ADPβS evokes microglia activation in the rabbit retina *in vivo*

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Abstract

To investigate whether stimulation of purinergic P2Y$_1$ receptors modulates the activation of microglial and Müller glial cells in the rabbit retina *in vivo*, adenosine 5′-O-(2-thiodiphosphate) (ADPβS; 2 mM in 100 μl saline), a non-hydrolyzable ADP analogue, was intravitreally applied into control eyes or onto retinas that were experimentally detached from the pigment epithelium. Both retinal detachment and application of ADPβS onto control retinas induced phenotype alterations of the microglial cells (decrease of soma size, retraction of cell processes) and had no influence on microglial cell density. ADPβS application onto detached retinas accelerated the process retraction and resulted in a strongly decreased density of microglial cells. The effects of ADPβS on microglia density and phenotype in detached retinas were partially reversed by co-application of the selective inhibitor of P2Y$_1$ receptors, MRS-2317 (3 mM in 100 μl saline). ADPβS apparently did not influence Müller cell gliosis, as determined by electrophysiological and calcium imaging records. It is concluded that rabbit retinal microglial cells express functional P2Y$_1$ receptors *in vivo*, and that activation of these receptors stimulates phenotype alterations that are characteristical for microglia activation.

Introduction

In the neural retina, pathogenic stimuli cause complex glial and immune responses that are characterized by activation of microglial cells, gliosis of macroglial cells (astrocytes and Müller cells), as well as breakdown of the blood–retina barrier and immigration of leukocytes. The main histological markers of microglial cell activation are cell proliferation and migration, and changes of the cellular phenotype [1]. In the normal non-injured neural tissue, resting microglial cells exhibit a ramified shape with multiple thin and complexely branched processes arising from slender bipolar cell bodies. Activation of microglial cells by pathogenic stimuli is associated with a morphological transformation (that includes cellular hypertrophy and process retraction) towards a coarse rod-like or amoeboid phenotype [1]. Müller cell gliosis is characterized by, among others, hypertrophy, proliferation, and upregulation of immunoreactivity of intermediate filaments [2]. Müller cell gliosis is associated with distinct physiological alterations such as downregulation of the main plasma membrane conductance, i.e., currents through inwardly rectifying potas-

sium (Kir) channels, and upregulation of the calcium and current responsiveness evoked by stimulation of purinergic P2 receptors by extracellular adenosine 5′-triphosphate (ATP) [3, 4]. Until now, it is not known whether microglial cell activation and Müller cell gliosis in the retina influences each other; partially, this is due to the absence of agents that specifically inhibit either microglia or Müller cell activation *in vivo*.

Despite of a large body of *in vitro* investigations, the exact mechanism(s) underlying the activation of glial cells *in vivo* is still largely unknown. Among the different signaling molecules which are assumed to cause glial cell activation, extracellular ATP which acts via P2 receptors may activate both microglial and macroglial cells [5, 6]. Cultured retinal microglial cells express metabotropic P2Y receptors as well as ionotropic P2X$_7$ receptors [7]. Under hypoxic conditions *in vitro*, activation of P2Y receptors induces proliferation of the cells while activation of P2X$_7$ receptors evokes the release of proinflammatory cytokines from the cells, and may cause microglial apoptosis [7]. However, it is not known whether this is also true for *in-vivo* conditions. The upregulation of the responsiveness of Müller cells to extracellular ATP under pathological conditions [4] may suggest that also in the case of macroglial cell activation, extracellular ATP may be an important signaling molecule. It is known that the extracellular concentration of ATP in neural tissues increases during

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inflammation and ischemia [8]. We used an *in-vivo* model of retinal detachment in the rabbit eye in order to determine whether adenosine 5′-O-(2-thiodiphosphate) (ADPβS), a non-hydrolyzable ADP analogue acting on various puringeric P2Y$_1$ receptor subtypes, may influence the detachment-induced activation of microglial and Müller cells. Detachment-induced neuronal degeneration and glial cell activation has been attributed to ischemic conditions present under this condition [9].

