activating $\approx 700$ 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$^+$]$_{ext}$ 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 $\Psi_m$ (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 $\Psi_m$ 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 tetraptetramethylnonium [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$^+$]. 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+]o 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+]o 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+]o 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+]. Alternatively, depolarization of the plasma membrane by elevated [K+]o 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 ψm [42], and therefore, as suggested previously [13], elevated [K+]o 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+]o. As previously stated, initiation of etoposide-mediated apoptosis has no known involvement at the plasma membrane, leading us to suggest that [K+]o affected [K+]o, by altering driving forces of K+ across the plasma membrane.

In conclusion, we have demonstrated that manipulation of [K+]o 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+]o acts at the plasma membrane, inhibiting DISC formation or activity or altering the electrochemical properties of the plasma membrane itself. Elevated [K+]o 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.

G. J. T. was in receipt of a Medical Research Council studentship. This work was in part funded by a European Union grant (QLG1-1999-07739).

REFERENCES

Elevated K+ inhibits apoptosis by multiple mechanisms


© 2001 Biochemical Society

Received 14 February 2001/27 March 2001; accepted 20 April 2001