Activity of protein kinase G (PKG) causes photoreceptor degeneration in two mouse models for Retinitis Pigmentosa

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Abstract

Purpose: Photoreceptor degeneration in retinitis pigmentosa (RP) is one of the leading causes of hereditary blindness in the developed world. Although causative genetic mutations are often known, the underlying neuronal degeneration mechanisms still remain to be elucidated. Gene mutations affecting the levels cyclic-guanosine-mono-phosphate (cGMP) are often associated with rapid photoreceptor cell death. We asked the question whether activity of cGMP-dependent protein kinase (PKG) was involved in retinal degeneration mechanisms.

Methods: We used custom made antibodies directed against different PKG isoforms to investigate expression in wild-type (wt) and rd1 retina. We then employed organotypic retinal explant cultures derived from wt, rd1, and rd2 animals to test compounds affecting the activities of either phospodeesterase 6 (PDE6) and/or PKG. To confirm the effects observed in vitro, we utilized three different in vivo application techniques (topical application, intravitreal injection, subtenon injection). For evaluation of the treatment outcomes histological staining, immunofluorescence, immunoblot, and TUNEL techniques were used.

Results: We found expression of PKG1 to be restricted mostly to the photoreceptor layer, whereas PKG2 was expressed in the inner nuclear layer and in ganglion cells. More importantly, we showed that activation of PKG hallmarks photoreceptor degeneration in rd1 and rd2 human homologous mouse models. When induced in wt retinae, PKG activity was both necessary and sufficient to trigger cGMP-mediated photoreceptor cell death. Target specific, pharmacological inhibition of PKG activity in both rd1 and rd2 retinae strongly reduced photoreceptor cell death in organotypic retinal explants and increased long-term photoreceptor survival. Likewise, inhibition of PKG in vivo, using three different application paradigms, resulted in photoreceptor protection in the rd1 retina.

Conclusions: These findings suggest a pivotal role for PKG activity in cGMP-mediated photoreceptor degeneration mechanisms and highlight the importance of PKG as a novel target for the pharmacological intervention in RP. PKG1 appears to be the more important isoform in this context. PKG-dependent cell death may be relevant also in other forms of inherited retinal degeneration.

Summary and Conclusions

1) cGMP accumulation and PKG activation hallmark *rd1* and *rd2* mouse retinal degeneration
2) PKG activation selectively killed wt photoreceptors, while PKG inhibition rescued *rd1* and *rd2* photoreceptors *in vitro*
3) PKG inhibition *in vivo* strongly and significantly protected *rd1* photoreceptors
4) PKG may constitute a novel and highly promising target for the treatment of Retinitis Pigmentosa and related diseases.

Selected References

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2) Sancho-Pelluz J, Arango-Gonzalez B, Kustermann S, Romero FJ, van Veen T, Zrenner E, Ekström P, Paquet-Durand F (2008) Photoreceptor cell death mechanisms in inherited retinal degeneration. *Mol. Neurobiol.* **38**:253-269.
3) Paquet-Durand F, Silva J, Talukdar T, Johnson L, Azadi S, Hauck S, Ueffing M, van Veen T, Ekström P (2007) Excessive activation of poly-ADP-ribose-polymerase (PARP) contributes to inherited photoreceptor degeneration in the rd1 mouse. *J. Neurosci.* **27**:10311-10319.

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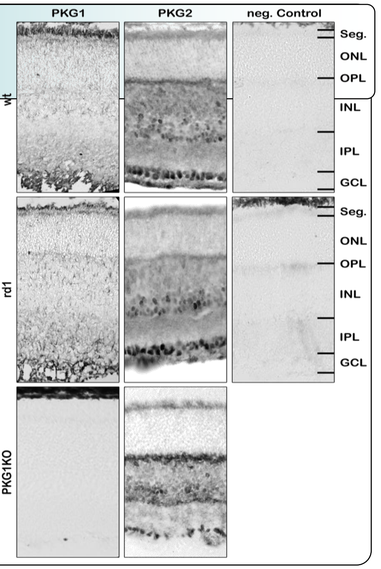
1 Expression of PKG isoforms in wild-type (wt) and *rd1* retina



Two custom made antibodies directed against either PKG1 or PKG2 where employed to investigate PKG isoform expression in wild-type (wt) and *rd1* retina at post-natal (PN) day 11.

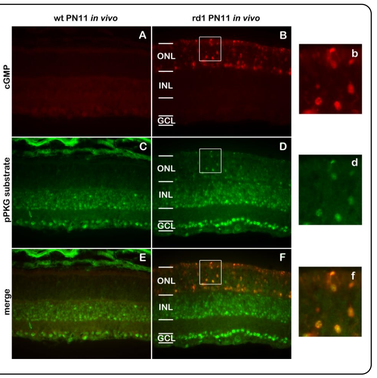
PKG1 appeared to be expressed predominantly in the photoreceptor outer segments of both wt and rd1 and to a lesser extent in cell bodies in the outer nuclear layer (ONL). Some PKG1 expression was also observed in Müller cells, in particular in processes, the outer limiting membrane and endfeet.