Materials and methods

Twenty-eight adult pigmented rabbits (2–3 kg, both sexes) were anaesthetized by i.m. injection of ketamine (50 mg/kg; Ratiopharm, Ulm, Germany) and xylazine (3 mg/kg; BayerVital, Leverkusen, Germany). After pars plana sclerotomy, a circumscript vitrectomy was performed in the area of the future detachment. Thin glass micropipettes attached to 250 µl glass syringes (Hamilton, Reno, Nevada) were used to create a local retinal detachment by subretinal injection of 0.25% sodium hyaluronate in saline (Healon; Pharmacia & Opion, Dübendorf, Switzerland). Central retinal areas (ventral to the medullary rays) with diameters of 8 to 10 mm were detached. In three animals, ADPβS (2 mM in 100 µl saline; Sigma-Aldrich, Taufkirchen, Germany) was applied both into the subretinal space (50 µl) and epiretinally into the vitreous body (50 µl) just after the retina was detached. In three animals, the selective anatgonist of P2Y$_1$ receptors, MRS-2179 (3 mM in 100 µl saline; Sigma-Aldrich) was applied around the detached retina. In other three animals, ADPβS (2 mM) and MRS-2179 (3 mM) were applied simultaneously to the detached retina. Three different types of sham-operated controls were used; (1) in three animals, ADPβS (2 mM in 100 µl saline) was intravitreally injected near the retinal surface after circumscribed vitrectomy, without detaching the retina; (2) in another three animals, saline (100 µl) was placed over the vitread surface of the undetached retina after vitrectomy ('saline control'); and (3) in three further animals, Healon (100 µl) was placed over the vitreal surface of the undetached retina after vitrectomy ("Healon control"). The Healon control was done to exclude the possibility that sodium hyaluronate alters the microglia activation state. After a survival time of 48 h, the animals were anaesthetized as described, and killed by i.v. application of T61 (3 ml; Hoechst, Unterschleißheim, Germany); then, the eyes were excised.

In order to label microglial cells, wholemounts of acutely isolated retinal pieces (3 mm^2) were placed, with their vitread surface up, into a perfusion chamber, and were incubated in extracellular solution containing Cy3-tagged *Griffonia simplicifolia* agglutinin (GSA; Sigma) isolectin I-B$_4$ (25 µg/ml) for 1 h at room temperature. The extracellular solution consisted of (mM) 110 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 1 Na$_2$HPO$_4$, 0.25 glutamine, 10 HEPES, 11 glucose, and 25 NaHCO$_3$, adjusted to pH 7.4 with Tris and bubbled with carbogen (95% O$_2$/5% CO$_2$). The severity of Müller cell gliosis was determined electrophys-

iologically by measuring the density of the Kir currents by using whole-cell patch-clamp records of acutely isolated Müller cells, as described previously [3], as well as by estimation of the incidence of Müller cells which showed intracellular calcium responses upon extracellular application of ATP in acutely isolated wholemounts. The calcium imaging experiments were made using the calcium-sensitive dyes Fluo-4/AM (11 µM) and Fura-Red/AM (17 µM; Molecular Probes, Eugene, Oregon), as described [4]. Fluorescence images were recorded using a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany), and determination of the incidence of responding cells was made according to a procedure described previously in detail [10].

The extent of microglial cell activation was estimated by counting the density of microglial cells at the vitreal surface (i.e., in the nerve fiber layer) of retinal wholemounts, and by determining two morphological key parameters of the cells, (1) check the rest the 'cross-sectional' area of their somata, and (2) check the rest the number of primary cell processes (the latter parameter was assessed by counting those processes which directly arose from the soma and which were longer than 10 µm, i.e., the average length/diameter of somata). Statistical analysis was made using the Prism program (Graphpad Software, San Diego, California); significant differences were determined by Student's *t*-test for two groups and by ANOVA followed by comparisons for multiple groups, respectively. Data are expressed as means ± SEM; n represents the number of retinal wholemounts or Müller cells investigated.

