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Multiple death pathways in retina-derived 661W cells following growth factor deprivation: crosstalk between caspases and calpains

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Abstract

During development of the mammalian retina, neurons that do not succeed in establishing functional synaptic connections are eliminated by apoptosis, allowing the formation of a finely tuned network. Growth factors play a crucial role in controlling the balance between apoptosis and survival signals not only at developmental stages but also in long-term preservation of retinal functions. In the present work, we explore the apoptotic mechanisms triggered by growth factor deprivation of retina-derived 661W cells. Under serum starvation conditions, these cone photoreceptors underwent cell death with participation of caspase-9, -3 and -12. Interestingly, inhibition of caspases did not prevent apoptosis but only resulted in a temporary delay. We show m-calpain activation in parallel with caspases, indicating that more than one execution pathway is available to cone photoreceptors. Moreover, crosstalk of the caspase and calpain pathways was detected, suggesting a loop that may act to amplify the apoptotic cascade.
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Abbreviations: PCD, programmed cell death; CNS, central neural system; APAF-1, apoptotic protease-activating factor-1; FITC, fluorescein isothiocyanate; PI, propidium iodide; DNA, deoxyribonucleic acid; PBS, phosphate-buffered saline; TBS, tris-buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, glycol ether diamine tetraacetic acid; DMSO, dimethyl sulphoxide; PARP, poly-(ADP-ribose) polymerase; kDa, kilo Dalton; mM, millimolar; μM, micromolar; WB, Western blot; SD, standard deviation

Introduction

Apoptosis is a form of cell suicide directly involved in the development and homeostasis of the mammalian central neural system (CNS). Differentiating neurons that fail to establish the appropriate synaptic connections or show impaired electrical activity are eliminated by this process, also referred as to programmed cell death (PCD).[1] The retina, as a part of the CNS, also undergoes a period of developmental apoptosis that allows adjustment of the final cell numbers, as well as stratification and proper connectivity among the different neuronal types. In this particular tissue, two waves of apoptotic cell death have been reported to occur, the first one coincident with the onset of cytogenesis/migration and the second one with synaptogenesis (for a review, see Vecino *et al.*[2]). Specifically, cone photoreceptors become postmitotic in the embryonic period but it is not until the second postnatal week that they reach their final position in the retina and maturation of the synaptic terminals takes place.[3]

Unlike apoptosis in the nematode *Caenorhabditis elegans*, where it is thought that developmental cell death is genetically predetermined, environmental cues such as trophic support are believed to influence neuronal survival during mammalian CNS development.[1,4–6] Indeed, various neurotrophins and growth factors have been reported to play a role in the protection of connecting neurons from apoptosis.[7] Likewise, a number of studies have related several trophic molecules to photoreceptor survival and differentiation. These include; insulin, fibroblast growth factor (FGF), epidermal growth factor (EGF), taurine, docosahexaenoic acid (DHA), glial-derived neurotrophic factor (GDNF) and pigment epithelium-derived factor (PEDF).[8–13] Additionally, the activation of phosphoinositide 3-kinase (PI3 K)/Akt and mitogen-activated protein kinase (MAPK) survival pathways during retinal development has been directly linked to growth factor stimulation,[14,15] highlighting the crucial role trophic support plays in cell death/survival balance.

Many of the typical morphological features associated with apoptosis can be orchestrated by activation of intracellular proteases. In particular, the caspase family of cysteine proteases are considered key executioners of PCD.[16] Caspases are synthesised in the cytosol as inactive pro-enzymes and in response to a severe stress become activated in a particular sequential cascade. Once activated, executioner caspases contribute to dismantle the cell via direct proteolysis of cell structures, repair enzymes and regulatory proteins. A second family of proteases implicated in PCD is the calpain family of calcium-activated cysteine proteases.[17] Like caspases, they are synthesised as inactive pro-enzymes and require autoprocessing to become active. In addition, calpains strictly depend on an increase in intracellular cytosolic Ca^{2+} levels for activation. Crosstalk of the calpain and caspase pathways has been previously reported in the literature. For instance, caspases facilitate calpain activity through the cleavage of its endogenous inhibitor calpastatin.[18,19] At the same time, calpains directly proteolyse executioner caspases, mediating their activation,[20,21] as well

as proapoptotic Bcl-2 family members that eventually cause the release of apoptogenic factors from the mitochondria involved in the triggering of the caspase cascade.[22–24]

While the time course of developmental apoptosis in the mouse retina has been extensively described in several studies,[10,25–27] the biochemical death pathways involved are not fully understood. In particular, relatively little is known about the molecular mechanisms of developmental apoptosis in cone photoreceptors, due to the paucity of cone-specific markers at early stages of development. An additional obstacle for the detection of cone apoptosis in the developing mouse retina arises from the fact that cones comprise less than 3% of the total photoreceptor population.[28] Knockout mice have, therefore, been indispensable in discerning the possible pathways executed by photoreceptors. A recent study by Zeiss *et al.*[29] employing caspase-3 knockout mice found that ablation of the later did not completely inhibit developmental apoptosis but resulted only in a temporary delay. These results indicate the existence of an alternative, possibly compensatory caspase-3 independent pathway.

