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Do calcium channel blockers rescue dying photoreceptors in the *Pde6b^{rd1}* mouse?

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Abstract

Retinitis pigmentosa (RP) is a genetically heterogeneous set of blinding diseases that affects more than a million people worldwide. In humans, ~5-8% of recessive and dominant RP cases are caused by nonsense mutations in the *Pde6b* gene coding for the β -subunit of the rod photoreceptor cGMP phosphodiesterase 6 (PDE6- β). The study of the disease has been greatly aided by the *Pde6b^{rd1}* (*rd1*) mouse model of RP carrying a null PDE6 β allele. Degenerating *rd1* rods were found to experience a pathological increase in intracellular calcium concentration ('Ca overload') when they enter the apoptotic process at postnatal day 10. A 1999 study suggested that the Ca²⁺ channel antagonist D-cis diltiazem delays the kinetics of *rd1* rod degeneration, conferring partial rescue on scotopic vision. Subsequent reports were mixed: whereas several studies failed to replicate the original results, others appeared to confirm the neuroprotective effects of Ca²⁺ channel antagonists such as diltiazem, nilvadipine and verapamil. We discuss the discordant results and the controversy they caused, and suggest potential causes for the discrepancy. We also discuss potential involvement of recently identified Ca²⁺-dependent mechanisms that include protective calcium ATPase mechanisms, ryanodine and IP3 calcium stores, and store operated channels in *Pde6b^{rd1}* neurodegeneration.

1.1 Introduction

Retinitis pigmentosa (RP), a genetically heterogeneous set of blinding diseases affecting more than a million people worldwide, derives its name from an accumulation of pigment clusters in the neural retina. Early symptoms associated with the main rod-cone dystrophy variants include night blindness and loss of peripheral vision, followed by eventual loss of central vision. One of the best-characterized forms of recessive RP in humans is caused by a nonsense mutation in the gene coding for the β subunit of the rod photoreceptor cGMP phosphodiesterase 6 (PDE6b) that has been linked to 5-8% cases of RP (McLaughlin et al., 1995). The essential features of photoreceptor degeneration in human RP have been replicated in rodent models, which can be studied over much shorter time frames. The *Pde6b^{rd1}* (*rd1*) mouse model carries a recessive nonsense mutation in PDE6b (reviewed in Farber, 1995). The mutation is characterized by rapid degeneration of rods beginning at late stages of photoreceptor differentiation, which in turn triggers apoptosis of cones. By P90, virtually all photoreceptors have disappeared (Carter-Dawson et al., 1978; Punzo et al., 2009) except ~3% of cone perikarya in the dorsal retina (Jimenez et al., 1996). While gene therapy and pharmacological treatments have been attempted to ameliorate photoreceptor degeneration in RP models, a significant bottleneck for developing treatment has occurred

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due to the large number of different genes that lead to RP (>46; www.sph.uth.tmc.edu/Retnet/) and the apparent inconsistency of pharmacological studies focused on treating RP. Hopes for a possible restorative pharmacological intervention were kindled by the report that D-cis diltiazem - an antagonist of voltage-activated Ca^{2+} channels which is commonly used as a cardioprotectant - partially rescues *rd1* photoreceptors (Frasson et al., 1999). Suppression of Ca^{2+} pathways that orchestrate apoptotic cascades in rods and cones promised to deliver a general therapeutic strategy that could be applied to treating many recessive retinal degenerations. Subsequent reports appeared to confirm (Read et al. 2002; Sanges et al., 2006) or repudiate (Pawlyk et al, 2002; Bush et al. 2000) the basic findings of the Frasson study. The aim of this paper is to review the literature, explore potential mechanisms involved in *Pde6b^{rd1}* neurodegeneration in the light of recent findings on Ca^{2+} signaling in vertebrate photoreceptors and explore the possibilities offered by the pharmacological approach to prevention of photoreceptor degeneration.

1.2. The *Pde6b^{rd1}* mouse and increased [cGMP]

The *Pde6b^{rd1}* (*rd1*) mouse phenotype was first identified in a Harvard albino mouse strain and subsequently found in wild and laboratory mice across the United States and Europe (e.g., Pittler et al., 1993). The mutation is associated with a nonfunctional rod-specific PDE6 gene located on the mouse chromosome 5. Every mouse strain with the *rd1* gene carries a murine leukemia provirus integrated into the first intron, combined with a point mutation which introduces a stop codon in exon 7 (Farber, 1995). At P10, 33% of mRNA is transcribed in *Pde6b^{rd1}* rods (Vicizian et al., 1991), however, the message is not expressed, presumably due to nonsense-mediated decay of PDEb6 mRNA and/or instability of the truncated PDE6b peptide (Bowes et al., 1990; W. Baehr, personal communication). Loss of the β subunit completely eliminates function of the PDE complex which consists of a catalytic dimer (non-identical α and β subunits) and two inhibitory γ subunits. PDE malfunction affects both amplitude and kinetics of the photocurrent and results in cGMP, Na^+ and Ca^{2+} overloads within *rd1* cells.

