Photoreceptor rescue and toxicity induced by different calpain inhibitors

François Paquet-Durand,* Daniela Sanges,† Julianne McCall,‡ José Silva,§ Theo van Veen,*¶ Valeria Marigo** and Per Ekström¶

*Division of Experimental Ophthalmology, Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany
†Telethon Institute for Genetics and Medicine (TIGEM), Napoli, Italy
‡Department of Neurosciences, University of California, San Diego, La Jolla, California, USA
§University of Lisbon (FCMUNL), Lisboa, Portugal
¶Department of Ophthalmology, University of Lund, Clinical Sciences, Lund, Sweden
**Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy

Abstract
Photoreceptor degeneration is the hallmark of a group of inherited blinding diseases collectively termed retinitis pigmentosa (RP); a major cause of blindness in humans. RP is at present untreatable and the underlying neurodegenerative mechanisms are largely unknown, even though the genetic causes are often established. The activation of calpain-type proteases may play an important role in cell death in various neuronal tissues, including the retina. We therefore tested the efficacy of two different calpain inhibitors in preventing cell death in the retinal degeneration (rd1) human homologous mouse model for RP. Pharmacological inhibition of calpain activity in rd1 organotypic retinal explants had ambiguous effects on photoreceptor viability. Calpain inhibitor XI had protective effects when applied for short periods of time (16 h) but demonstrated substantial levels of toxicity in both wild-type and rd1 retina when used over several days. In contrast, the highly specific calpain inhibitor calpastatin peptide reduced photoreceptor cell death in vitro after both short and prolonged exposure, an effect that was also evident after in vivo application via intravitreal injection. These findings highlight the importance of calpain activation for photoreceptor cell death but also for photoreceptor survival and propose the use of highly specific calpain inhibitors to prevent or delay RP. Keywords: apoptosis, calcium, CAST, CNX295, retina. J. Neurochem. (2010) 115, 930–940.

Retinitis pigmentosa (RP) designates a group of genetic diseases causing photoreceptor cell death and blindness in humans. RP usually follows a two-stage process in which first the rod-type photoreceptors and then the cone-type photoreceptors degenerate (Kennan et al. 2005). In the developed world, RP is the prevalent cause of hereditary blindness in the working age population (Farrar et al. 2002). Although RP causing mutations have been identified in 36 genes to date (Retnet database: http://www.sph.uth.edu/retnet, information retrieved in May 2010), the mechanisms responsible for photoreceptor cell death are still largely unknown and currently no treatment is available (Sancho-Pelluz et al. 2009), and is characterized by a loss-of-function mutation in the gene encoding for the β-subunit of rod photoreceptor cGMP phosphodiesterase 6 (Pde6b) (Bowes et al. 1990). This causes cGMP to accumulate in rd1

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Address correspondence and reprint requests to François Paquet-Durand, Institute for Ophthalmic Research, University of Tübingen, Röntgenweg 11, 72076 Tübingen, Germany. E-mail: francois.paquet-durand@klinikum.uni-tuebingen.de; francois.paquet-durand@med.lu.se

Abbreviations used: CAST, calpastatin peptide; CNG, cyclic nucleotide gated ion channel; CPI XI, calpain inhibitor XI; CRB, calpain reaction buffer; DIV, days in vitro; HDAC, histone deacetylase; ONL, outer nuclear layer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PKG, protein kinase G; PN, postnatal day; PTX, phosphate buffered saline containing 0.2% Triton X100; rd, retinal degeneration; RP, retinitis pigmentosa; TUNEL, terminal dUTP nick end labelling; wt, wild-type.
photoreceptors and eventually triggers cell death (Farber and Lolley 1974; Paquet-Durand et al. 2009), via pathways that are now starting to be revealed (e.g., Sanges et al. 2006; Sancho-Pelluz et al. 2008).