In order to explore the cell death pathways available to immature cone photoreceptors, we have employed the photoreceptor cell line 661W. These recently characterised cells express markers of cone photoreceptors, including blue and green opsins, transducin and X-arrestin, and were derived from a postnatal day 8 mouse retina transformed with the SV40 T-antigen.[30] We demonstrate that following growth factor deprivation, these cells execute an apoptotic death programme that involves parallel activation of calpains and caspases. These results substantiate the existence of more than one death pathway in cone photoreceptors and identify calpains as alternative executioners of cell death. Interestingly, inhibition of either protease is not sufficient to prevent cell death, indicating a compensatory role in the execution of the apoptotic programme.

Results

Serum deprivation induces apoptosis in 661W cone photoreceptors

Previous studies have described the withdrawal of growth factors from the medium as an apoptosis-inducing event in retinal cell cultures.[31,32] To investigate the effects of serum deprivation on 661W cone photoreceptors, we cultured these cells in the absence of trophic support for different periods of time and measured death by labelling apoptotic cells with fluorescein isothiocyanate (FITC)-conjugated annexin V. Cells undergoing apoptosis exhibit disorganisation of the plasma membrane, followed by the externalisation of certain phospholipids such as phosphatidyl serine. In the presence of Ca^{2+}, annexin V binds phosphatidyl serine with high affinity, allowing the detection of the apoptotic population by flow cytometry. In order to differentiate early-apoptotic from late-apoptotic cells, we performed a double staining with propidium iodide (PI), a fluorescent indicator of plasma membrane integrity. Annexin V-positive but PI-negative events represent cells in the early stages of apoptosis, whereas positive events for both markers correspond to cells in a later phase, when plasma membrane permeability has been compromised. Upon growth factor withdrawal, 661W photoreceptors underwent PCD externalising phosphatidyl serine in their plasma membrane (Figure 1a). Apoptosis occurred as soon as 12 h after insult; however, in order to study in detail the entire process, a time-course of up to 96 h (when approximately 60% of the cells were apoptotic) was followed in further

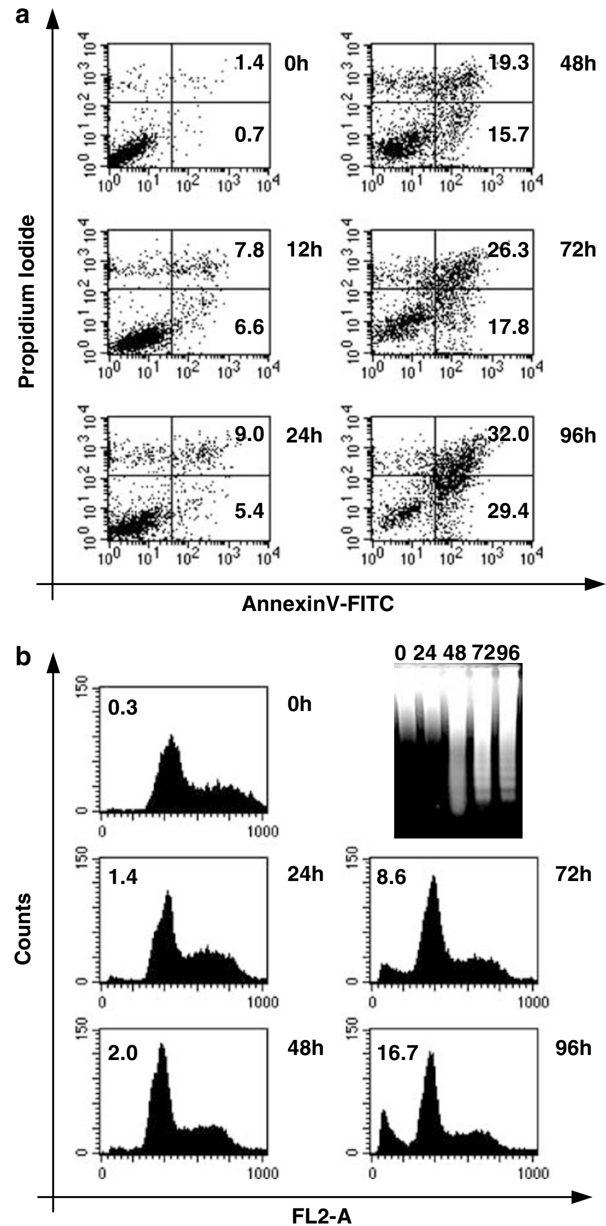

Figure 1 Serum starvation triggers apoptosis in 661W photoreceptors. (**a**) The time course of apoptosis was determined by annexin V *versus* PI staining of serum-starved 661W cells. Annexin V^+/PI^- events (lower right quadrants) represent early stages of apoptosis, whereas annexin V^+/PI^+ events (upper right quadrants) stand for late apoptotic or secondary necrotic cells. The figure is representative of three independent experiments run in triplicate. (**b**) DNA fragmentation was detected by agarose gel electrophoresis (right panel) and flow cytometric analysis of the cell cycle (left panel). The detection of a DNA ladder from 48 h of treatment coincides with the accumulation of events in the sub-G_1 region of the histogram. The figures are representative of two and three independent experiments, respectively

experiments. To confirm apoptotic death, 661W cells were subjected to serum deprivation and then, total deoxyribonucleic acid (DNA) was extracted and analysed by electrophoresis. Biochemically, apoptosis is characterised by internucleosomal DNA cleavage producing fragments that are multiples of 180–200 base pairs, which appear in conventional agarose gels as a ladder pattern. The presence of this typical ladder at 48 h after insult (Figure 1b) verified apoptotic cell death in this model. Additionally, the DNA contents of serum-starved 661W photoreceptors was analysed by flow cytometry. Internucleosomal cleavage was revealed by an accumulation of events, after 48 h of starving, in the hypodiploid or sub-G_1 region of the histogram representing the different phases of the cell cycle (Figure 1b).