Although it is clear that *rd1* phenotype is caused by mutated PDE6, the causal relationship between abnormally high [cGMP] and apoptosis is still not well understood. cGMP content in the *Pde6b^{rd1}* retina increases days before signs of degeneration become apparent, reaching its maximum around the eye opening when rod degeneration is at its peak (Farber and Lolley 1974). Early signs of *rd1* expression in photoreceptors such as retarded growth of OS and IS are seen 4-8 days after birth (Sanyal and Bal, 1973). Swelling of rod mitochondria and appearance of vacuoles in the IS occur at P8 (Farber and Lolley 1974). While cellular integrity and thickness of P10 *rd1* ONL is comparable to the WT, OS disks show signs of disruption, chromatin is fragmented and the P10 increase in TUNEL-positive outer nuclear layer (ONL) cells is followed by rapid degeneration of rods by P14. At P18-P21, rods are gone, leaving a single row of cone perikarya (Carter-Dawson et al., 1978) and a total loss of image-forming vision by P40.

1.3. Calcium regulation and overload in the photoreceptor inner segment

Rod photoreceptors are formed by two compartments, the OS and the IS, that communicate through a thin nonmotile cilium. PDE6 in the wild type retina is located in the OS whereas the pro-apoptotic cascades are confined to the IS. *Pde6b^{rd1}* retinas express a rudimentary OS, hence it is not clear whether a messenger mechanism transmits a 'degenerate and die' message from the OS to the IS and/or the entire holoenzyme complex remains 'stuck' in the IS. Such a messenger could be the metabolic stress caused by depletion of ATP expended to combat excessive Na^+ and Ca^{2+} influx or stress caused by accumulation of proteins in the ER. An alternative, which is not mutually exclusive, is that the signal is mediated by

elevated $[Ca^{2+}]$ itself as Ca^{2+} regulates all aspects of photoreceptor function, including degeneration (Szikra and Krizaj, 2009).

There is little doubt that degenerating photoreceptors experience Ca^{2+} overload. Elevated cytosolic Ca^{2+} concentrations in *rd1* rods have been measured directly with Ca^{2+} indicator dyes and indirectly by observing compensatory changes in Ca^{2+} -dependent proteins. Around P10, $[Ca^{2+}]_i$ elevations are observed in acutely dissociated *rd1* rods and in cultured *rd1* explants (Doonan et al., 2005; Sanges et al., 2006), accompanied by upregulated CaM kinase II and migration of phospholipases & associated $G_{\beta 1 \gamma 1}$ subunits (Donovan and Cotter, 2002; Hauck et al., 2005) whereas the calpain inhibitor calpastatin is strongly downregulated (Paquet-Durand et al. 2006). Other parallel signs of Ca^{2+} -dependent changes in *rd1* rods include prominent increases in m and μ calpain proteases and cleavage of caspases-3 & -12 and AIF (apoptosis-inducing factor; Sharma and Rohrer, 2004). Mitochondria are depolarized by excess Ca^{2+} (Doonan et al., 2005), possibly leading to activation of the permeability transition pore (He et al., 2000) and translocation of AIF and caspase 12 into the nucleus (Sanges et al., 2006). Another Ca^{2+} store, the endoplasmic reticulum (ER), activates pro-apoptotic Bid, Bax, PERK, IRE1 α and ATF4 pathways (Lin et al., 2007). Given that *rd1* rods experience an enormous degree of ER stress due to the traffic jam of proteins normally destined for the OS, it is very likely that there is a concomitant malfunction of SERCA-mediated Ca^{2+} sequestration and ryanodine & IP3 receptor mediated release of ER Ca^{2+} (see below).

In early 90-ies Mark Tso's group at the University of Illinois showed that the diphenylpiperazine flunarizine, a non-specific antagonist of Ca^{2+} channels (Edward et al., 1991) but not the dihydropyridine nimodipine, a specific L-type channel antagonist (Li et al., 1991), protect photoreceptors stressed by exposure to strong light. However, two L-type antagonists, D-cis diltiazem and verapamil, were soon afterwards found to inhibit photoreceptor degeneration in the *Drosophila rdbB* mutant (Sahly et al., 1992) and to confer partial protection on *Pde6b^{rd1}* photoreceptors (Frasson et al., 1999; Takano et al., 2004; Sanges et al., 2006). These studies initiated interest in pharmacological approach to protecting vision through management of the Ca^{2+} overload.

