

# Apoptosis: A Potential Therapeutic Target for Retinal Degenerations

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**Abstract:** Many retinal degenerations both inherited and induced are characterized by a loss of vision that is associated with death of photoreceptors. Inherited retinal diseases, which include Retinitis Pigmentosa (RP), form the largest single cause of blindness in the developed world. The genetics of RP is complex and approximately 48 genes have been implicated in the pathology of this disorder, in addition to the numerous mutations that exist within each gene (e.g. rhodopsin has <100). An attempt to overcome each individual mutation provides an overwhelming challenge. However targeting apoptosis, which represents a highly controlled, final common pathway to photoreceptor cell death, may provide a more practical approach. Caspases have been considered the primary executioners of apoptosis in many systems, however it is now apparent that other proteases such as calpains and cathepsins are affiliated with apoptosis. Conflicting data regarding the role of caspases in the execution of apoptosis in retinal degenerations will be critically discussed in light of reports demonstrating that key components of this pathway are downregulated during retinal development. This may control susceptibility to apoptosis in the developing retina and indeed during the maturation of other post-mitotic cells such as neurons and heart and skeletal muscle. Mitochondria function as central regulators of the intrinsic pathway of apoptosis through their role in energy production, calcium homeostasis and compartmentalization of cell death activators. The potential to control release of these apoptogenic proteins from mitochondria will also be examined with particular emphasis on the role of Bcl-2 family proteins and the regulators of calcium influx.

**Key Words:** photoreceptor, apoptosis, caspase, calpain, mitochondria, Bcl-2.

## RETINAL DEGENERATIONS

Inherited retinal degeneration is a broad term applied to dystrophies including Retinitis Pigmentosa (RP), Age-related Macular Degeneration (AMD), Ushers Syndrome and others, which result in a progressive loss of vision. RP is the most widespread of these disorders, affecting an estimated 1.5 million individuals worldwide, with an incidence of 1 in 4000 (Bunker, *et al.*, 1984). It is believed to be the most prevalent cause of registered blindness in working populations in non-tropical countries. Initial symptoms of RP include night-blindness or nyctalopia, which results from rod photoreceptor degeneration. As rod cells die, cone photoreceptor viability is compromised and the disease progresses towards loss of peripheral fields, tunnel vision and finally blindness. Other characteristic features include pigmented deposits in advanced RP and attenuation of the retinal blood vessels Fig. (1).

## Molecular Genetics of RP

RP is a heterogeneous disease; inheritance can follow an autosomal dominant (adRP), autosomal recessive (arRP), X-linked (xlRP) or digenic pattern. AdRP and arRP comprise approximately 20% of RP cases while xlRP is responsible for approximately 10%. Although rare, digenic inheritance of the disease does occur with the co-inheritance of mutations within the genes encoding peripherin and ROM 1 (rod outer

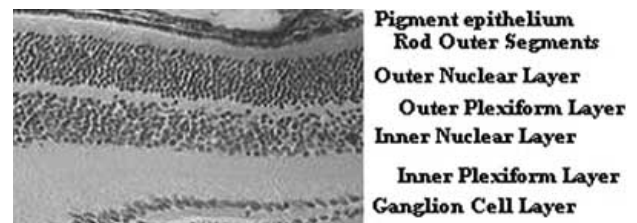


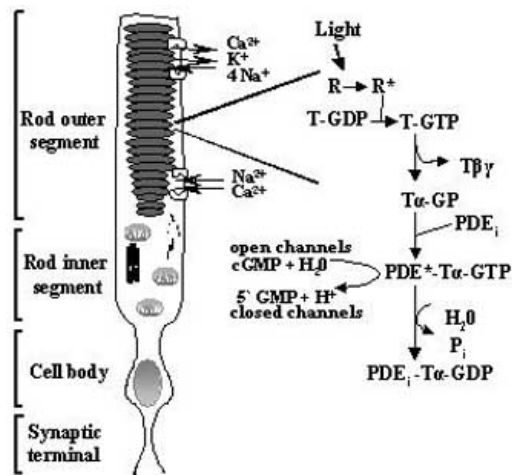
Fig. (1). A hematoxylin-eosin section of the mouse retina.

The retina is a seven-layer structure, with light entering the ganglion cell layer (GCL) first. From there it must penetrate each layer before reaching the rods and cones, the outer segments of which transduce the light. It is this layer of rod and cone photoreceptors, which degenerates during RP. The INL contains cell bodies of bipolar, horizontal and amacrine cells.

segment protein 1). While the majority of RP cases are inherited, isolated instances are also common, occurring in about 40% of RP cases. The genetics of these retinopathies is complex, with around 48 genes implicated in RP many encoding photoreceptor-specific proteins of the visual transduction cascade (<http://www.sph.uth.tmc.edu/Retnet/home.htm>). Visual stimuli are processed in membrane discs of rod outer segments via a tightly controlled signaling cascade Fig. (2). The primary event in this cascade is absorption of light by the photoreactive pigment rhodopsin. Once activated, rhodopsin triggers the G-protein transducin, which further activates a cGMP phosphodiesterase (cGMP PDE). The resultant hydrolysis of cGMP closes cGMP-gated channels, leading to hyperpolarization of the cell and

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signaling. Termination of phototransduction requires phosphorylation of rhodopsin by rhodopsin kinase, followed by binding of arrestin.



**Fig. (2).** A schematic diagram of a rod photoreceptor cell and the phototransduction cascade.

The photoreceptor is divided into an outer segment region containing the machinery of phototransduction and an inner segment region containing mitochondria, ER, nucleus and synaptic terminal. The outer segment consists of a stack of flattened membrane sacs called discs, of which the photopigment rhodopsin is the primary constituent. Activation by rhodopsin, of this G-protein cascade causes stimulation of cGMP PDE, increased hydrolysis of cGMP and hence decreased cytoplasmic concentrations of cGMP. cGMP-gated channels are no longer held open, reducing the influx of cations ( $\text{Na}^+$  and  $\text{Ca}^{2+}$ ) and thus the cell becomes hyperpolarized.

Genes implicated in RP include, rhodopsin (RHO), the catalytic PDE subunits (PDE6A) and (PDE6B), the subunit of rod cGMP-gated channel (CNGA1) and arrestin (SAG) (Danciger, *et al.*, 1995, Dryja, *et al.*, 1995, Dryja, *et al.*, 1990, Dryja, *et al.*, 1990, Huang, *et al.*, 1995, McLaughlin, *et al.*, 1995, McLaughlin, *et al.*, 1993, Nakamachi, *et al.*, 1998). Mutations in the rhodopsin gene are the most common and may affect protein folding, stability or trafficking (Sung, *et al.*, 1991). Structural components of photoreceptors, such as peripherin also harbour mutations causative of RP. Peripherin is located in rod and cone outer segment discs and appears to be critical for the formation and stabilization of photoreceptor outer segments (Goldberg, *et al.*, 1998). Over 40 mutations have been identified within the peripherin gene of patients with retinal dystrophies (Keen and Inglehearn, 1996). Mutations in genes such as 11-*cis* retinal dehydrogenase (Yamamoto, *et al.*, 1999) and cellular retinaldehyde binding protein (Maw, *et al.*, 1997), required for recycling of the rhodopsin chromophore 11-*cis*-retinaldehyde, also result in RP.