Apoptosis of 661W cells proceeds with participation of caspases

Given the contradictory results in the literature regarding caspase involvement in photoreceptor PCD, we investigated caspase activity in these cells following growth factor withdrawal. As serum depletion has been described as a mitochondrial pathway-triggering stimulus,[33] we initially performed Western blot (WB) analyses of caspase-9 and -3 in 661W whole-cell extracts. Upregulation of caspase-9 (49 kilo Dalton (kDa)) and processing into 39/37-kDa fragments was detected from 24 h of treatment, providing evidence for a caspase-dependent death pathway (Figure 2a). Accordingly,

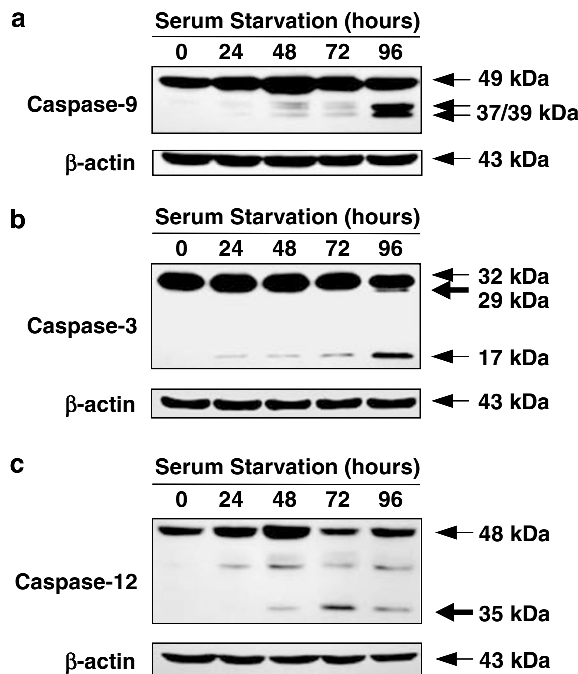

Figure 2 Biochemical analysis of apoptotic machinery: caspases. Whole lysates from 661W cells starved for 24, 48, 72 and 96 h were subjected to Western blot (WB) with antibodies against caspase-9, -3 and -12. Thin arrows indicate procaspases and caspase-mediated cleavage products. Thick arrows in (**b**) and (**c**) correspond to calpain-mediated cleaved forms. Detection of β-actin was used as loading control. Figures are representative of at least three independent experiments

caspase-3 underwent cleavage into a 17-kDa active form that could be identified by immunoblot from 24 h of starving. An additional 29-kDa band, previously described as a calpain-mediated breakdown product,[21,34] was detectable at 96 h, suggesting crosstalk between these two families of proteases (Figure 2b). We also determined the activation status of caspase-12, an endoplasmic reticulum-associated caspase, previously related to serum deprivation-induced apoptosis in other systems.[35] We detected an early upregulation of caspase-12 (48 kDa) and cleavage to an approximately 35-kDa peptide (Figure 2c), which has also been reported to be consequence of calpain-mediated proteolysis.[24,36] Together, these results indicate that caspase-dependent pathways are implicated in apoptosis of 661W cone photoreceptors.

Calcium-dependent protease m-calpain is activated in 661W cells apoptosis after serum depletion

Since the presence of 29- and 35-kDa bands in caspase-3 and -12 immunoblots, respectively (Figure 2b and c), suggested calpain activity, we explored the role of this protease in apoptosis of 661W cells under serum deprivation conditions. The increase in intracellular Ca^{2+} concentration is an absolute requirement for calpain activation, therefore, we analysed Ca^{2+} levels by using the cell-permeable indicator Fluo3-AM. Untreated or serum-deprived 661W cells were loaded with this probe, which fluoresces brightly upon Ca^{2+} binding, and fluorescence was measured in a flow cytometer. As expected, a shift in the fluorescence peak occurred (Figure 3a) indicating an increase in the levels of free cytoplasmic Ca^{2+} as a result of the treatment. Upon increase in Ca^{2+} levels, calpains undergo autocleavage in a similar manner to that of caspases, becoming active proteases. In parallel, degradation of the endogenous inhibitor of calpains, calpastatin, takes place. WB analyses of m-calpain and calpastatin showed a decrease in both, indicating activation and inactivation, respectively (Figure 3b). To further confirm m-calpain activity, we examined two of its substrates, looking for characteristic breakdown products. Besides caspase-mediated fragments of 86 and 25 kDa, poly-(ADP-ribose) polymerase (PARP) (113 kDa) can also be cleaved by calpain during apoptosis, generating a 40-kDa peptide.[37] Similarly, fodrin (α-II-spectrin) (240 kDa) undergoes calpain-mediated degradation into 150 and 140 kDa fragments, in addition to the caspase-dependent products of 150 and 120 kDa.[38] In serum-starved 661W photoreceptors, an approximately 40-kDa PARP band and a 140-kDa fodrin band, consequence of calpain activity, were detected by WB (Figure 3c and d). As expected, the above-described caspase products were detected too. This implies that, in addition to caspases, m-calpain is also activated during serum starvation-induced apoptosis of 661W photoreceptors.