Results

Microglia activation

Microglial cells in control retinas exhibited the typical ramified morphology of resting microglia, as illustrated by the example shown in Figure 1A (left side). These cells have, in the mean, three primary processes evolving from the cell soma (Figure 2B), and multiple thin and long side branches (not determined). Application of Healon onto the vitread surface of the retina did not change the mean density of microglial cells (Figure 1B) nor the morphology of the cells (Figures 2A, B) two days after surgery when compared with saline application. Application of ADPβS into the vitreous of control eyes did not significantly change the density of GSA lectin-stained cells (Figure 1B) but significantly ($P < 0.05$) reduced the cell soma size (Figure 2A) and caused a significant process retraction (Figure 2B). The number of cell processes decreased to 1.0 ± 0.2 in the presence of ADPβS, which is significantly smaller ($P < 0.001$) when compared to the values in the saline (3.1 ± 0.4) or Healon control retinas (3.2 ± 0.3). This alteration reflects the morphological transformation from ramified to rod-like cells which accompanies microglial activation [1].

Detachment of the neural retina from the pigment epithelium caused similar alterations as intravitreal appli-
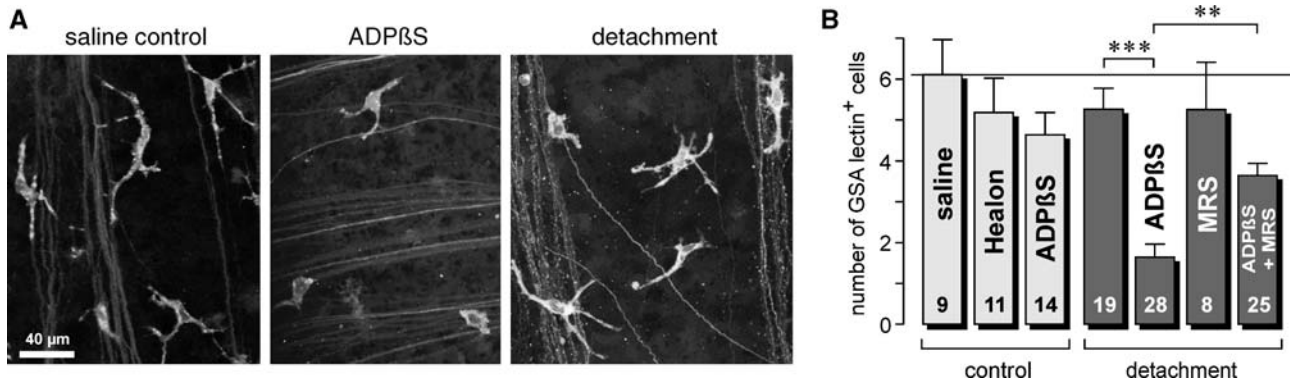
Figure 1. Effect of ADPβS on the density of microglial cells in the nerve fiber layer in control and detached rabbit retinae. (A) View onto the vitreal surface (i.e., onto the nerve fiber layer) of control detached retinas. The images show GSA lectin-labeled microglial cells and thin and long structures which represent axon nerve fiber bundles. The images were taken 48 h after sham-operation with application of saline (*left*) or of ADPβS (2 mM in 100 µl saline) onto the vitread surface (*middle*), and from a retina which was detached for 48 h (*right*). (B) Mean (±SEM) number of GSA lectin-labeled cells per unit area of vitread retinal surface (230 × 230 µm). The effects were measured 48 h after surgery in acutely isolated wholemounts. The saline and Healon controls were measured at 48 h after placement of 100 µl of saline and Healon, respectively, over the vitreal surface of undetached retinas. The selective inhibitor of $P2Y_1$ receptors, MRS-2317 (3 mM in 100 µl saline), was applied with or without ADPβS to the detached retinas. Numbers of investigated wholemounts within the bars. ** $P < 0.01$; *** $P < 0.001$.