However, in spite of our growing knowledge of the genetics of RP, many aspects of the disease remain unclear. For example, RP displays variable expressivity in that the

same genetic mutation can have very different consequences for each individual. In addition, the mechanism by which the many mutations described leading to photoreceptor cell death remains to be determined.

### Animal Models of RP

The direct study of human RP is hindered by difficulty in obtaining the relevant tissues samples and thus animal models are essential to advance our understanding of this disease. A number of animal models exist that sustain the same mutations found in humans, thereby representing the human disease both genetically and phenotypically. Mammalian models in particular provide us with a wealth of information, by helping to identify candidate genes, understand the disease mechanism and ultimately develop treatments. A defect in the  $\alpha$  subunit of cGMP PDE causes photoreceptor degeneration in the long recognized, naturally occurring retinal degeneration (rd) mouse (Bowes, *et al.*, 1989, Bowes, *et al.*, 1990, Bowes, *et al.*, 1993, Pittler and Baehr, 1991). The retinal degeneration slow (rds) mouse provides a second naturally occurring model with a mutation in the gene coding for peripherin (Sanyal and Jansen, 1981). More recently, transgenic mice have been generated, that develop the characteristic features of RP, for the most part with targeted disruption of the rhodopsin gene (Humphries, *et al.*, 1997, Naash, *et al.*, 1996) but also of the peripherin gene (McNally, *et al.*, 2002).

Induced retinal injuries, resulting from the administration of N-methyl-N-nitrosourea (MNU),  $\text{Ca}^{2+}/\text{Pb}^{2+}$  or excessive light exposure represent another useful class of animal model. Of particular relevance is the light-induced model, given that photoreceptors from retinal degeneration mutants are more susceptible to the damaging effects of light and it is likely that continuous stimulation of the phototransduction cascade in this model mimics events occurring in other models. A recent review offers an excellent summary of animal models of retinal degeneration (Fauser, *et al.*, 2002).

### APOPTOSIS

In spite of the complex genetics underlying its pathology, programmed cell death or apoptosis is a feature common to all cases of RP both in humans and in animal models (Li Z. and Milam, 1995). Apoptosis was first attributed a role in photoreceptor cell death in 1993, on the basis of nuclear fragmentation and chromatin condensation (Chang, *et al.*, 1993). These features and others including mitochondrial depolarization, alterations in phospholipid asymmetry, membrane blebbing, cell shrinkage and formation of membrane bound vesicles termed apoptotic bodies are now considered indicators of apoptosis (Kerr, *et al.*, 1972).

Apoptosis is a tightly controlled death program, where the cell actively participates in its own demise. Classically, caspases have been considered the key executioners of this death program and in many instances of apoptosis this is the case. The caspases are a family of cysteine proteases comprised of at least 13 members, which has been subdivided into three categories based on sequence homology and substrate-specificity. All caspases possess an active thiol group necessary for activity and have a specific requirement for an aspartate residue at target cleavage sites (Thornberry

and Lazebnik, 1998). These proteases are synthesized as inactive precursors composed of three domains, a highly variable NH<sub>2</sub> terminal regulatory domain and two heterologous enzyme subunits (Margolin, *et al.*, 1997). Proteolytic processing and activation requires cleavage of the N-terminal domain and the formation of a tetramer of two heterodimers Fig. (3). To date, three caspase mediated death pathways have been described Fig. (4). Receptor-mediated pathways require ligand binding to receptor, subsequent receptor oligomerization, followed by recruitment and activation of caspase-8 (Peter and Kramer, 1998). The intrinsic pathway involves release of cytochrome-c from the mitochondrial intermembrane space, which leads to formation of the apoptosome consisting of cytochrome-c, caspase-9 and apoptotic protease activating factor-1 (Apaf-1) (Slee, *et al.*, 1999). Evidence has shown that endoplasmic reticulum (ER) stress can trigger a novel apoptotic pathway in which caspase-12 functions as an initiator caspase (Morishima, *et al.*, 2002). It has been reported, at least *in vitro*, that caspase-9 is a direct substrate of caspase-12 and resultant caspase-9 processing is independent of cytochrome-c release. In each instance, cleavage of the initiator caspase usually leads to a downstream cascade of caspase activation and completion of the death program.

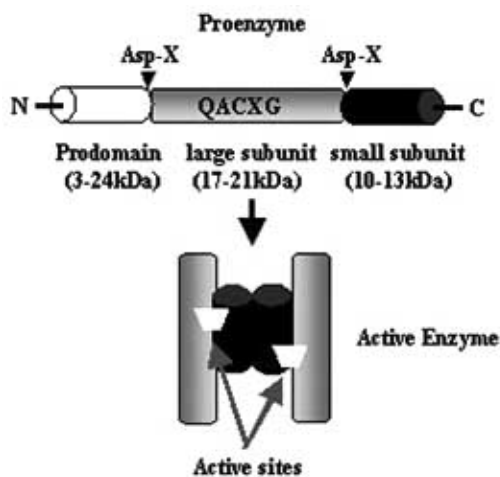


Fig. (3). Procaspase and caspase structure.

Caspases are synthesized as inactive proenzymes that require proteolytic cleavage at aspartic acid residues between their three domains. The large and small subunits then reconstitute to form an active caspase heterodimer.

Caspases have been successfully utilized in the suppression of apoptosis in a range of disease models. For example, the broad-spectrum caspase inhibitor *z*-VAD-fmk effectively reduced myocardial-reperfusion injury *in vivo*, probably by attenuating cardiomyocyte apoptosis (Yaoita, *et al.*, 1998). Administration of the caspase-9 inhibitor, LEHD-CHO into the brain of rat pups during hypoxia-ischemia reduced neuronal loss (Feng, *et al.*, 2003). In addition, airway inflammation has been successfully attenuated in a murine asthma model, using *z*-VAD-fmk (Iwata, *et al.*, 2003). Suppression of caspase-8 using small interfering RNA (siRNA) improved hepatocyte survival in an animal

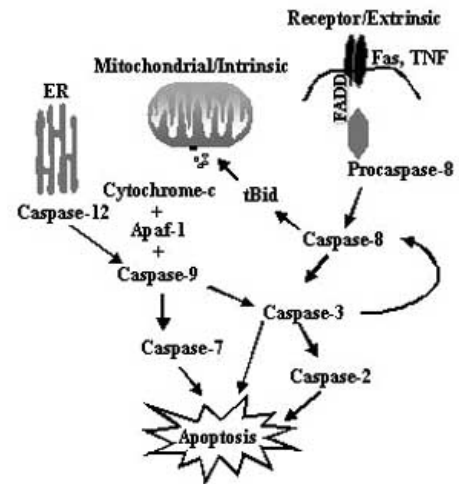


Fig. (4). Caspase-mediated cell death pathways.