Caspase inhibition leads to partial apoptosis blockade

The crosstalk between caspase and calpain systems has been broadly reported in the literature. We have demonstrated that

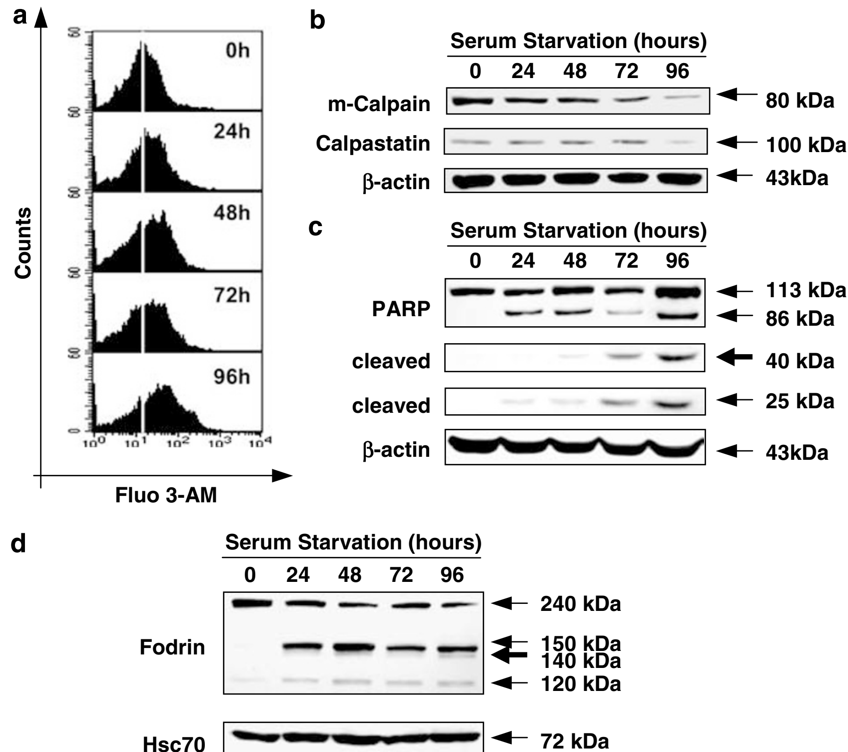

Figure 3 Biochemical analysis of apoptotic machinery: calpains. (**a**) Following serum starvation of 661W cells for 24, 48, 72, and 96 h, an increase in intracellular Ca^{2+} levels was verified by flow cytometry with the Ca^{2+} indicator Fluo 3-AM. An increase in fluorescence is indicative of the presence of free cytosolic Ca^{2+}. The experiment was performed three times with similar results. Whole lysates from 661W cells were obtained and resolved by SDS-PAGE. Blots were probed with (**b**) m-calpain and calpastatin, (**c**) PARP and (**d**) fodrin antibodies. Different exposure times were employed for PARP WB, in order to pick up all cleavage products. Blots were reprobed with β-actin or Hsc70 antibodies to verify equal loading of protein. Thin and thick arrows specify caspase and calpain-dependent cleavage fragments, respectively. The experiment was performed at least three times with identical results

in PCD of 661W photoreceptors both proteases are implicated, but the exact contribution of these individual proteases is unclear. To elucidate the sequence of molecular events taking place following trophic support depletion, we cultured 661W cells for 96 h in serum-free medium, in the presence of increasing concentrations (5–80 μM) of benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), a broad range caspase inhibitor. The rate of cell death, as determined by PI uptake, was significantly lower (ANOVA, $P<0.01$) in samples treated in the presence of the inhibitor (Figure 4a), but the increase in z-VAD-fmk concentration did not correlate with an enhanced protection from PCD, suggesting a saturation in the kinetics of inhibition. Treatment with z-VAD-fmk also decreased the number of hypodiploid cells (ANOVA, $P<0.01$), although DNA fragmentation was never completely abrogated, not even in the presence of 80 μM z-VAD-fmk (Figure 4b). This indicates the likely contribution of an additional caspase-independent pathway. In order to discard the possibility of partial caspase inhibition, we performed *in vitro* caspase-9 and -3 activity assays with their chromogenic substrates, Ac-LEHD-ρNA and Ac-DEVD-ρNA, respectively. In serum-starved cell homogenates, these substrates were hydrolysed, revealing caspase activity. On the contrary, in extracts of cells that had been starved for 96 h in the presence of 50 μM z-VAD-fmk, caspase activity dropped to basal levels (Figure 4c), indicating successful inhibition (*t*-test $P<0.01$). Immunoblot analysis of fodrin also verified caspase blockade, as shown by the absence of a caspase-mediated product (120 kDa) in samples incubated with the inhibitor (Figure 4d). Nevertheless, the calpain-derived fragments of fodrin (140 and 150 kDa) were not affected. Caspase-12 WB confirmed that m-calpain activation was not abrogated when caspases were inhibited, since the calpain-dependent 35-kDa band was detectable in samples treated in the presence or absence of z-VAD-fmk (Figure 4e). These observations indicate that caspases are not the sole executioners of apoptosis in 661W cone photoreceptors, and that the involvement of calpains in the programme does not require caspase activity.