2.1. D-cis-diltiazem and neuroprotection in the retina

The dihydropyridine diltiazem is a competitive antagonist of L-type calcium channels with an IC_{50} of $\sim 5 \mu M$ (Koch and, Kaupp 1985). The D-cis stereoisomer is the active compound in several commonly used prescription drugs. Three other L-type blockers, verapamil, nilvadipine, nimodipine and nifedipine are also designated for human use.

The first important study using D-cis diltiazem was performed by Serge Picaud's group in Strasbourg and published in Nature Medicine in 1999 (Frasson et al., 1999). The protocol involved intraperitoneal (IP) injections of D-cis-diltiazem two times a day, starting at P9, reaching a daily dose of 54 mg per kg body weight, a dose ~ 6 fold higher than the maximal allowed human daily dose ($< 1 mg/kg$ 4 times daily; Bush et al., 2000). Retinal wholemounts and slices were labeled with an antibody against rhodopsin and outcomes were measured indirectly via the scotopic ERG b-wave which showed a remaining b-wave ($\sim 20 \mu V$) in 4 out of 10 animals treated at P36. Though the amplitude was small (compared to $\sim 800 \mu V$ in wild type eyes), this result was highly significant as the *rd1* scotopic b-wave is basically flat around P21 (Pawlyk et al. 2002). Additionally, the diltiazem-treated cohort at P36 had ~ 18600 surviving rods, a 2-3 fold increase compared to 7500 rods in non-treated animals; a modest protective effect on cone survival was also observed. These early results prompted a clinical study which found that diltiazem, supplemented with vitamin E, improves vision in human RP patients (Pasantes-Morales et al. 2002).

2.2. Criticism of the Frasson study

The immediate concern voiced by Tso was that the rhodopsin antibody used by Frasson et al. might have depicted ongoing rod degeneration rather than healthy, “protected”, cells (Edward and Tso, 2000). Several groups, which attempted to replicate the original study using D-cis-diltiazem in different models of RP degeneration, reported mostly discouraging results. A histological & ERG study by Sieving's group found no significant rescue in the P23H rat that carries a dominant negative opsin mutation (Bush et al. 2000). A follow-up study using the same methodology in the *rd1* dog that carries a stop codon in its PDE6b gene found no neuroprotection (Pearce-Kelling et al. 2001). Yamazaki et al. (2002), Fox et al. (2003) and Takano et al. (2004) observed no effect of D-cis diltiazem on *Pde6b^{rd1}* rods after treating P9 mice for 7 days with 1 mg/ml diltiazem (i.e., at ~50 times lower dose than the Frasson study). Finally, Pawlyk et al (2002), using a different *rd1* strain, found no significant differences in a double blind study that reproduced the original study conditions. There were no changes in rhodopsin and cone (S and M) opsin immunoreactivity nor were changes observed in the ERG, leading the authors to conclude “there is no compelling rationale for testing D-cis-diltiazem (Cardizem, Hoechst-Marion Roussel) as a possible treatment for RP.”

2.3. Subsequent evidence shows L-type channels are involved in degeneration

As D-cis diltiazem in the Frasson study only partially (~50%) blocked L-type channels, a stronger effect would be expected with full channel block or *in vitro*. A subsequent study found that elimination of the β_2 subunit of the L-type calcium channel results in degeneration at a (delayed) rate similar to that in the Frasson study (Read et al., 2002). A 40% increase in the number of surviving cells at P21 was observed in *Pde6b^{rd1}* - β_2 KO animals, directly and unambiguously confirming involvement of L-type channels in *rd1* degeneration. Subsequently, Sanges et al. (2006) showed that the D-cis stereoisomer blocked Ca^{2+} overload, resulting in a decreased number of TUNEL-positive *rd1* rods. Translocation of AIF and caspase-12 to the nucleus of remaining rods was inhibited, confirming the effectiveness of D-cis diltiazem in retinal *rd1* explants.