An important mechanistic finding relates to calpain-type proteases, which have been shown to be expressed and hyperactive in degenerating rd1 photoreceptors (Paquet-Durand et al. 2006, 2007a). The calpains are a group of calcium (Ca\(^{2+}\))-activated proteases with 14 known isoforms (Croall and Jaartela 2001; Blomgren et al. 2007) that have been implicated in neurodegeneration in a number of different tissues (Leist and Paquet-Durand 2006; Sanges et al. 2006). Throughout cellular life certain calpains are regulated by the highly specific, endogenous calpain inhibitor, calpastatin (Goll et al. 2003; Hood et al. 2004). The dissociation of the calpain-calpastatin complex allows for Ca\(^{2+}\)-dependent activation of the protease (Hood et al. 2004; Li et al. 2004; Suzuki et al. 2004). Over-expression of calpastatin has been associated with an increased resistance to injury and reduced neuronal cell death in neurodegenerative disease models (Wingrave et al. 2004; Higuchi et al. 2005). Similarly, in rd1 mouse photoreceptors but also in other neurodegenerative diseases such as Alzheimer’s, excessive activation of calpains and cell death correlates with a strong down-regulation of calpastatin (Paquet-Durand et al. 2006; Rao et al. 2008).

Together these findings offered two important hypotheses for RP research: (i) that uncontrolled calpain activity strongly contributes to photoreceptor cell death and (ii) that specific calpain inhibitors may prove useful for neuroprotective retinal therapy. We therefore tested different calpain inhibitors in rd1 and wild-type (wt) retinae to further investigate the importance of calpain for photoreceptor cell death. Our results show that the low molecular weight calpain inhibitor XI (CPI XI) can have both beneficial and detrimental effects on photoreceptor viability, depending on whether it is applied by an acute or chronic treatment, respectively. On the other hand, the highly specific inhibitor calpastatin peptide (CAST) consistently exerted strong neuroprotective effects across different in vitro paradigms. Furthermore, CAST reduced rd1 photoreceptor cell death in vivo, suggesting a role for certain calpain inhibitors in the development of novel RP therapies.

Experimental procedures

Animals

Animals were housed under standard white cyclic lighting, had free access to food and water, and were used irrespective of gender. C3H rd1/rd1 (rd1) and control C3H wt mice (Sanayal and Bal 1973; Sanges et al. 2006) were used for in vivo and in vitro experiments. All procedures were performed in accordance with the Swedish National Animal Care and Ethics Committee (permits # M213/03, # M225/04, # M242/07), with the Italian Animal Care Ethics Committee (PRTO. No. 6916/07), and with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and visual research. Because of the critical changes at postnatal day (PN) 11 (Hauke et al. 2006; Sancho-Pelluz et al. 2008), comparisons between rd1 and wt were carried out at this age.

Organotypic retinal explant culture

For in vitro experiments, PN5 animals were killed by decapitation and retinal explants were generated in principle as described previously (Caffe et al. 2001). In the present study, explants were allowed to adjust to culture conditions for 2 days in vitro (DIV) before being treated for another 4 days for what will be referred to here as chronic treatment, or for the last 16 h of the 6 day total incubation, which is hereafter addressed as acute treatment. The end point for the experiments thus corresponds to PN11 in vivo (5 + 2 + 4 days or 5 + 5 days + 16 h). In addition to these two different short-term treatments, a long-term treatment was used in which cultures where explanted at PN5, allowed to adjust to in vitro culture for 2 DIV, and then treated for another 12 DIV until PN19. Explants were maintained under serum-free conditions in R16 nutrient medium (Invitrogen, Paisley, Scotland), and the culture medium with or without treatment was changed every 2 days.

Two calpain inhibitors were used: CPI XI [Z-l-Abu-CONH(CH2)3-morpholine; sometimes referred to as CX295; Calbiochem, San Diego, CA, USA] and CAST (Sigma, Stockholm, Sweden). Calpain inhibitor XI concentration ranged from 0.5 μM to 100 μM, whereas 20 μM was used for CAST treatment (Movsesyan et al. 2004). Controls for CPI XI treated retinae were exposed to equivalent amounts of vehicle (dimethylsulfoxide), whereas controls for water soluble CAST received regular R16 medium. Upon completion of each experiment, the preparations were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h, followed by rinsing with PBS and cryoprotecting with 25% sucrose in PBS. Some preparations were left unfixed to enable the calpain activity assay (below).