Initiation of the intrinsic mitochondria-mediated pathway requires cytochrome-c, Apaf-1 and caspase-9 to constitute the apoptosome, resulting in caspase-9 autolysis and caspase-3 activation. Caspase-9 is cleaved directly by caspase-12 in response to ER stress, while caspase-8 is responsible for activation of caspase-3 in the receptor-mediated pathway. Activation of caspase-3 results in a cascade of downstream caspase processing. However these pathways are not mutually exclusive, caspase-3 can activate caspase-8 in the form of a feed-back loop and caspase-8 can cleave Bid, inducing cytochrome-c release and caspase-9 activation.

model of acute liver failure and in an animal model reflecting the symptoms of human acute viral hepatitis (Zender, *et al.*, 2003). With this technique, double-stranded RNA induces sequence-specific posttranscriptional gene silencing by a process known as RNA interference (RNAi). 21- and 22-nt RNA fragments readily mediate RNAi *in vitro*, however the application of this technique *in vivo* presents a number of challenges. Functional optimization and delivery of these molecules to complex environments such as the nervous system may prove difficult (Wood, *et al.*, 2003). Indeed, application of 'naked' siRNA targeting dopamine receptors directly to the rat brain appears inefficient (Isacson, *et al.*, 2003).

A number of good reviews have been published, which discuss the potential use of caspase inhibitors and Inhibitor of Apoptosis Proteins (IAPs) in disease models (Bilsland and Harper, 2002, Holcik, *et al.*, 2001, Rideout and Stefanis, 2001). In fact, small molecule caspase inhibitors for the potential treatment of neurodegenerative disease, stroke and inflammation are currently being developed by the pharmaceutical industry.

### Apoptosis in Development

Programmed cell death plays an important part in the sculpture of tissues and organs in both embryonic and postnatal development. Much of our knowledge came from the study of *Caenorhabditis elegans*, an organism that begins

life with 1090 cells of which 131 are removed by apoptosis during their maturation. Both the brain and the retina are organs, which undergo dramatic reshaping as they mature. The retina develops from a single layer of undifferentiated neurons to a mature retina made up of three layers of fully differentiated cells. Superfluous cells unable to make functional neural connections are eliminated by waves of apoptosis occurring during the first three postnatal weeks.

Bax and Bak, members of the Bcl-2 family, are the first apoptotic proteins to be identified as essential for developmental apoptosis in the retina (Hahn, *et al.*, 2003). In Bax<sup>-/-</sup>Bak<sup>-/-</sup> double mutant mice developmental apoptosis is almost entirely abolished. Apoptotic cells were completely absent in the inner nuclear layer (INL) at postnatal day 7 (P7), normally the peak of developmental apoptosis. In Bax<sup>-/-</sup> or Bak<sup>-/-</sup> single knockouts, this removal of unwanted cells continued, indicating that either one or other pro-apoptotic protein is essential for apoptosis. These findings are substantiated by additional evidence that Bax<sup>-/-</sup>Bak<sup>-/-</sup> mice exhibit defective development in a wide range of tissues (Lindsten, *et al.*, 2000). Indeed, only 10% of these animals survive into adulthood and those that do survive exhibit persistent interdigital webs and an accumulation of cells in both the central nervous and hematopoietic systems. It appears that once Bax fulfils its role in development it is rapidly downregulated in the adult rat brain, therefore brain mitochondria from undifferentiated neural cells are more sensitive to Bax-mediated cytochrome-c release than their fully differentiated counterparts (Polster, *et al.*, 2003). These studies suggest that at least developmental apoptosis appears to proceed via a classical caspase-dependent, mitochondria-mediated mechanism, although to date there is no direct evidence of caspase activation.

## CASPASES AND RP

The involvement of the caspase family of cysteine proteases in photoreceptor cell death is a topic of continuing debate and consequently a case for both caspase-dependent and caspase-independent photoreceptor apoptosis will be presented. Caspase-independent apoptosis is now widely accepted and has been described in neuronal systems in response to UV (McCullum, *et al.*, 2002), amyloid- $\beta$  (Selznick, *et al.*, 2000) nitric oxide (Okuno, *et al.*, 1998) and following seizure-induction (Fujikawa, *et al.*, 2002) or traumatic brain injury (Zhang, *et al.*, 2002). Moreover, this laboratory has reported caspase-independent photoreceptor apoptosis in the rd mouse model of RP, where the insult is chronic and increases in an age-dependent manner, inducing a relatively slow rate of photoreceptor death. A pathway that does not require activation of caspase-9, -8, -7, -3 and -2 or release of cytochrome-c is described. Indeed, the absence of cytochrome-c release is highlighted as an apical event in caspase-independent apoptosis in this *in vivo* model (Doonan, *et al.*, 2003). Caspase cleavage fragments were readily detected in treated mouse cell line positive controls, with the same level of apoptosis (<10%) observed in the rd mouse, validating the antibodies used in this study.

These results are in direct contrast to another report demonstrating caspase activation during retinal degeneration

in the same model (Jomary, *et al.*, 2001). This study demonstrated activation of caspase-8 with resultant Bid cleavage, cytochrome-c release and activation of caspase-3. However, careful consideration should be given to the interpretation of immunohistochemistry experiments that utilize polyclonal antibodies, in addition, some of the antibodies applied did not discriminate between inactive and active forms of caspases. Furthermore, Bid contains a region, which is extremely susceptible to proteolysis, not only by caspase-8 but also by calpain generating a 14-15 kD truncated Bid (tBid) (Mandic, *et al.*, 2002). Subretinal or intravitreal injection of the pan-caspase inhibitor z-VAD-fmk would clarify this discrepancy. In this regard, a transient delay in photoreceptor apoptosis in response to intraperitoneal (i.p.) application of the caspase-3 inhibitor Ac-DEVD-CHO has been shown (Yoshizawa, *et al.*, 2002). However, after P13 the therapy was no longer successful and photoreceptors underwent apoptosis, supporting the existence of an alternative death pathway. A further study evaluates the use of z-VAD-fmk as a treatment for photoreceptor loss in rd mouse retinal cell explants (Caffe, *et al.*, 2001). This broad-spectrum caspase inhibitor did not rescue rd photoreceptor cells *in vitro*. Certainly, there is doubt concerning penetration of such inhibitors and therefore their efficacy when applied i.p. should be questioned, however the insurmountable technical difficulties encountered when applying peptide inhibitors to early PND mice ensure that this important result remains elusive. Caspase-independent apoptosis has also been described in the light-induced model of retinal degeneration (Donovan and Cotter, 2002). Photoreceptors of balb/c mice exposed to white fluorescent light (5000 lux) die rapidly by apoptosis mediated by nitric oxide (Donovan, *et al.*, 2001). Elevated calcium and oxidative stress contribute significantly to this apoptotic program without the activation of caspase -9, -8, -7, -3 and -1. In addition, sub-retinal injection of zVAD-fmk, did not protect against this light-induced damage.