In contrast to this, PKG2 expression was restricted to the inner retina with a more prominent staining in the outer plexiform layer (OPL), the lower part of the inner nuclear layer (INL) and in the ganglion cell layer (GCL).

The immunostaining for PKG1 and 2 isoforms thus is suggestive of a mutually exclusive expression in the different retinal cell types.

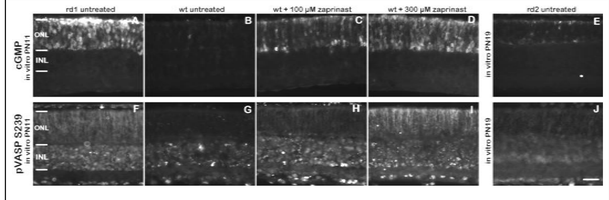
Omission of primary antibody (neg. control) and sections from PKG1 KO animals were used as controls.

2 *In situ* detection of cGMP and PKG activity dependent substrates



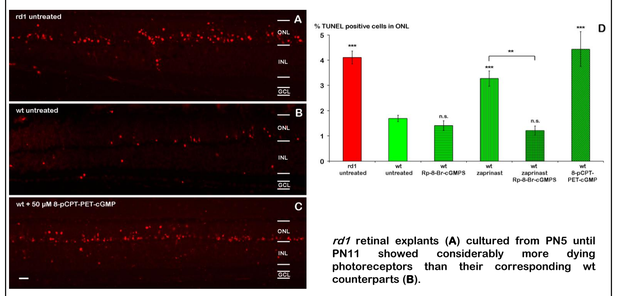
rd1 photoreceptors at PN11 (**B**) exhibited a strong immunoreactivity to cGMP antibody when compared to wild-type (wt; **A**).
The antibody directed against phosphorylated PKG substrates (pPKG, **C, D**) showed a differential staining of *rd1* photoreceptor cell bodies and processes and labeled *rd1* photoreceptor segments.
Merged cGMP and pPKG substrate pictures showed a high degree of overlap between the two stainings (**E, F**).
White squares indicate magnifications shown in **b, d, f**. Some of the inner retina labeling in **C** through **F** relates to endogenous IgG in blood vessels.

3 cGMP and pVASP immunoreactivity in wt, *rd1* and *rd2* retinal explants



Untreated *rd1* retinal explants at PN11 exhibit high levels of cGMP immunoreactivity (**A**) in the ONL. pVASP (**F**) is detected in all layers of the *rd1* retina, including the ONL. Untreated wt retinal explants occasionally show cGMP positive photoreceptor cells (**B**) and almost no pVASP immunoreaction (**G**) in the ONL. wt explants exposed to 100 or 300 µM zaprinast display a marked increase in cGMP positive cells (**C, D**) which affects predominantly the ONL and is concomitant with a corresponding increase in pVASP detection (**H, I**). *rd2* retinal cultures show some cGMP positive photoreceptors (**E**) while pVASP staining (**J**) appears similar to the wt situation with occasional detection of individual photoreceptors.

4 PKG activity is necessary and sufficient to induce cGMP-mediated photoreceptor cell death

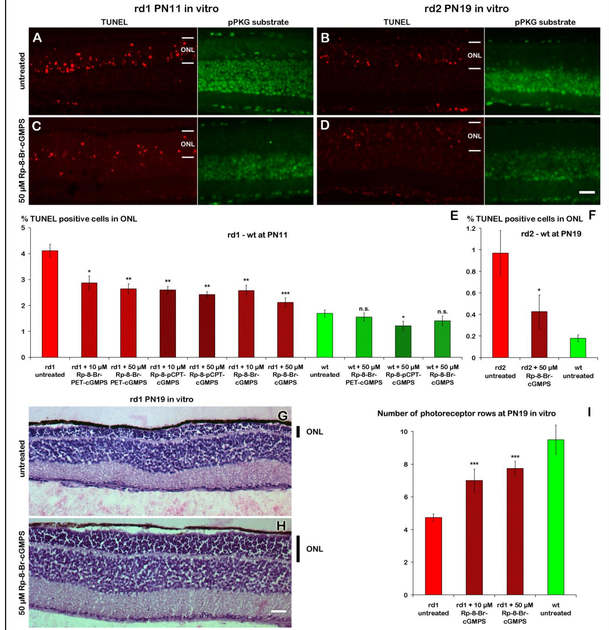


rd1 retinal explants (**A**) cultured from PN5 until PN11 showed considerably more dying photoreceptors than their corresponding wt counterparts (**B**).

Inhibition of PKG with 50 µM Rp-8-Br-cGMPS did not significantly affect the number of TUNEL positive cells in wt explants. Conversely, inhibition of PDE5/6 with 100 µM zaprinast led to a significant increase (quantification in **D**). The effect of zaprinast was reversed by co-treatment with PKG inhibitor (10 µM), suggesting that PKG activity was necessary for induction of cGMP-dependent cell death.