In vivo experiments

Because of the rapid progression and early onset of the rd1 degeneration, intravitreal injections were performed at PN10, approximately 2 days before eye opening. Animals were anesthetized with an intraperitoneal injection of 2 mL/100 g body weight of avertine [1.25% (w/v) 2,2,2-tribromoethanol and 2.5% (v/v) 2-methyl-2-butanol; Sigma]. The eye lid was opened carefully, a small incision made below the ora serrata and 1 μL of a solution containing 100 μM CAST in PBS was injected into the eye. Sham-treated animals received only PBS. The capillary was maintained in the eye for approximately 3 s to avoid reflex. As the free intraocular volume of the mouse eye at this age is approximately 5 μL, the effective inhibitor concentration was estimated to be at 20 μM. Animals were killed between 4 h and 16 h (approx. PN11) after injection. The eyes were enucleated and immediately snap frozen on dry ice without fixation. The whole eyes were then embedded in Tissue Tek® (Sakura Finetek, Zoeterwoude, The Netherlands) and sectioned (12 μm) in a cryotome (HM560 Microm, Walldorf, Germany). The fresh, unfixed sections were then used for enzyme activity assays. A total of 12 animals were used for intravitreal injection.
Calpain activity assay

Tissue sections from unfixed retinas were incubated for 15 min in calpain reaction buffer (CRB: 25 mM HEPES, 65 mM KCl, 2 mM MgCl₂, 1.5 mM CaCl₂, 2 mM dithiothreitol, pH 7.2). The fluorescent calpain substrate CMAC, t-BOC-Leu-Met (A6520, Invitrogen, Carlsbad, CA, USA) was then added to CRB at a final concentration of 2 μM and incubated in the dark for 2 h at 37°C. The sections were washed twice for 10 min each in CRB and then mounted with Vectorshield (Vector, Burlingame, CA, USA). The activity assay generally labeled the cell membranes, while calpain-activity-positive cells additionally showed a bright labeling of the nucleus and perinuclear cytoplasm.

Terminal dUTP nick-end labeling

Sections were fixed in 4% PFA, and washed four times in PBS, then incubated for 1 h in 20% goat serum. Terminal dUTP nick-end labeling (TUNEL) staining was done using an in situ cell death detection kit (Roche, Mannheim, Germany) conjugated with tetramethylrhodamine red. Controls were performed by omitting the terminal deoxynucleotidyl transferase enzyme from the labelling solution (negative control) and in pre-treating the sections for 30 min with DNase I (Roche, 3 U/mL) in 50 mM Tris–HCl, pH 7.5, 1 mg/mL bovine serum albumin to induce DNA strand breaks (positive control). The negative control gave no staining at all, while the positive control resulted in general staining of all nuclei in all sections of the retina (Paquet-Durand et al. 2007b).

Staining protocol

Frozen tissue sections or PFA fixed retinas were stained for general histological light microscopic analysis with hematoxylin-eosin according to standard protocols.

Immunostaining was performed on fixed tissue sections. These were washed four times for 10 min each in PBS containing 0.2% Triton X100 (PTX). Blocking solution containing PTX and 5% normal serum from the host animal, from which the secondary antibody was obtained, was applied for 1 h. Primary mouse antibody against rhodopsin antibody (MAB5316, Millipore, Solna, Sweden) was diluted (1: 800) in PTX containing 2% normal serum and applied overnight at 4°C. Sections were then washed four times for 10 min each in PTX and incubated with secondary anti-mouse antibody labelled with Alexafluor 488 fluorescent dye (1: 250, Invitrogen). After three more washing steps in PBS, the sections were mounted with Vectashield (Vector) or Glycergel (DAKO, Carpinteria, CA, USA). Controls consisted of sections processed in parallel without primary antibody and application of the fluorescence detection system.

Microscopy, cell counting and statistics

Histological work was performed on 12 μm frozen sections. Morphological observations and routine light microscopy were performed on a Zeiss Axioshot (Zeiss, Jena, Germany) microscope equipped with a Zeiss Axiovision digital camera. Fluorescence excitation was provided by a HBO 100W halogen lamp. Images were captured using Zeiss Axiovision 4.2 software; image overlays and contrast enhancement were done using Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA).