Clearly there is ample evidence to support a role for either caspase-dependent or caspase-independent apoptosis in retinal cell death, however a recent publication from this laboratory offers interesting results. In the light-induced model of retinal degeneration, caspases were not activated due to an absence of mitochondrial cytochrome-c release. Moreover, addition of cytochrome-c and dATP to retinal lysates could not activate caspase-9 or -3. Reduced expression of Apaf-1 was observed during retinal maturation and this correlated with defective cytochrome-c dependent caspase activation (Donovan and Cotter, 2002). Expression of caspase-3 in the adult mouse retina was also significantly reduced presumably as a supplementary mechanism to safeguard against execution of apoptosis. On the other hand, cultured 661W photoreceptor cells exposed to visible light do exhibit caspase-1 activation (Krishnamoorthy, *et al.*, 1999). Yet this is unsurprising when you consider that these cells were harvested from P2 mice, prior to downregulation of caspase expression and thus the pathway is available. This crucial regulation of apoptotic susceptibility observed in the developing retina has been documented in other postmitotic tissues such as the brain (Yakovlev, *et al.*, 2001). Rapid caspase activation and cell death, which occur normally during neuronal development, could have serious

implications in the mature brain. This is clearly demonstrated by the many neurological diseases in which excessive apoptosis is observed. Likewise, photoreceptor cells are terminally differentiated, post-mitotic neurons required to retain tight control of apoptosis.

Nevertheless, it is possible that in response to a particular stress, caspases could be re-expressed making the pathway available once again. Just such an increase in caspase-3 and Apaf-1 expression has been demonstrated in the brain in response to traumatic brain injury (Yakovlev, *et al.*, 2001). Another study has provided evidence of increased levels of caspase-3 mRNA that correlated with an increase in caspase-3 activity during blue light-induced photoreceptor apoptosis (Wu, *et al.*, 2002). Thus a caspase-mediated pathway, dependent on the re-expression of key proteins, remains possible in the mature retina. On the other hand, photoreceptors evidently possess an alternative way to die, which from the evidence presented may be as important if not more important than a caspase-dependent pathway.

Clearly there are several examples from the literature highlighting both caspase-dependent and independent photoreceptor apoptosis. However, we cannot ignore the evidence that post-mitotic cells, including cells of the retina downregulate their apoptotic machinery and therefore caspase inhibitors would not be therapeutically effective in this instance. Under conditions where caspases are re-expressed in response to death stimuli caspase inhibitors may have therapeutic value, however the longevity of such an effect is uncertain given that alternative executioners are available. Evidently, targeting caspase pathways is not ideal and for this reason it could be of greater utility to focus our attention upstream at the initiation stages of apoptosis.

## CASPASE-INDEPENDENT APOPTOSIS

### Cathepsins

Armed with the growing evidence that caspases are not the only family of proteases implicated in apoptosis, it is clear that other candidates must be considered. The cathepsins are a family of cysteine, aspartate and serine proteases, of which cysteine cathepsins B and L and the aspartate cathepsin D are linked to apoptosis. These proteases are normally localized to lysosomes, however during programmed cell death they translocate to either the cytosol or the nucleus where they can participate in either caspase-dependent or caspase-independent cell death. Cathepsin B is the dominant protease involved in tumour necrosis factor (TNF) induced tumour cell apoptosis. While caspase inhibitors did not protect, a range of cathepsin B inhibitors rescued cells from death (Foghsgaard, *et al.*, 2001). In addition, cathepsin D has the ability to cleave and activate Bax, resulting in the selective release of AIF from mitochondria and caspase-independent commitment to death (Bidere, *et al.*, 2003). However, in some cell types it is thought that cathepsins may be essential for survival. It has been proposed that an aspartic protease, similar or identical to cathepsin D, is the enzyme responsible for the proteolysis of photoreceptor outer segments. Indeed, cathepsin D knockout mice exhibit photoreceptor degeneration, shortened outer segments and build up of photoreceptor breakdown products, modelling the degenerative condition AMD

(Rakoczy, *et al.*, 2002). The role of cathepsin D in the lysosomal digestion of phagocytosed photoreceptor outer segments suggests that cathepsins may be essential for maintaining normal function, however the potential importance of aberrant cathepsin function in the retina is as yet unknown.

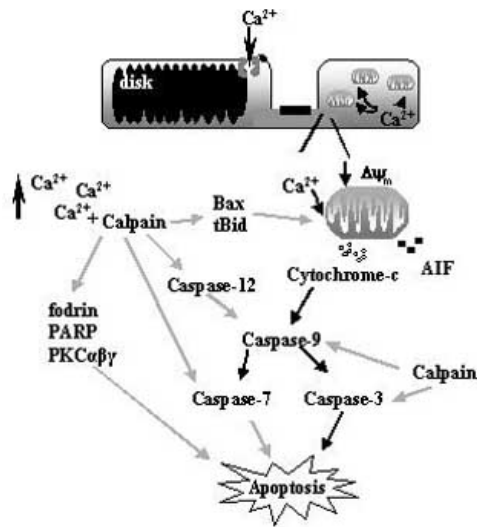
### Granzymes

A well-described pathway by which cytotoxic T lymphocytes (CTLs) and natural killer cells eliminate pathogenic cells involves the granzyme family of serine proteases. Once delivered to the target cell granzyme B activates a caspase-dependent pathway (Quan, *et al.*, 1996), however it is also capable of inducing apoptosis in the presence of a caspase blockade (Shi, *et al.*, 1996). One study in particular, demonstrated that Bid is also a substrate for granzyme B *in vitro*, cleaving Bid under conditions where caspase activity is inhibited (Barry, *et al.*, 2000). It is possible therefore, that granzyme B may be responsible for the generation of tBid observed in the rd model (Jomary, *et al.*, 2001). Recently, a new caspase-independent granzyme-A(GrA)-mediated pathway was described, which exhibited not DNA fragmentation but single-stranded DNA nicks and other features of apoptosis such as chromatin condensation and loss of mitochondrial membrane potential (Beresford, *et al.*, 2001). The endonuclease involved has been identified as GrA-activated Dnase (GAAD), which is localized to the ER and once activated translocates to the nucleus (Fan, *et al.*, 2003). However, little else is known about the pathway involved or indeed the presence and/or relevance of granzyme activity in photoreceptor apoptosis.