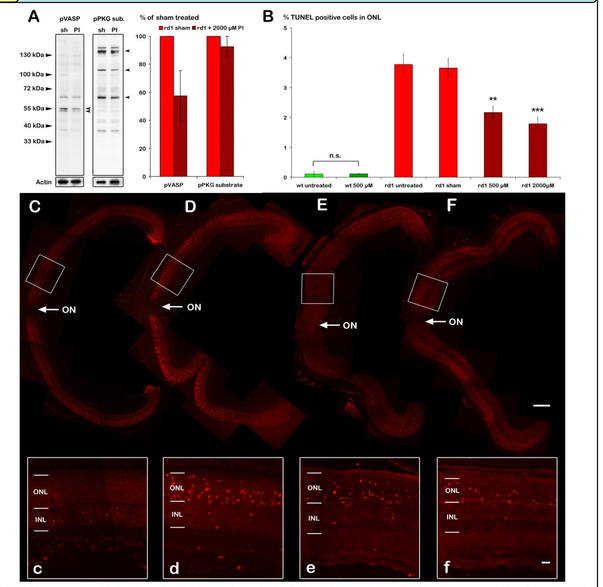
Accordingly, selective PKG activation with 50 µM 8-pCPT-PET-cGMP alone (**C**) was sufficient to cause a strong and highly significant increase in the number of TUNEL positive cells in the wt ONL.

5 PKG inhibition is neuroprotective in *rd1* and *rd2* retina *in vitro*



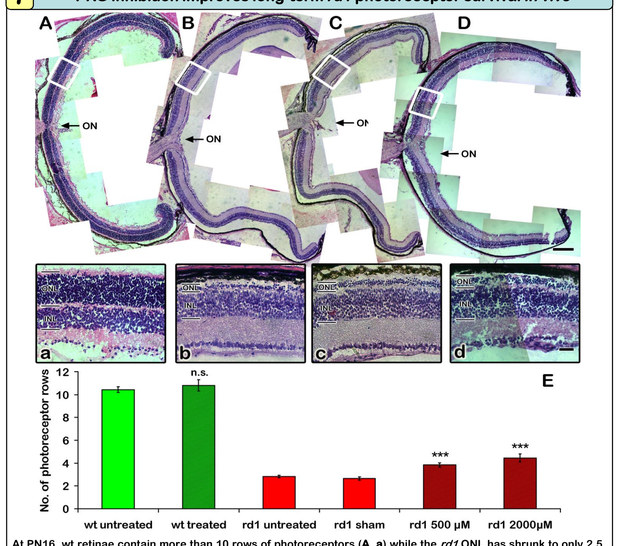
Explanted retinae were treated with 50 µM of the PKG inhibitor Rp-8-Br-cGMPS until PN11 (*rd1*) or PN19 (*rd2*). At these stages treated retinal explants (**C, D**) showed a substantial reduction in dying, TUNEL positive cells (red) and in pPKG substrate immunostaining (green) in the *rd1* and *rd2* ONL compared to untreated ones (**A, B**). Three different PKG inhibitors were found to significantly decrease the number of TUNEL positive cells in *rd1* retinae in a concentration dependent fashion (**E**). At the same time wt retinal explants treated with PKG inhibitors did not display adverse effects. PKG inhibition also resulted in a significant decrease of TUNEL positive cells in *rd2* explants cultured until PN19 (**F**). *rd1* explants treated until PN19 (**G, H**) displayed an increased number of surviving photoreceptors when compared to untreated counterparts (Quantification shown in **I**).

6 Subtenon PKG inhibitor injection protects *rd1* photoreceptors *in vivo*



At PN11, subtenonal injection of 500 µM or 2000 µM Rp-8-Br-cGMPS (PI) resulted in decreased pVASP and pPKG substrate WB immunoreactivity, compared with sham control (sh) (**A**). The percentage of TUNEL positive cells in the ONL was significantly reduced by the treatment (**B**). While wt retinae displayed only few TUNEL positive cells in the ONL (**C**, **c**), *rd1* retinae showed a dramatic increase in their numbers (**D**, **d**). PKG inhibitor caused a concentration dependent reduction of TUNEL positive cells in *rd1* retinae (500 µM: **E**, **e**; 2000 µM: **F**, **f**).

7 PKG inhibition improves long-term *rd1* photoreceptor survival *in vivo*



At PN16, wt retinae contain more than 10 rows of photoreceptors (**A**, **a**) while the *rd1* ONL has shrunk to only 2.5 rows of photoreceptors (**B**, **b**). *rd1* animals that had received a single subtenonal injection of either 500 µM (**C**, **c**) or 2000 µM (**D**, **d**) Rp-8-Br-cGMPS at PN9, showed a significant increase in their respective ONL thicknesses at PN16. Quantification shown in (**E**).