Percentages of both calpain activity and TUNEL-positive cells were assessed and calculated in a blinded fashion as reported previously (Paquet-Durand et al. 2006, 2009). For each animal the central areas (in proximity to the optic nerve) of at least three sections were quantified to yield an average value, and at least three different animals were analyzed for each time-point and genotype. The number of photoreceptor rows remaining after long-term in vitro culture (PN5–19) was counted manually on hematoxylin-eosin stained specimens. At least nine values from different sectioning levels in central areas of the retina were taken down and averaged for each explant, and these values were then used for further calculations and comparisons. Values are given as mean ± standard error of the mean (SEM), except for long-term in vitro treatment where lower n-values required the use of median instead of mean. In cases where single sample groups were compared to a control group, statistical significance was tested using unpaired, two-tailed, Student’s t-test and Microsoft Excel Software. For multiple comparisons, statistical significance was tested using GraphPad Prism 4.01 Software (GraphPad Software, La Jolla, CA, USA) and employing one-way ANOVA followed by Bonferroni’s multiple comparison post-test. Significance levels were: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)

Results

Effect of calpain inhibitor XI on photoreceptor viability varies by treatment

We had previously established that, when included in the calpain assay system, CPI XI potently reduced the calpain activity that could be visualized in rd1 photoreceptors ex vivo (Paquet-Durand et al. 2006). We therefore tested the efficacy of this inhibitor to rescue rd1 photoreceptors in an in vitro organotypic retinal explant system. Two different treatment paradigms were utilized: chronic and acute, as described in Experimental Procedures (PN5 + 2 + 4 DIV and PN5 + 5 DIV + 16 h in vitro, respectively). The TUNEL assay for dying cells identified a significant, and concentration dependent, difference between the outcomes of the chronic and acute treatments (Fig. 1). As CPI XI concentration increased, the chronically-treated retinas showed more TUNEL-positive cells, particularly in the outer nuclear layer (ONL), but opposite to this, in acutely-treated retinas, their number decreased (Fig. 1a, e; b, f). Thus, at the highest CPI XI concentration (100 μM), chronic treatment resulted in a 195% increase in TUNEL-positivity in the ONL (8.3 ± 0.8% SEM, n = 4, p < 0.001) compared to vehicle treated (dimethylsulfoxide) rd1 retinae (4.3 ± 0.2%, n = 14), while after acute treatment the ONL of retinai exhibited 43% less TUNEL-positive cells (CPI XI treated: 2.5 ± 0.4%, n = 4; control: 4.4 ± 0.3%, n = 12, p < 0.01; Fig. 1i). The discrepancy between the effects of chronic and acute treatment was even more obvious when the percentage of TUNEL-positive cells was plotted against CPI XI concentration, and a linear regression fitted through the data points (Fig. 1j).

Wild-type retinas responded to CPI XI treatment in similar ways (Fig. 1c, g; d, h). A comparatively low number of TUNEL-positive cells were seen in the vehicle treated
ONL (1.6 ± 0.1%, n = 8), representing a low level of photoreceptor degeneration caused by the culture procedure. However, the levels of cell death were significantly increased by chronic CPI XI treatment (4.3 ± 0.4%, n = 10, p < 0.001), while acute treatment led to a numerical reduction of TUNEL-positive cells in the ONL (CPI XI treated: 0.9 ± 0.2%, n = 6; control: 1.5 ± 0.3%, n = 4, p = 0.08; Fig. 1i).

Calpastatin peptide protects rd1 photoreceptors in vitro

The results of the chronic CPI XI treatment prompted the need to look for a different, less toxic calpain inhibitor. When calpastatin peptide (CAST), at the concentration of 20 μM, replaced CPI XI under the identical chronic treatment, the percentage of TUNEL-positive cells in the rd1 ONL drastically diminished from 3.9% (± 0.4 SEM, n = 6) in untreated preparations to 1.8% (± 0.2, n = 12, p < 0.001) in CAST treated specimens. This was statistically equivalent to wt control (1.6 ± 0.1%, n = 8) and correlated with a statistically significant (p < 0.001) decrease of calpain activity-positive cells in the ONL (Fig. 2). Beyond the ONL, cell death labeling in the inner nuclear layer (INL) and ganglion cell layer of CAST-treated rd1 and wt retinae did not identify detrimental effects of CAST treatment on cultured tissue.