### Calpains

Neuronal calpains are calcium triggered proteolytic enzymes that are activated by autolytic processing in several instances of necrosis but also in some forms of apoptosis. This family includes two ubiquitous forms of calpain, calpain-1/ $\mu$ -calpain and calpain-2/ $m$ -calpain, which are stimulated in response to different calcium concentrations.  $\mu$ -calpain and  $m$ -calpain are heterodimers consisting of 28 kD and 80 kD subunits. The 28 kD subunit is identical in the two isoforms, whereas the 80 kD subunits are only 50% homologous. Calcium binding induces a conformational change, that promotes cleavage of the 28 kD subunit and results in activation of the enzyme. These calcium-dependent proteases appear to be activated in an uncontrolled and prolonged manner in a number of neurodegenerative conditions including Alzheimer's disease (Boland and Campbell, 2003), a model of Parkinson's disease (Crocker, *et al.*, 2003) and during chronic ethanol exposure in the rat brain *in vivo* (Rajgopal and Vemuri, 2002). Evidence from the literature also suggests that calpains may be involved in the proteolysis of crystallins and thereby progression of cataracts (Takeuchi, *et al.*, 2001).

In many cases determining the relative roles of calpains and caspases is further complicated by evidence of cross-talk between the two proteolytic pathways (Neumar, *et al.*, 2003) Fig. (5). Evidence suggests that caspase-12, -7 and -3 can be cleaved and activated by calpain (Blomgren, *et al.*, 2001, Nakagawa and Yuan, 2000, Ruiz-Vela, *et al.*, 1999). In



**Fig. (5).** Calcium can induce activation of calpains or caspases or both in combination.

Sustained increases in intracellular calcium can lead to apoptosis by a number of different mechanisms. Increased calcium may induce mitochondrial changes by disrupting membrane integrity, promoting release of apoptogenic proteins such as cytochrome-c or AIF. Elevated cytosolic calcium activates calpains, which proteolyse specific substrates leading directly to the death of the cell. In some instances they may cleave certain caspases rendering them insensitive to further cleavage, thus promoting a calpain-only form of apoptosis. In other instances calpains may feed into a caspase-dependent pathway, through cleavage of Bax, Bid and a number of the caspases.

contrast, calpain activation in response to glutamate excitotoxicity can prevent activation of a caspase-dependent pathway thereby promoting a caspase-independent death mechanism (Lankiewicz, *et al.*, 2000). It may achieve this by partial cleavage of caspase-3 to a 30 kD form, which cannot be processed further into its active subunits. The same appears to be true for an *in vitro* system where calpains have been shown to cleave caspase-9 and -7, at sites distinct from those of the caspases generating catalytically inactive fragments (Chua, *et al.*, 2000). Calpains also function upstream of mitochondria through induction of Bax cleavage, giving rise to a potent pro-apoptotic 18 kD fragment (Wood, *et al.*, 1998). This fragment is localized to mitochondria, is unable to interact with Bcl-2 and possesses a cytochrome-c releasing activity (Choi, *et al.*, 2001, Gao and Dou, 2000). As previously mentioned, a calpain cleavage site has been mapped to a region within Bid, giving rise to fragments either 14 kD (Mandic, *et al.*, 2002) or 8 kD (Chen, *et al.*, 2001) in size that are capable of inducing cytochrome-c release from mitochondria. There is also evidence that caspases may regulate calpain activity, through cleavage of calpastatin, the endogenous inhibitor of calpain (Porn-Ares, *et al.*, 1998). In spite of the complex interactions of these pathways it is clear that calpains can act independently of

other proteases or alternatively they have the capacity to feed into caspase-mediated pathways either upstream or downstream of mitochondria.

Calpain isoforms have been implicated in retinal cell death induced by ischemia-reperfusion in rat retinas *in vivo* (Sakamoto, *et al.*, 2000) and during hypoxia-induced retinal damage *in vitro* (Tamada, *et al.*, 2002). Indicators of calpain activity in these systems included increased intracellular calcium, cleavage of calpain-specific substrates and protection from apoptosis provided by the calpain inhibitor SJA6017. Moreover, there is an indication that calpains play a role in the light-induced model of retinal degeneration and potentially in the rd model. Calpain activity in the light-induced model was assessed by monitoring cleavage of the fluorogenic substrate Suc-Leu-Tyr-AFC, which indicated a 3-fold induction of calpain activity (Donovan and Cotter, 2002). Calpain substrates cleave preferentially at Val, Leu or Ile in the P2 position of the target protein whereas the amino acids at the P1 position are relatively diverse. There is a sizeable overlap among caspase and calpain substrates that includes fodrin, actin, vimentin, FAK, PARP and others. In this laboratory, an alternative poly-ADP-ribose polymerase (PARP) cleavage fragment was detected in both the rd mouse and in MNU-treated mice, which is not typical of caspase-mediated processing (Doonan, *et al.*, 2003). PARP (116 kD) is ordinarily cleaved by caspase-3 producing a fragment 85 kD in size however in this case the detected fragment was approximately 40 kD, which is indicative of calpain-specific cleavage (McGinnis, *et al.*, 1999). Thus, a role for calpains in the execution of apoptosis in the rd mouse could also be postulated.

## MITOCHONDRIA

Mitochondria, under normal conditions are responsible for aerobic respiration, however they also function in the suppression of apoptosis by the compartmentalization of proapoptotic factors. This highlights the maintenance of mitochondrial integrity and the prevention of mitochondrial depolarization as critical to the inhibition of apoptosis. Thus, early intervention through inhibition of these initiating events will target both caspase-dependent and independent death programs.

Factors confined to mitochondria include cytochrome-c, Smac/Diablo, Omi/HtrA2, apoptosis inducing factor (AIF) and endonuclease G (endoG). As discussed previously, cytochrome-c is generally confined to mitochondria, however its release into the cytosol upon rupture of the mitochondrial membrane permits it to interact with Apaf-1, and caspase-9, resulting in a cascade of caspase activation. Smac/Diablo interacts directly with IAPs, neutralizing their activity and releasing caspases from their inhibition (Srinivasula, *et al.*, 2000). Omi/HtrA2 has a similar pro-apoptotic function, however it can also mediate apoptosis in a caspase-independent manner, which requires its protease activity (Cilenti, *et al.*, 2003). Neither of these proteins has been examined as yet in models of photoreceptor apoptosis, however Smac/Diablo is released during lactacystin-induced apoptosis in retinal pigment epithelial (RPE) cells (Kim, *et al.*, 2003).