Inhibition of calpains attenuates serum withdrawal-induced apoptosis of 661W cells

In view of the fact that caspases were not acting upstream of calpains in this model, we investigated the effects of blocking calpains using diverse pharmacological inhibitors. We cultured 661W cone photoreceptors without trophic support for 96 h, in the presence of increasing concentrations (0.5–8 μM) of calpain blockers, and then assessed cell death by measuring PI uptake. As illustrated in Figure 5a, the peptide aldehydes ALLN and MDL28170 induced a U-shaped

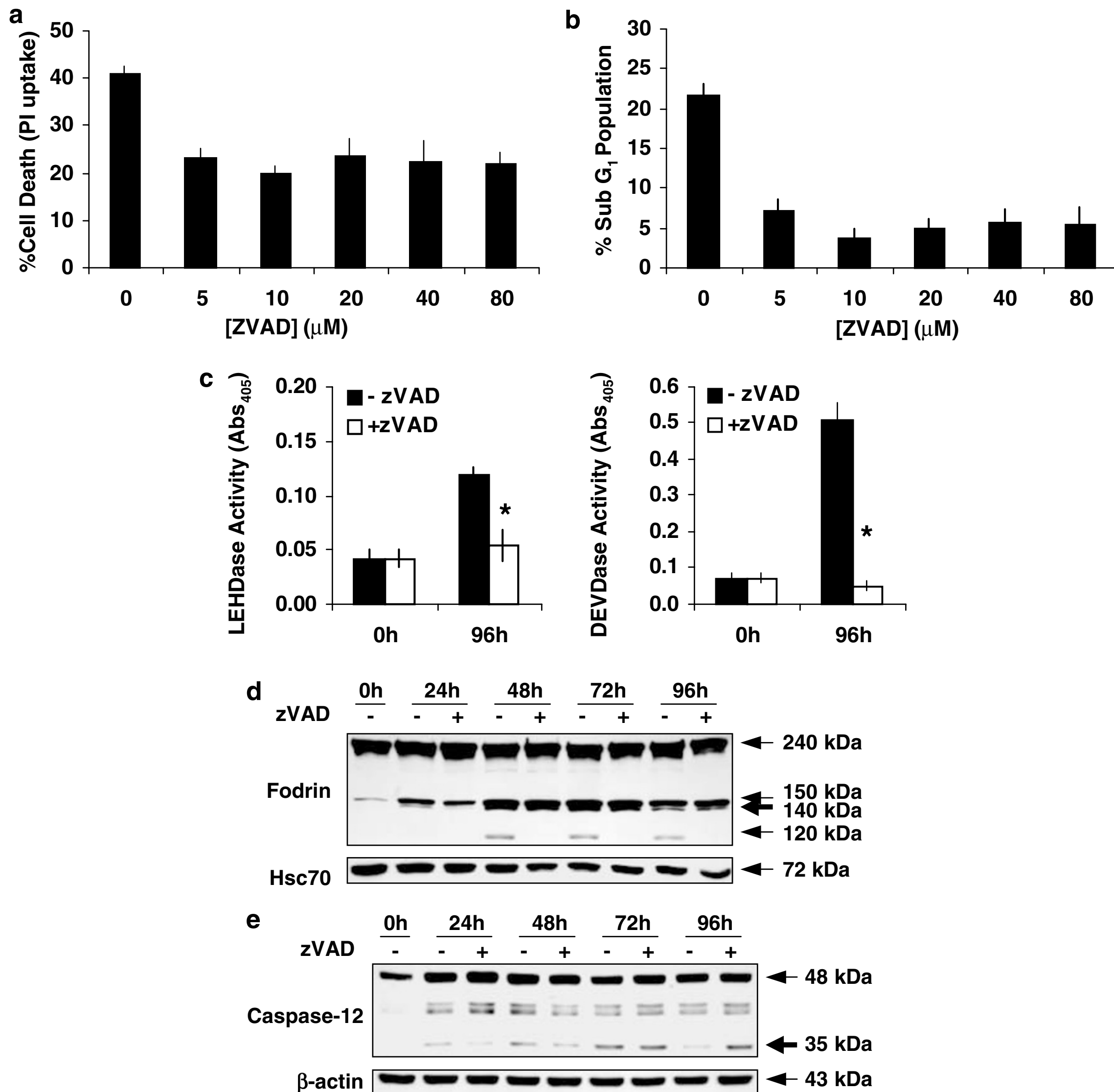

Figure 4 Calpain activation under caspase-inhibiting conditions. 661W cells were cultured in serum-free medium for 96 h, with increasing concentrations of z-VAD-fmk. Cell death was assessed by flow cytometry measuring PI uptake (**a**) or percentage of cells with hypodiploid DNA contents (**b**). Values are mean $\pm$ S.D. of three independent experiments run in duplicates. (**c**) *In vitro* caspase-3 and -9 activity assays with ρNA-conjugated substrates (Abs_{405}) of 661W lysates serum starved for 96 h with or without 50 μM z-VAD-fmk. Data represent mean $\pm$ standard deviation (S.D.) of three independent experiments run in duplicates. Asterisks indicate significant difference (*t*-test, $P<0.01$.) (**d**) WB analysis of fodrin cleavage under caspase-inhibiting conditions (50 μM z-VAD-fmk), showing caspase- and calpain-dependent breakdown products. (**e**) Immunoblot analysis of caspase-12 showing calpain-dependent breakdown products in the presence of 50 μM z-VAD-fmk. Detection of β-actin or Hsc70 demonstrates equal loading of protein. Blots are representative of at least three independent experiments

dose–response in 661W cells subjected to serum deprivation. At concentrations known to have a potent inhibitory effect over calpains, and in a lesser extent over cathepsins B and L but not over other cysteine proteases, serine proteases or the proteasome,[39,40] these peptides were protective against PCD; nevertheless, doses higher than 4 μM were cytotoxic, increasing cell death levels to 1.68- and 1.84-fold, and DNA fragmentation to 2.32- and 2.96-fold, respectively (Figure 5b). On the contrary, the calpain blockers SJA6017 and z-LLY-fmk successfully attenuated cell death without inducing a cytotoxic response at the same doses (ANOVA, $P<0.01$). This reduction in death was accompanied by a significant decrease in the numbers of hypodiploid cells (ANOVA, $P<0.01$), as shown in Figure 5b. The protective effect of calpain inhibitors in this model demonstrates the participation of calpains in PCD of 661W cone photoreceptors. At the same time, the incomplete protection observed points towards a parallel proteolytic pathway.