As judged by TUNEL analyses, CAST had a neuroprotective effect and CPI XI a photoreceptor toxic effect in the chronic treatment, in cultures ending at a time-point corresponding to PN11. However, at this time-point the rd1 degeneration had not yet led to an obvious decrease in the number of ONL cell rows, and by an experiment of this kind it was thus not possible to conclude that such treatments affect the long-term survival of photoreceptors. To study this, we prolonged the in vitro treatment to 14 DIV until a time-point corresponding to PN19 (PN5 + 2 + 12). Under these conditions, control wt retinae displayed 9.3 rows of photoreceptors in the ONL (± 0.3 SEM, n = 3; Fig. 3a) which was significantly decreased by CPI XI (40 μM) treatment to 6.8 (± 0.7, n = 3, p < 0.05; Fig. 3b). Vehicle treated degenerating rd1 retinae exhibited 3.3 (± 0.3, n = 9; Fig. 3c) rows of photoreceptors at PN19. This was further reduced by CPI XI treatment (2.8 ± 0.2, n = 6; Fig. 3d), although this difference did not attain statistical significance. In clear opposition to this, CAST (20 μM) treatment significantly increased the number of surviving rd1 photoreceptor rows by almost 70% (CAST: 5.5 ± 0.9, n = 3, p < 0.05; Fig. 3e, quantification in 3f).

To obtain a further index on the status of the photoreceptors, the expression of rhodopsin was analyzed (Fig. 3g–k). Healthy, wt retinae in vivo show rhodopsin expression restricted to the outer segments of rod photoreceptors (Fig. 3g), while rd1 retinae, where outer segments do not develop properly, present with a partial mislocalization in the cytoplasm of photoreceptors, that is, staining is here readily
seen in the ONL (Fig. 3i) (Bowes et al. 1988; Sancho-Pelluz et al. 2010). In degenerating rd1 retinae cultured until PN19, the size of rod outer segments and rhodopsin immunofluorescence was strongly reduced when compared to wt (Fig. 3g, i). In wt retinae, CPI XI appeared to increase rhodopsin immunoreactivity in the ONL (Fig. 3g, h). In rd1 explants after long-term CAST treatment, survival of photoreceptors was increased as judged by the number of ONL cell rows (Fig. 3f) with most ONL cells expressing rhodopsin (Fig. 3i, k) thus confirming rod photoreceptor identity. In the untreated rd1 control, however, many of the remaining ONL cells were rhodopsin-negative and hence most likely cone photoreceptors. Still, we cannot exclude the possibility that among the rhodopsin-negative cells, in the untreated rd1 situation, there might have been occasional rods which had lost rhodopsin expression.

The use of the rhodopsin staining also illustrated a remarkable photoreceptor neurite outgrowth into the inner nuclear layer of the untreated rd1 specimens (20.9 ± 2.4 neurites/mm) when compared to wt retinae (5.0 ± 0.6 neurites/mm), which resembled what has been reported previously for degenerating human rod photoreceptors (Li et al. 1995). However, such rod neurite sprouting was strongly reduced in CPI XI treated rd1 retinae (11.2 ± 3.0 neurites/mm) but not affected by CAST treatment (24.3 ± 2.9 neurites/mm), suggesting a potential dependency of this process on the activity of only certain types of calpains (Fig. 3i–l).

Taken together, CAST treatment in both short- and long-term retinal culture demonstrated a strong pro-survival effect, suggesting a major contribution of calpastatin targets to mutation induced rd1 photoreceptor death. At the same time, chronic application of CPI XI clearly had detrimental effects on photoreceptors, while the inner retina appeared to be less, or not at all, affected.