Both AIF and endoG can translocate from mitochondria to nuclei under certain conditions, where they mediate

chromatinolysis (Susin, *et al.*, 1999, van Loo, *et al.*, 2001). EndoG is a 30 kD nuclease which had a proposed function in mitochondrial DNA replication based on its location and substrate specificity. However, the release of this nuclease from mitochondria under apoptogenic conditions and its ability to induce DNA fragmentation independent of caspase activation have defined a new role for endoG. There are indications that apoptosis can proceed in the absence of caspase activity in response to mitochondrial damage, initiating a program of cell death parallel to caspase activation. Caspase-independent apoptosis in mast cells is mediated by translocation of endoG from mitochondria to nuclei (Yoshikawa and Tasaka, 2003). It also appears that endoG may compensate for Caspase-Activated DNase (CAD) in cells deficient for this protein (Li, *et al.*, 2001). Whilst, no research has been done concerning the potential function of endoG in photoreceptor apoptosis, the presence of DNA fragmentation in the absence of Inhibitor of CAD (ICAD) cleavage could point towards this nuclease.

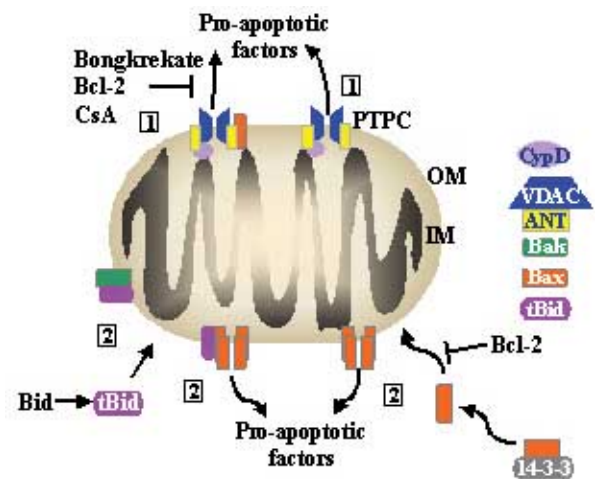
AIF is a 57 kD flavoprotein with an important survival function under normal conditions, as demonstrated by the harlequin (hq) mutant mouse which has a proviral insert in this gene causing an 80% reduction in expression. This leads to progressive degeneration of cerebellar and retinal neurons, due to the inability of AIF to carry out its function in mitochondria, which relates to oxygen scavenging. In this regard, hq mice become increasingly sensitive to peroxide and exhibit increased ROS-induced neuronal damage, highlighting a vital role for AIF in neuronal survival in the ageing mouse (Klein, *et al.*, 2002). However it has also been attributed a role in apoptosis, becoming active upon translocation from mitochondria to nuclei, where it initiates chromatin condensation and large-scale DNA fragmentation. These effects occur in the presence of saturating doses of z-VAD-fmk, demonstrating that AIF can act in a caspase-independent manner. AIF translocation has been observed in T lymphocytes, Jurkat cells, HeLa cells and in cerebral ischemia in the neonatal rat brain (Bidere, *et al.*, 2003, Murahashi, *et al.*, 2003, Pardo, *et al.*, 2001, Susin, *et al.*, 1999, Zhu, *et al.*, 2003). Importantly, relocation of AIF has been demonstrated in retinal detachment (RD), a clinically relevant *in vivo* model of RP. RD is induced by subretinal injection of sodium hyaluronate, causing photoreceptor apoptosis, which correlates with diminished electroretinogram (ERG) amplitudes (Hisatomi, *et al.*, 2002). While both AIF and cytochrome-c were relocalized during RD, AIF relocalized to the nucleus and cytochrome-c to the cytosol, apoptosis was not inhibited by Z-VAD-fmk (Hisatomi, *et al.*, 2001). In contrast, administration of brain-derived neurotrophic factor (BDNF) to the subretinal space inhibited both AIF relocalization and nuclear events. This indicates a pathway mediated by mitochondria yet caspase-independent, recognizing a potential function for AIF in photoreceptor apoptosis. In light of reports describing DNA fragmentation in the absence of ICAD cleavage and caspase activation, it will be interesting to determine if AIF has an important function in other models of retinal degeneration. In view of the single location of these proteins, it is clear that the mitochondrion is a central point from which many different pathways may be controlled. It is pertinent therefore, to consider the means by which mitochondria may be

ruptured during photoreceptor apoptosis with a view to developing good therapeutic strategies. An informative review published in 2003, examines the role of mitochondria as potential targets for both neuroprotection and cardioprotection (Mattson and Kroemer, 2003).

### Bcl-2 Family and Control of Mitochondrial Integrity

The release of proteins such as those mentioned above, requires permeabilization of the outer mitochondrial membrane (OMM), an event that is controlled in part by members of the Bcl-2 family. This family of proteins consists of both death antagonists (Bcl-2, Bcl-X<sub>L</sub>) and death agonists (Bax, Bak, Bim, Bad, Bid) that elicit opposing effects on mitochondria. Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains, BH1-4. Anti-apoptotic members tend to possess all four domains, while it appears that the BH3 domain serves as a critical death domain in pro-apoptotic members. This is supported by the emergence of a subset of pro-apoptotic members including Bid, Bad, and Bim, which possess only a BH3 domain. Additional characteristics of these proteins include their ability to form homodimers as well as heterodimers and to integrate into membranes.

Mitochondrial membrane permeabilization (MMP) is clearly a pivotal event in the progression of apoptosis in many systems. At least two mechanisms of MMP have been described, both of which will be briefly discussed Fig. (6). One widely accepted theory involves the assembly of the so-called permeability transition pore complex (PTPC), which



**Fig. (6). Mitochondrial membrane permeabilisation.**

Two basic mechanisms of MMP are proposed here. 1 VDAC, ANTI and cypD come together to form the PTPC. This pore complex may also associate with Bax, Bak or Bim, which accelerate channel opening or Bcl-X<sub>L</sub>, which causes closure. 2 Bax is released from its interaction with 14-3-3 and translocates from cytosol to mitochondria in response to diverse signals. Here it oligomerizes forming protein permeant pores. Pore formation can also take place in conjunction with the BH3-only Bcl-2 family member Bid upon proteolysis to tBid. Bak is located at mitochondria and has similar pore forming properties to Bax, via its oligomerization and association with tBid.

forms at a contact site between the outer membrane (OM) and the inner membrane (IM). The primary constituents of this complex are voltage-dependent anion channel (VDAC) on the OM, adenine nucleotide transporter (ANT) on the IM and ANT associated protein cyclophilin D (Vieira, *et al.*, 2000). The pore behaves as a voltage-gated channel that becomes activated in response to high calcium concentrations, oxidative stress or low transmembrane potential. Under these conditions the pore opens persistently allowing not only calcium but also low-molecular weight compounds to leave the mitochondria. It has also been proposed that Bcl-2 family members can regulate PTPC formation; Bax and Bak induce opening of the pore, while Bcl-2 and Bcl-X<sub>L</sub> facilitate closure. Bax, Bak and Bcl-X<sub>L</sub> interact directly with VDAC, while Bax and Bcl-2 bind ANT demonstrating the potential for PTPC modulation by Bcl-2 family members (Brenner, *et al.*, 2000, Marzo, *et al.*, 1998, Shimizu, *et al.*, 2000, Shimizu, *et al.*, 1999). In addition, Bim is capable of direct interaction with VDAC resulting in its activation (Sugiyama, *et al.*, 2002).