Discussion

The aim of this work was to dissect the biochemical and molecular events associated with cone photoreceptor cell death after trophic factor withdrawal in the 661W cell line. In this way, we hope to gain an insight into the mechanisms of

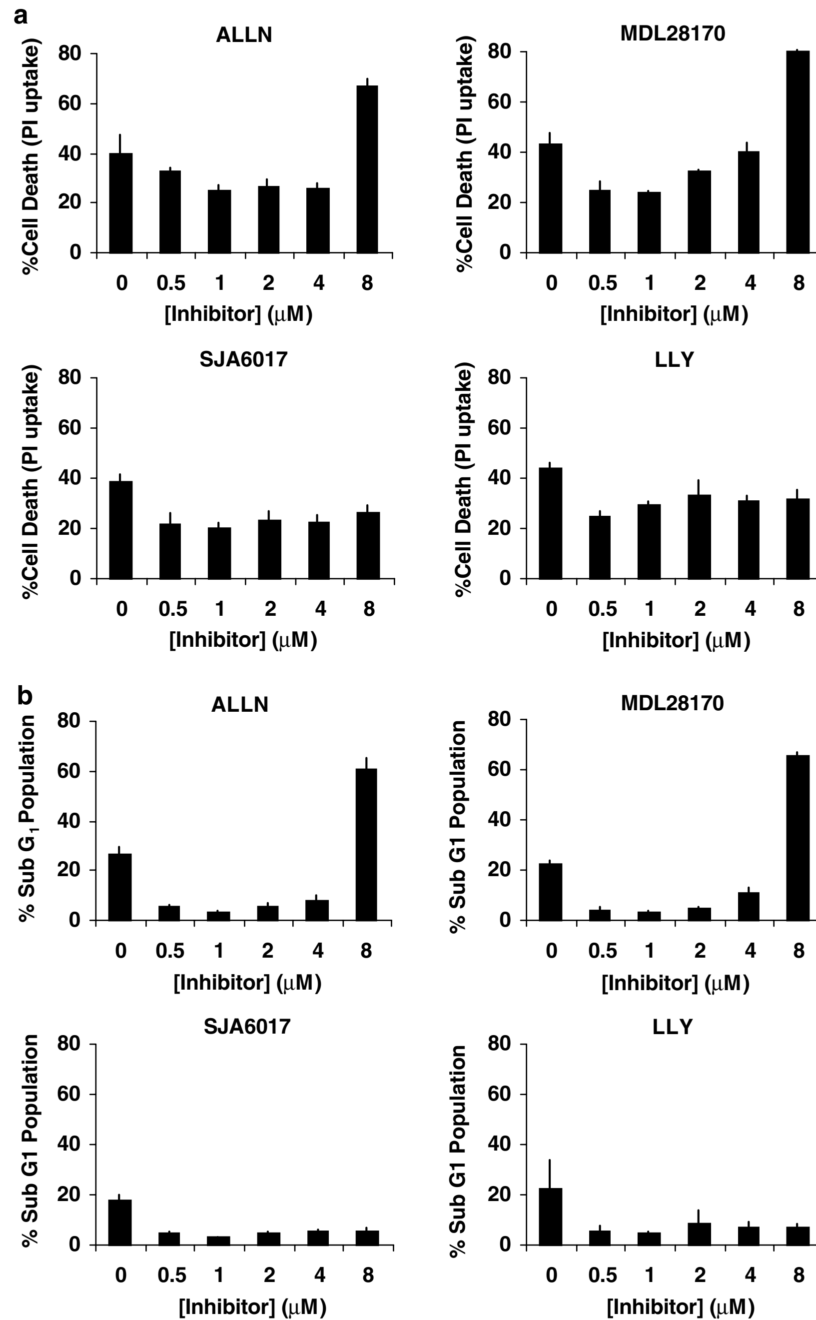

Figure 5 Calpain inhibition. 661W cells were cultured in serum-free medium for 96 h with increasing concentrations of the calpain inhibitors ALLN, MDL28170, SJA6017 and LLY. Cell death was assessed by flow cytometry measuring PI uptake (**a**) or percentage of cells with hypodiploid DNA contents (**b**). Values are mean ± S.D. of three independent experiments run in duplicates

postnatal developmental apoptosis in the retina, where photoreceptor PCD may occur by a similar mechanism due to lack of essential growth factors. Although studies *in vitro* cannot undoubtedly reproduce the physiological conditions of an organism or a tissue, they constitute a useful tool in order to characterise molecular events taking place under tightly controlled conditions. Moreover, the heterogeneity of the retina regarding numerous cell types makes indispensable the use of a cell line in order to dissect the apoptotic pathways in a specific phenotype. Retina-derived 661W cells have been characterised as photoreceptor neurons, which express specific markers of cones, the cells responsible for colour perception and bright-light vision.[30,41] They represent an invaluable tool for the study of cone PCD, given the detection problems that can arise in studying dynamic events in cells that account for a mere 2.8% of total photoreceptors in the mouse retina.[28]