**Inhibition of calpain reduces calpain activity and cell death in vivo**

To explore the outcome of calpain inhibition in vivo in rd1 mice, CAST was injected into the vitreous body of the eye. In the first series of experiments, CAST was injected at PN10 and the percentage of dying photoreceptors was assessed using the TUNEL assay at different time-points post-injection. When compared to untreated specimens (Fig. 4a), at 4 h post-injection there was no significant effect, but at 8 h and 16 h (Fig. 4b) post-injection a strong and significant decrease in the number of TUNEL-positive cells was observed (Fig. 4c). At 48 h an obvious difference between treated and untreated specimens could no longer be noted, presumably because of drug clearance and/or degradation. As the damage to the early post-natal mouse eye caused by intravitreal injections is substantial, repeated
Injection traumas would result in severely increased retinal cell death. The considerable delay in the neuroprotective effects of CAST treatment in vivo could be because of the fact that at the time of treatment a certain number of cells were already compromised to an extent that did not allow rescue anymore.

As the beneficial effect of the treatment was most pronounced at 16 h post-injection, in a second set of experiments the effects of CAST treatment on calpain activity and TUNEL assay were assessed using this treatment-analysis interval, which hence ends the test at an age roughly corresponding to PN11. When compared to untreated or...
sham-treated specimens, CAST injection in vivo led to a clear decrease in the number of cells positive for either calpain activity (rd1 CAST: 1.5 ± 0.3%, n = 3; rd1 sham: 3.6 ± 0.5%, n = 4, p < 0.01) or for TUNEL (rd1 CAST: 2.1 ± 0.2%, n = 3; rd1 sham: 3.2 ± 0.2%, n = 4, p < 0.01).

The reduction in the percentage of dying, TUNEL-positive cells in the rd1 ONL in vivo corresponds to the previous in vitro observations and thus confirms the neuroprotective effects of CAST treatment.

Discussion

Calpains have been widely addressed as key agents in processes of degeneration, especially in the CNS (Movsesyan et al. 2004; Paquet-Durand et al. 2007a; Vosler et al. 2008). The present study supports these notions and exhibits the therapeutic potential of calpain inhibition in photoreceptor degeneration. Our work further underlines the complexity of the calpain system, as the data also demonstrate that certain calpain isoforms or related proteases may be necessary components for photoreceptor survival.

Mechanisms of photoreceptor cell death and activation of calpains

The mutation in the murine homologue of the phosphodiesterase-6, PDE6B gene causes excessive accumulation of cGMP in the rd1 mouse model for RP (Farber and Lolley 1974; Paquet-Durand et al. 2009). The cGMP targets include cGMP-dependent protein kinase G (PKG), which is activated in rd1 photoreceptors, and cGMP activated cyclic nucleotide gated (CNG) ion channels (Pilz and Broderick 2005). While CNG channels may allow for increased Ca²⁺-influx from extracellular sources (Frasson et al. 1999), PKG dependent phosphorylation of inositol 1,4,5-trisphosphate receptors could lead to Ca²⁺ release from intracellular stores (Wagner et al. 2003). In either case, elevated cytosolic Ca²⁺ levels would permit activation of calpains. As PKG is 100-fold more sensitive to cGMP than are CNG channels (Lincoln and Cornwell 1993), rising cGMP levels might induce a biphasic increase in cytosolic Ca²⁺, first by PKG-dependent mobilization of Ca²⁺ from intracellular stores and then by CNG channel mediated extracellular Ca²⁺ influx. Interestingly, a cGMP-PKG-dependent activation of calpains was demonstrated in osteoclasts (Yaroslavskiy et al. 2007), and this may happen also in rd1 photoreceptors, as the increase in PKG activity in these cells (Paquet-Durand et al. 2009) is concomitant with calpain activation (Paquet-Durand et al. 2006).

In addition, we have recently shown that rd1 photoreceptor degeneration involves high activity of poly-ADP-ribose-polymerase (Paquet-Durand et al. 2007b), which may have a bearing on Ca²⁺ homeostasis and cause activation of calpains (Vosler et al. 2009). Rising intracellular Ca²⁺ levels may cause activation of histone deacetylases (HDACs) (Qui and Ghosh 2008), which was indeed observed in rd1 photoreceptors (Sancho-Pelluz et al. 2010). HDAC activation strongly impacts gene transcription (Gallinari et al. 2007), and correlates with down-regulation of neuroprotective genes.