Nilvadipine, an L-type antagonist often used in Japan, was effective in decreasing photoreceptor cell loss in the RCS rat (Yamazaki et al. 2002) and *Pde6b^{rd1}* (Takano et al. 2004) & *rds* mice (Takeuchi, et al. 2008). The effect of nilvadipine vs. the lack of effect seen with diltiazem treatment was ascribed to greater permeability of dihydropyridines vs. benzothiazepines (Takano et al. 2004). Surprisingly, while nilvadipine appeared to preserve photoreceptor structure, it had no effect on ERG b-wave amplitudes. Microarray analysis after nilvadipine treatment showed downregulation of proapoptotic genes coding for caspases-3, -9 and -14 as well as and ADP-ribosyl cyclase, the product of which acts as an allosteric modulator of the ryanodine receptor (RyR). RyR itself, a major cellular mediator of calcium-induced calcium release in the ER, was upregulated (Takano et al. 2004), implicating Ca^{2+} stores in *rd1* degeneration.

D-cis-diltiazem inhibits not only L-type calcium channels, but also CNG channels and vice versa, the L-cis isomer inhibits L-type channels with an $\text{IC}_{50} \sim 75 \mu\text{M}$ (Koch and Kaupp, 1985; Baumann et al., 2004). A slight advantage of L-cis over the D-cis isomer was suggested for retinas treated with zaprinast (Vallazza-Deschamps et al. 2005) and lead (Fox et al., 2003). In conclusion, while elimination of L-type-mediated Ca^{2+} entry delays, but does not prevent, *rd1* rod degeneration, the accumulated evidence supports the notion that Ca^{2+} overload mediated by some type of Ca^{2+} channel potentiates degeneration.

3. Other players may be involved

The ineffectiveness of L-type channel blockade in rat and dog might have been due to drug dosing and accessibility issues (some dihydropyridines are notoriously difficult to get into solution let alone across the blood-brain barrier). Alternatively, the rhodopsin mutation in the P23H mutant and the *Mertk* mutation in the RCS rat may involve different degeneration mechanisms unrelated to Ca^{2+} influx. A final possibility is that Ca^{2+} regulation in degenerating photoreceptors involves players that were not directly affected by L-type and/or CNG channel antagonists.

Edward et al. (1991) hinted at the contribution of IP3-sensitive intracellular Ca^{2+} stores localized in the ER. Indeed, suppression of Ca^{2+} sequestration into the ER kills differentiating photoreceptors (Linden, 1999; Chiarini et al. 2003), presumably due to global suppression of mRNA translation that occurs in cells with depleted Ca^{2+} stores (Brostrom and Brostrom, 2003). Conversely, at P10-P12, Ca^{2+} overload in *rd1* cells coincides with peak expression of pro-apoptotic ER markers such as caspase-12, phospho-pancreatic ER kinase (p-PERK), phospho-eukaryotic initiation factor 2 α (p-eIF2 α) and glucose-regulated protein-78 (GRP78/BiP) (Yang et al., 2007, Lin et al., 2007), possibly resulting in their amplification. This suggests that a critical junction for preventing Ca^{2+} -mediated apoptosis might be Ca^{2+} stores and/or store-operated channels rather than L-type/CNG channels.

Store-operated channels belonging to different canonical TRPC classes are prominently expressed in rods and cones (Szikra et al., 2008; Krizaj et al., 2008a) together with their activators, STIM1 proteins (Szikra et al., 2009). TRPCs play an increasingly recognized role relative to Ca^{2+} overloads that drive calpains and apoptosis (Marasa et al., 2006; Shan et al., 2008) and neuroprotection (Bollimuntha et al., 2006; Yamamoto et al., 2007). In normal retinas, Ca^{2+} influx through these channels is likely to serve a neuroprotective function by preventing pathological decreases in $[\text{Ca}^{2+}]_i$ in cells exposed to saturating light, which when prolonged was shown to trigger photoreceptor degeneration (Leconte and Barnstable, 2000; Woodruff et al., 2003). TRPC transcription and expression is dramatically affected in *Pde6b^{rd1}* degeneration together with upregulated expression of the major protective PMCA1 calcium transporters (Krizaj et al., 2008b). This suggests that *rd1* rods are battling Ca^{2+} overload by upregulation of endogenous Ca^{2+} clearance mechanisms. It remains to be seen whether upregulation of these ATPases prolongs rod survival. In summary, the *Pde6b^{rd1}* mouse model has greatly facilitated studies of retinal degeneration caused by defects in expression and function of PDE6. This degeneration is intimately associated with chronic elevation in $[\text{Ca}^{2+}]_i$ which has a causal role in activating pro-apoptotic pathways within the inner segment. The conflicting interpretations of pharmacological effects may stem partly from lack of drug specificity and partly from our lack of a comprehensive understanding of Ca^{2+} regulation and trafficking in the photoreceptor IS. Successful therapeutic interventions will depend on our understanding of signaling pathways involved in the degeneration process.

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