In spite of the fact that previous studies in our group ruled out the involvement of caspases in several *in vitro* and *in vivo* models of photoreceptor degeneration,[42–44] caspase-9, -3, -7

(data not shown) and -12 were active during apoptosis of 661W cells. This conflicting data can readily be explained if one considers the differences between the models employed. Firstly, 661W photoreceptors are derived from retinal tumours of a postnatal day-8 mouse. At this age, developmental apoptosis in the retina peaks and the downregulation of apoptotic machinery, including apoptotic protease-activating factor-1 (APAF-1) and caspase-9 and -3, observed at later stages of normal retinal development[45] has not yet taken place. These cells, unlike mature neurons, are therefore 'competent to die' and it is not surprising that they execute a caspase-dependent cascade following a stimulus that triggers the intrinsic mitochondrial pathway. Supporting this, our findings are in agreement with two previous studies that report caspase-3 involvement in early postnatal retinal development.[24,46] Secondly, 661W cells represent a pure cone population, whereas the models mentioned above were referring to the entire population of photoreceptors and/or the whole retina. We can therefore not exclude the possibility that cone and rod photoreceptors die by different mechanisms. Finally, the nature of the insult, slow and progressive, allows some protein synthesis *de novo*, as demonstrated by caspase-9 and -12 upregulation (Figure 2a and c). Acute insults such as DNA damage or oxidative stress, employed in some of the other models, may result in the cell using the 'stand-by' machinery instead.

Importantly, in this present study, we demonstrate that despite their activation, caspases do not play an essential role in cone photoreceptor apoptosis. This is evident since caspase inhibition does not completely rescue cells from apoptosis nor prevents calpain activation. Recently, Methot *et al*.[47,48] demonstrated that high fractional caspase inhibition was needed to block DNA fragmentation *versus* other apoptotic markers, leading to an overestimation of putative caspase-independent apoptotic mechanisms.[47,48] In our system, we demonstrate that caspases are fully inhibited by the pan-caspase inhibitor z-VAD-fmk, using a substrate cleavage assay (Figure 4c). Moreover, we observe saturation in the kinetics of inhibition from 10 μM doses and our results are in agreement with previous studies that describe the complete blockade of cell death in caspase-dependent *in vitro* models of apoptosis at the same concentrations we used.[49,50] Therefore, we have shown that a caspase-independent mechanism is responsible, in part, for serum deprivation-induced apoptosis of 661W cells. We report an additional role for m-calpain and demonstrate parallel activation of calpain and caspase proteases in serum starvation-induced PCD of 661W cells. The fact that calpains are able to orchestrate a photoreceptor apoptotic programme in these cells further supports the existence of a nonconventional route to apoptosis, which may be orchestrated in cases when the archetypal caspase pathway is unavailable due to physiological blockades. Indeed, we have previously reported evidence of calpain activation in caspase-independent photoreceptor apoptosis during light-induced retinal degeneration, where the caspase pathway appears to be blocked due to downregulation throughout development of essential apoptotic machinery.[45]

In summary, in the current study, we have described cone photoreceptor PCD pathways that take place in conditions resembling those of early postnatal retinal development. We demonstrate that caspases participate in cone photoreceptor apoptosis, although they are not essential to accomplish the programme. We also provide evidence for a calpain-mediated pathway, which is independent of and occurs in parallel with caspase activation.

Materials and Methods

Reagents and antibodies

The pan-caspase inhibitor z-VAD-fmk was purchased from Bachem (Saffron Waldon, UK). Caspase-3 and -9 chromogenic substrates, acetyl-Asp-Glu-Val-Asp-ρ-nitroaniline (Ac-DEVD-ρNA) and acetyl-Leu-Glu-His-Asp-ρ-nitroaniline (Ac-LEHD-ρNA), respectively, were purchased from Alexis Biochemicals (Läufefingen, Switzerland). The Ca^{2+} indicator 1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, pentaacetoxymethyl ester (Fluo 3-AM) was supplied by Molecular Probes (Leiden, The Netherlands). Calpain inhibitors 4-fluorophenylsulfonyl-Val-Leu-CHO (SJA6017; calpain inhibitor VI), benzyloxycarbonyl-Leu-Leu-Tyr-fluoromethylketone (z-LLY-fmk; calpain inhibitor IV), *N*-acetyl-Leu-Leu-Nle-CHO (ALLN; calpain inhibitor I) and carbobenzoxy-Val-Phe-CHO (MDL28170; calpain inhibitor III) were obtained from Calbiochem (Nottingham, UK). Cell Signaling Technology (Hertfordshire, UK) provided the following antibodies: PARP (#9542), caspase-3 (#9662), -9 (#9504) and -12 (#2202). Fodrin (α-II-spectrin #FG6090) antibody was obtained from Affinity Bioreagents, m-calpain (#208755) from Calbiochem and calpastatin (#C8363) from Sigma (Dublin, Ireland). Secondary antibodies anti-rabbit, -rat or -mouse peroxidase-coupled were obtained from Dako (Glostrup, Denmark).

Cell culture

The 661W photoreceptor cell line was generously provided by Dr Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). These cells were routinely cultured in Dubelcco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere with 5% CO_2. For induction of apoptosis, 75 000 cells per well were seeded in tissue culture six-well plates (NalgeNUNC International, Hereford, UK) and allowed to attach for 16 h. The cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4) and 2 ml of serum-free medium was added to each well. After incubation for different periods of time, the cells were detached with a trypsin- ethylene diamine tetraacetic acid (EDTA) solution (Sigma) and, together with their supernatants, washed once with ice-cold PBS.