Fig. 4 CAST treatment reduces calpain activity and cell death in vivo: CAST (20 μM) was injected into the vitreous body at PN10. When compared to untreated rd1 retina, 16 h following CAST treatment the number of cells positive for cell death (TUNEL assay; a, b) and calpain activity (c, d) was strongly reduced. The merged image (e, f) illustrates the high degree of colocalization between TUNEL (red) and calpain activity (blue) positive cells. The protective effect of CAST treatment was maximal at 16 h post-injection (g). At this time-point, CAST decreased the number of cells positive for calpain (blue bars) and cell death (red bars) significantly (h). For each time-point at least three different rd1 animals were analyzed, error bars represent SEM, scale bar in (b) = 100 μm. Significance levels were: *p < 0.05, **p < 0.01.
such as cyclic AMP response element binding protein and calpastatin (Kitagawa 2007) in the degenerating rd1 retina (Azadi et al. 2006; Paquet-Durand et al. 2006). Down-regulation of calpastatin in turn facilitates activation of calpains. Together, cGMP-dependent activation of PKG, CNG channels, poly-ADP-ribose-polymerase and HDAC might therefore constitute an auto-feedback loop that, once triggered, culminates in excessive activation of calpains and eventual cell death.

Calpastatin peptide and calpain inhibitor XI

The effect of calpain inhibitors on photoreceptor cell death in the rd1 mouse was approached using both in vitro and in vivo techniques. For the in vitro studies, an organotypic retinal explant culture system (Caffe et al. 2001) was used with both acute and chronic treatment schedules, and revealed that the outcome on photoreceptor viability depended on both the inhibitor and the treatment paradigm used.

At PN11, under acute (16 h) application used here, CPI XI promoted photoreceptor survival in a concentration dependent manner. However, chronic inhibition (4 days), which was six times longer in treatment duration, resulted in the complete opposite effect, causing more cells to undergo cell death than in untreated explants, again in a concentration dependent fashion. The detrimental effect by CPI XI was further corroborated in long-term, PN19 cultures of wt retinae. The opposing results from acute and chronic inhibition suggest that, apart from being able to induce photoreceptor degeneration, calpain activity may simultaneously be required for normal functioning of photoreceptors in both rd1 and wt retinae.

In contrast to CPI XI, CAST was found to counteract photoreceptor death in all in vitro and in vivo paradigms tested and thus served a neuroprotective role regardless of treatment design. The difference between the outcomes from the two inhibitors could originate in the manner of inhibition. The dipeptidyl-alpha-ketoimide CPI XI (CX295) inhibits the active site of calpains (Donkor 2000) and is hence predicted to act on all known calpain isoforms. In addition, it may also weakly inhibit cathepsin B (Blomgren et al. 2001). On the other hand, the peptidic calpain inhibitor CAST is based on the sequence of the endogenous calpain inhibitor calpastatin (Maki et al. 1989) and has thus a different operation mode, as it prevents the autolytic cleavage that leads to calpain activation (Goll et al. 2003). CAST is generally regarded to be the most specific inhibitor of calpains, and displays high selectivity for calpain isoforms associated with the small calpain subunit (calpain 4, CAPN4), which include the two main isoforms calpain 1 and 2 (μ- and m-calpain, respectively) as well as calpain 9 (Suzuki et al. 2004). This distinction in selectivity and mechanisms may be at the root of our differing results in the sense that the photoreceptors may, for their survival, rely on the continuous activity of calpain isoforms that can be inhibited by CPI XI, but not by CAST. With prolonged time of inhibition by CPI XI, a pro-survival effect would then be outweighed by the detrimental loss of the activities of some calpains. Alternatively, CPI XI may have unwanted side-effects such as inhibition of other proteases like cathepsins or the proteasome, which again with time might render photoreceptor survival impossible. While both inhibitors may score similarly well in inhibiting calpains in cell-free or other test systems, our results from a complex neuronal tissue thus strongly indicate vital differences in their respective mode of action and/or specificity. Another aspect worth considering is that we previously reported a reduction in calpastatin mRNA in the retina of the rd1 mouse (Paquet-Durand et al. 2006). As CAST in essence mimics calpastatin, its addition to the rd1 retina may be seen as a way of counteracting the calpastatin discrepancy, which again fits with a better protective effect than when inhibiting calpains in general. The fact that the CAST effect was lost relatively soon after in vivo injection, could be because of metabolism or to increased demand after the time of eye opening (PN12–13). Additional in vivo studies with improved delivery methods will be needed to further establish neuroprotection mediated by calpastatin or structurally related inhibitors. Nevertheless, the results obtained with CAST treatment underline the benefits of calpain inhibition during photoreceptor degeneration, provided the right conditions are applied.