Alternative mechanisms demonstrate the ability of pro-apoptotic members of the Bcl-2 family to form pores either singly or collectively. Pro-apoptotic Bax translocates from the cytosol to mitochondria in response to various signals, where it oligomerizes creating protein permeable pores in the OM. Furthermore, Bax is capable of forming heterodimers with Bcl-2, thereby negating its anti-apoptotic activity (Nouraini, *et al.*, 2000). Oligomerization of Bax can also be induced by truncated Bid (tBid), a death ligand that acts in combination with monomeric Bax, inducing the release of twice as much cytochrome-c as either Bax oligomers or tBid alone. In this instance cytochrome-c release is insensitive to cyclosporin A plus ADP, inhibitors of PTPC (Brustovetsky, *et al.*, 2003). As discussed, Bax has also been shown to enhance PTPC, which may explain why, in some cases the effects of Bax are inhibited by bongkrekate and CsA, both inhibitors of PTPC components (Vieira, *et al.*, 2000). In the absence of mitochondrial Bax, tBid activates the mitochondrial resident Bak inducing oligomerization and pore formation (Korsmeyer, *et al.*, 2000). A recent study has also shown that Bax oligomerization may trigger a Bak conformational change and homo-oligomerization indicating some degree of functional cooperation (Mikhailov, *et al.*, 2003). There is ample evidence in the literature to support either mechanism (Degli Esposti and Dive, 2003, Halestrap, *et al.*, 2002, Tsujimoto and Shimizu, 2002), however, it is likely that they may be utilized independently or in combination depending on the cell type and/or stimulus. It is unknown whether these channels are selective or non-selective in their release of apoptogenic proteins however, there is evidence to support the release of all soluble intermembrane pro-death factors (Sutton, *et al.*, 2003, Van Loo, *et al.*, 2002). Nonetheless, from the standpoint of exploiting mitochondria to arrest apoptosis, preventing MMP is of greater importance than determining which factors are released.

### Targeting Mitochondria in RP

It is known that MMP is central to the pathogenesis of many acute and chronic neurodegenerative diseases therefore attempts to modulate the proteins that control this event

could be very useful. The Bcl-2 family of proteins play a complex role in the regulation of programmed cell death, specifically through modulation of mitochondrial integrity. The effect of Bcl-2/Bcl-X<sub>L</sub> over-expression on photoreceptor survival has been examined in a number of models of RP, with widely conflicting results. A number of studies have been undertaken, some of which show promising results of Bcl-2 mediated photoreceptor survival (Nir, *et al.*, 2000). In the rds mouse a two-fold preservation of photoreceptors was observed in Bcl-2 over-expressors at 3 months when compared with nontransgenic mice at the same age. The progression of photoreceptor apoptosis in response to low-level lead exposure in combination with Bcl-X<sub>L</sub> over-expression has also been studied (He, *et al.*, 2003). In this model Bcl-X<sub>L</sub> over-expression preserved rod viability up to P90 at which time 25% of rod photoreceptors remained in lead-exposed mice. The authors hypothesize that Bcl-X<sub>L</sub> in lead-exposed rods complexes with Bax preventing its insertion into mitochondria.

A comprehensive study from 1996 provides evidence of transient protection mediated by over-expression of Bcl-2 (Chen, *et al.*, 1996). Transgenic mice over-expressing Bcl-2 were generated and crossed with mice carrying defective opsin, with rd mice, and with normal albino mice, subsequently exposed to damaging light. Benefits were experienced with respect to each model, with the number of surviving photoreceptors significantly increased in each case. However, it appears that the degeneration was slowed rather than completely halted, given that Bcl-2 transgenic mice eventually became indistinguishable from their transgene negative littermates. Interestingly, over-expression of Bcl-2 resulted in increased levels of apoptosis in wild type mice. The evidence presented in this paper was substantiated by a similar report of transient photoreceptor rescue in Pdegtm1 knockout mice, homozygous for a mutant allele of the gamma subunit of cGMP PDE (Tsang, *et al.*, 1997). Another study shows that co-expression of appropriate levels of Bcl-2 and Bag-1 (a Bcl-2 binding protein) synergistically retard degeneration in a rhodopsin mutant (Eversole-Cire, *et al.*, 2000). Equally in this case the pattern of degeneration was temporarily delayed but not prevented. Moreover, conflicting reports show an absolute lack of protection provided by Bcl-2 and even suggest that it may contribute to the degeneration observed. The effects of both Bcl-2 and Bcl-X<sub>L</sub> in two different models were measured and neither transgene could prevent or delay photoreceptor apoptosis (Joseph and Li, 1996). In mice expressing SV40 T antigen (tag), transgenic Bcl-2 initially blocked apoptosis but itself ultimately resulted in the death of photoreceptors (Quiambao, *et al.*, 2001).

Recently, a study carried out to determine if the temporary protection afforded by Bcl-2 may result from insufficient levels of functional complexes between anti-apoptotic Bcl-2 and its proapoptotic counterparts, yielded some interesting results. The evidence suggested that endogenous Bax was downregulated during normal retinal development between P16 and P24 and persisted into adulthood (Eversole-Cire, *et al.*, 2002). This downregulation of Bax correlated with the diminished ability of Bcl-2 to protect against photoreceptor cell death. It is therefore reasonable to postulate that Bax may not play an important

role in retinal degenerative disease and as a consequence Bcl-2 is unable to afford protection in this instance.

In fact, alterations in Bax expression may provide an explanation for such vastly conflicting results. The abundance of the various pro and anti-apoptotic Bcl-2 family members and their ability to form functional complexes could vary depending on the age of the animal and the stimulus inducing apoptosis. It is possible that changes in the expression of Bcl-2 family proteins, for example Bax or Bak, may occur in order to compensate for Bcl-2/Bcl-X<sub>L</sub> over-expression. Evidently there is a fine line between the protection afforded by Bcl-2 and its deleterious effects, which might also explain the differences experienced by each researcher. It is possible that too much Bcl-2/Bcl-X<sub>L</sub> could disrupt the balance of Bcl-2/Bcl-X<sub>L</sub>-Bax heterodimers, increasing the concentration of free Bax. The transient nature of the protection observed in some of the studies could be explained in a number of ways. Over-expression of Bcl-2/Bcl-X<sub>L</sub> may disrupt the activation of one pathway, for example a caspase-mediated mitochondria-dependent pathway, however death pathways not regulated by Bcl-2/Bcl-X<sub>L</sub> are probably at work in photoreceptors. Nevertheless, it is important to remember that partial rescue of weeks to months over the lifespan of a mouse could extrapolate into a significant rescue in humans.