Cell death measurements: PI uptake and annexin V staining

Quantification of cell death was performed with the vital dye PI (Sigma). Following treatments, the cells were collected as described above and resuspended in 300 μl of PBS. PI was added to a final concentration of 50 μg/ml and samples were immediately analysed by flow cytometry. A double staining with fluorescein isothiocyanate (FITC)-conjugated annexinV and PI was performed for quantification of apoptosis. Cells were harvested, washed once with ice-cold PBS and resuspended in 100 μl of calcium-binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1 : 10 annexinV-FITC solution (IQ Products,

Groningen, The Netherlands). After a 10-min incubation in the dark at room temperature, the cells were diluted with 200 μl of binding buffer and PI was added immediately before flow cytometric analysis. Samples were analysed in a FACScan equipment (Becton Dickinson) using the software CellQuest (Becton Dickinson) for subsequent data treatment. A total of 10 000 events per sample were acquired.

DNA isolation and agarose gel electrophoresis

Total DNA from 3×10^6 untreated or serum-deprived cells was extracted with the GenElute Mammalian Genomic DNA Purification kit (Sigma) as per the supplier's instructions. When necessary, DNA was concentrated by ethanol precipitation using Pellet Paint (Novagen). Then, it was resolved in a 2% agarose Tris-acetate-EDTA (TAE, pH 7.6) gel, stained by soaking in an ethidium bromide solution and visualised under UV light.

Detection of hypodiploid (Sub-G_1) population by flow cytometry

After the induction of apoptosis, the cells were harvested as described in 'cell culture', resuspended in 200 μl of PBS containing 25 μg/ml RNase A and incubated at 37°C for 30 min. Then, the cells were permeabilised and stained with a PI/NP40 solution (50 μg/ml and 0.1% (v/v) final concentrations, respectively) for 5 min at room temperature. Following this, distribution of the cell-cycle phase with different DNA contents was determined with a FACScan flow cytometer. In each sample, 20 000 gated events were acquired. Analysis of the cell-cycle distribution was performed with CellQuest software.

Measurement of intracellular free Ca^{2+}

Cytoplasmic Ca^{2+} levels were determined using the probe Fluo 3-AM. After trypsinisation, cells were washed once with ice-cold PBS and resuspended in 200 μl of fresh buffer containing 250 nM Fluo 3-AM. After a 30-min incubation in the dark at 37°C, fluorescence was measured in a FACScan flow cytometer. A total of 10 000 events per sample were acquired.

Western blotting

Cells were plated in tissue culture flasks, allowed to attach overnight and apoptosis was induced by replacing the routine medium with serum-free medium. After the appropriate incubation times, whole-cell extracts were obtained and resolved by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, cells were scraped and, together with the supernatant, washed once with ice-cold PBS followed by resuspension in cell lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM glycol ether diamine tetraacetic acid (EGTA), 1% NonidetP-40 (NP40), 0.25% sodium deoxycholate, 0.2 mM AEBSF (Calbiochem), 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml pepstatin and 0.1 μg/ml leupetin). After incubation on ice for 20 min, debris was pelleted by a 10-min centrifugation ($10\,000 \times g$) at 4°C and protein concentration in the supernatants was normalised with the Bio-Rad (Hemel Hempstead, UK) assay, using bovine serum albumin as standard. In total, 20–40 μg of protein were diluted in 2 × sample buffer (10% SDS, 100 mM dithiothreitol (DTT), glycerol, bromophenol blue, Tris–HCl) and resolved on 6–12% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline/0.1% Tween-20 (TBS/T, pH 7.6), for 1 h at room temperature. Membranes were incubated at 4°C overnight, with the appropriate dilution of primary antibody (1 : 5000 anti-m-calpain, 1 : 1000 all others). After three, 5-min washes with TBS/T, blots were incubated with the corresponding peroxidase-conjugated secondary antibody (dilution 1 : 1000) for 1 h at room temperature. They were then washed again three times with TBS/T, rinsed briefly with PBS, and developed with the enhanced chemiluminescence reagents (ECL, Amersham, Buckinghamshire, UK). Detection of β-actin (1 : 5000, Sigma) or Hsc70 (1 : 5000, Stressgen) was used as control for equal loading of protein.

Caspase activity assay

Pellets from 3×10^6 untreated or serum-deprived 661W cells were resuspended in 50–100 μl of chilled lysis buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 0.1% 3-[(3-cholomidopropyl) dimethylammonio] propane-1-sulphonic acid (CHAPS), 1 mM DTT, 100 μM EDTA and 0.1% NP40) and incubated on ice for 10 min. Following this, samples were sonicated for 20 s and centrifuged ($10\,000 \times g$) at 4°C for 10 min to eliminate debris. Assay buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT and 100 μM EDTA) was added to 50 μg of total protein to make a final volume of 100 μl. The reaction was started by addition of caspase-3 or -9 ρNA-conjugated substrate to a final concentration of 200 μM, from a 10 mM stock solution in dimethyl sulphoxide (DMSO). After a 20-h incubation at 37°C, absorbance was determined (405 nm) in a microtitre plate reader (SpectraMax-340, Molecular Devices, CA, USA).

Statistical analysis

For multiple comparisons, a one-way ANOVA followed by a Tuckey's test was employed to detect significant differences among samples. In the case of two-sample comparisons, a Student's *t*-test assuming unequal variances was used to determine whether there was a significant difference between the two sample means.

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