Calpain activity in photoreceptor physiology

High intracellular Ca\textsuperscript{2+}-concentrations are known to occur in many degenerative processes and may lead to an excessive activation of calpains, which in turn may cause cell death (Suzuki et al. 2004; Vosler et al. 2008). By comparison, the functions of calpains under physiological Ca\textsuperscript{2+}-concentrations are less understood, although reports from a range of studies suggest involvement in several and diverse processes (Croall and Ersfeld 2007). An extended absence of CPI XI sensitive calpain activities – even if this is only partial, as with lower inhibitor concentrations here – could thus lead to a cumulative disruption of calpain-dependent cellular activities. As photoreceptors have one of the highest protein turn-over rates of any cell type (Young 1976), they may be particularly vulnerable to inhibition of proteolysis and improper disposal of surplus proteins. Interestingly, calpains of the types Lp82/85, which appear to be insensitive to inhibition by calpastatin (Goll et al. 2003), are found to play a role in rodent eye lens maturation (Ma et al. 2000). We have reported the presence of Lp82/85 calpain in the mouse retina (Paquet-Durand et al. 2006), and these might therefore be amongst the calpains inhibited by CPI XI but not by calpastatin. Furthermore, calpains have been implicated in proteolysis of arrestin in photoreceptors (Azarian et al. 1995) and
prolonged deactivation of the phototransduction cascade. CPI XI seemingly induced an increase in rhodopsin immunofluorescence in the wt ONL. Although this may simply reflect general protein accumulation in photoreceptors, it would also be compatible with an involvement of calpains in the hydrolysis and/or trafficking of rhodopsin. Dysregulated arrestin breakdown, alone or in combination with effects on rhodopsin, could thus promote degeneration of photoreceptors in ways similar to those causing retinal degeneration via abnormal rhodopsin/arrestin complexes (Chuang et al. 2004; Chen et al. 2006). Independent of the exact cause for the observed effects, the non-photoreceptor cells of the retina appeared to be much more tolerant to calpain inhibition.

Neurite sprouting in retinal degeneration has been found also in the human retina (Li et al. 1995), and may be linked to phenomena associated to degeneration in general, such as the presence of activated Müller cells, which are known to produce factors that could act stimulatory on various neuronal processes (Bringmann et al. 2006). In addition to such a mechanism, the excessive neurite sprouting in rd1 photoreceptors may be boosted by their high levels of cGMP (Paquet-Durand et al. 2009), as a role of cGMP-PKG signalling in neurite outgrowth has been described (Ditlevsen et al. 2007). Interestingly, the sprouting effect was blocked by CPI XI, while it was unaffected after CAST treatment. Apart from the possibility that this result could be secondary to effects on, for instance, calpain sensitive Müller glia events, calpains are also known to regulate filopodial motility, neurite outgrowth (Robles et al. 2003), cell migration and adhesion (Glading et al. 2004; Franco and Huttunencher 2005), which may all be relevant in this context. Although the mechanisms of neurite outgrowth are outside of the scope of this report, the results again demonstrate the different properties of the two inhibitors and emphasize the importance of studying them in parallel.

Conclusion

While numerous studies have suggested calpain inhibition as a potential approach to prevent neurodegeneration in many different experimental systems, including the retina, our study for the first time provides data that calpain inhibition may have not only beneficial but also detrimental effects. The results bring about important considerations for the implementation of calpain inhibitors as therapeutic agents for RP and highlight the need to more precisely define calpain substrates. Nevertheless, the strong protective effect of CAST both in vitro and in vivo suggests that restriction of calpain inhibition to specific calpains is a feasible approach for the treatment of inherited retinal degeneration. In the future, carefully targeted and highly selective calpain inhibitors might further improve this.

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