Cyclosporin A (CsA) is thought to promote its potent neuroprotective effects in animal models of neurodegenerative disease at the level of the mitochondrion (Buki, *et al.*, 1999, Keep, *et al.*, 2001, Sullivan, *et al.*, 2000). This immunosuppressive drug targets either cyclophilin D, which plays a decisive role in PTPC function or calcineurin, preventing calcineurin-mediated dephosphorylation of Bad. IBMX-induced calcium overload in rat retinas *in vitro* results in the release of cytochrome-c from mitochondria. This release of cytochrome-c was only partially blocked by L-*cis*-diltiazem consistent with its partial protection against apoptosis (see calcium section) (Fox, *et al.*, 2003). However, CsA and its nonimmunosuppressive equivalent NIM811 completely blocked release of cytochrome-c, indicating that the effect of rod calcium overload was mediated by PTPC opening. The authors suggest that therapeutic agents such as NIM811, which block cytochrome-c release and subsequent caspase activation, could be of use in the treatment of photoreceptor apoptosis. However, it is important to note that inhibition of PTPC by CsA is believed to be transient and cannot be overcome by increasing calcium concentrations and thus it is not an irreversible pore blocker.

## Calcium

A sustained increase in the level of intracellular calcium has been linked with apoptosis in a number of model systems (Fain and Lisman, 1999, Stridh, *et al.*, 1999). Under normal conditions, mitochondrial Ca<sup>2+</sup> fluxes are integral parts of cellular calcium signalling. It is understood that mitochondria can also buffer non-toxic levels of calcium, extruding these through the sodium/calcium exchanger. However accumulation of mitochondrial calcium eventually results in mitochondrial dysfunction and release of pro-apoptotic proteins. Thus modulation of calcium channels, in order to prevent a lethal calcium influx, should also be

considered as a possible site of intervention upstream of proteases.

Elevation of intracellular calcium has been identified as a key mediator of apoptosis in the light-induced model of retinal degeneration moreover, administration of the calcium channel blocker D-*cis*-diltiazem completely inhibited cell death (Donovan and Cotter, 2002). The drug was administered i.p., in a single dose prior to light-induction and afforded protection up to 48 hours after treatment. While the mechanism of protection is not yet established, the most reasonable hypothesis is that this drug modulates calcium influx via a block on L-type voltage-gated channels. IBMX-induced calcium overload of rat retinas *in vitro*, increases the number of open cGMP-gated channels, thereby inducing photoreceptor apoptosis through disturbance of mitochondrial membrane potential (He, *et al.*, 2000). Using this model, the authors have shown L-*cis*-diltiazem provided partial neuroprotection through blockade of cGMP-activated channels (Fox, *et al.*, 2003). However, inhibition of L-type calcium channels with D-*cis*-diltiazem or verapamil afforded no protection, indicating the limited importance of calcium influx via this particular channel in this model.

The efficacy of D-*cis*-diltiazem in protecting against photoreceptor apoptosis in the rd model is controversial. An initial study from 1996, provided evidence of photoreceptor rescue and preserved visual function (Frasson, *et al.*, 1999). In contrast, these promising results could not be repeated in the same model, with failure to detect either a delayed rate of degeneration or a significant improvement in ERGs (Pawlyk, *et al.*, 2002). Similar negative results were recorded for a canine model of retinal degeneration with a mutation in PDE6B (Pearce-Kelling, *et al.*, 2001) and in P23H rhodopsin transgenic rats (Bush, *et al.*, 2000). Arguably, aberrant levels of cGMP in the rd mouse could lead to increased calcium influx through cGMP-gated channels and based on this supposition, the L-isomer of the drug could prove more beneficial. However, it has also been shown that photoreceptor degeneration was delayed in rd mice lacking L-type voltage-dependent calcium channels (Read, *et al.*, 2002), indicating calcium entry to photoreceptors via this mechanism. As a result targeting both channels types with a combination of L and D isoforms may be necessary to afford consistent protection, since the route by which calcium enters photoreceptors in this model is unknown. Therefore, despite the long record of diltiazem use in the successful treatment of cardiac disorders these conflicting results do not present a compelling rationale for testing this drug. However, alternative targets of calcium-mediated apoptosis may present themselves as viable options.

## CONCLUSION

It is apparent from the literature reviewed here that the issue of caspase-dependent or independent photoreceptor apoptosis is one of continuing dispute. Accumulating evidence suggests that early developmental apoptosis is indeed dependent on the caspase machinery to remove superfluous, unwanted cells. However, components of the classical caspase-dependent apoptotic machinery are down regulated immediately after the completion of developmental apoptosis. This correlates with the withdrawal of

photoreceptor cells from mitosis and the need for tight control of apoptosis, since rapid caspase activation and cell death would have grave implications for the mature retina. It has been proposed that retinal degenerations involve a pathologic reactivation of the developmental apoptotic program, however this would necessitate the re-expression of not one but a number of genes. It is reasonable to postulate that under these conditions alternative proteases may be employed to execute apoptosis under conditions of stress. Candidates include cathepsins, granzymes and calpains, however considering that a role for calcium has been established in a number of models calpains may represent a clear choice.

However, in the absence of a unanimous downstream target mitochondria may be considered a viable upstream target of both caspase-dependent and caspase-independent apoptosis. Translocation of proapoptotic proteins from mitochondria into the cytosol is in many cases a key initiating event in apoptosis. Cytochrome-c has traditionally been attributed primary significance, however the identification of additional mitochondrial proteins, such as AIF, endoG, Smac/Diablo and Omi/HtrA2 which have important death functions, has altered this view. The anti-apoptotic effects of Bcl-2 seem to be negated by the near absence of its heterodimerization partner Bax in mature photoreceptors, indicating that this may not represent a useful target. However, Bcl-2 gene therapy is of continuing interest to the pharmaceutical industry, indeed the development of techniques that will deliver local, high concentrations of the Bcl-2 gene is ongoing. It is conceived that this will afford protection against neurodegenerative disease, cardiovascular disease and ischemic disorders. In this regard, Bcl-2 delivery using viral vectors has been achieved in an attempt to rescue both dopaminergic and thalamic neurons *in vivo* (Caleo, *et al.*, 2002, Natsume, *et al.*, 2002). Conversely, Bcl-2 antisense or Genasense is in Phase I clinical trials in an attempt to enhance the effectiveness of existing cancer treatments in the elimination of cancer cells by apoptosis. Inhibitors of the PTPC such as cyclosporin A and NIM811 have yielded positive results in one model of retinal degeneration, indeed mitochondria have been targeted for neuroprotection in this way in models of ischemic stroke and TBI. Consequently, if we could better understand the mechanism or more likely mechanisms by which mitochondria are penetrated, it is likely that this knowledge could be exploited. Overall it is evident that a clearer understanding of the fundamental control points in photoreceptor apoptosis is of vast importance if we are to identify novel and successful therapeutic targets.

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