cation of ADPβS, i.e., it did not change the density of the microglial cells at the vitreal retinal surface (Figures 1A, B), it significantly ($P < 0.05$) decreased the cell soma size (Figure 2A), and it resulted in retraction of cell processes (Figure 2B). In the mean, the number of primary processes per cell was 1.6 ± 0.2 that is significantly ($P < 0.001$) smaller when compared to saline or Healon controls. Appli-

Figure 2. Effect of ADPβS on the microglial cell phenotype in control and detached rabbit retinae. (A) Mean (±SEM) cross-sectional area of the microglial cell somata (in µm²). (B) Mean (±SEM) number of processes which evolve from the soma of microglial cells. Numbers of investigated wholemounts within the bars. n.s., not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

cation of ADPβS onto both sides of the detached retinas at the time of surgery facilitated the transformation into unipolar rod-like cells, resulting in a significant ($P < 0.05$) reduction of the number of primary cell processes (to 1.0 ± 0.2) (Figure 2B). However, unlike retinal detachment or ADPβS application to control retinas which both did not alter the density of microglial cells, application of ADPβS to detached retinas resulted in a significantly ($P < 0.001$) decreased number of cells at the vitreal surface (Figure 1B). Co-application of ADPβS and the selective inhibitor of $P2Y_1$ receptors, MRS-2179, resulted in a partial, but significant reversal of the effects of ADPβS on microglial density (Figure 1B) and phenotype (Figures 2A, B). The data suggest that the effect of ADPβS is, at least in part, mediated by activation of $P2Y_1$ receptors. Slices of GSA lectin-stained retinas revealed that ADPβS did not stimulate the migration of the cells into inner retinal layers not shown.
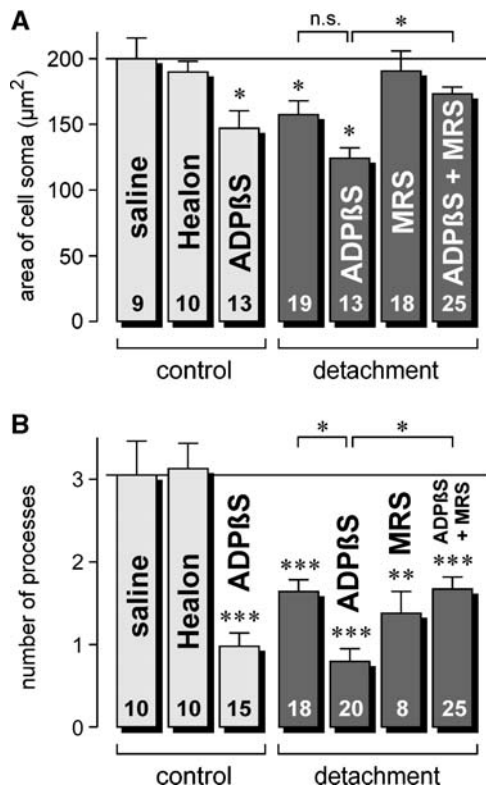
Müller cell gliosis

The severity of Müller cell gliosis was estimated by measuring two key parameters of cell activation: the amplitude of the Kir currents in acutely isolated cells, and the incidence of cells that show calcium responses to purinergic stimulation. One of the main markers of Müller cell gliosis in detached retinas is an upregulation of the incidence of cells that respond to extracellular application of ATP with a transient elevation of the intracellular calcium concentration (Figure 3A) [4]. In untreated control retinas, $14.4 \pm 3.7\%$ of the Müller cells investigated in acutely isolated wholemounts showed a calcium response upon application of ATP (200 µM) (Figure 3B). In retinas that were detached for 48 h, the incidence increased significantly to $55.0 \pm 9.8\%$ ($P < 0.001$). When ADPβS was applied to detached retinas at the time of surgery, a similar high incidence of responding cells was observed ($63.6 \pm 8.4\%$). When ADPβS was applied into control eyes, a slight but non-significant elevated incidence was observed
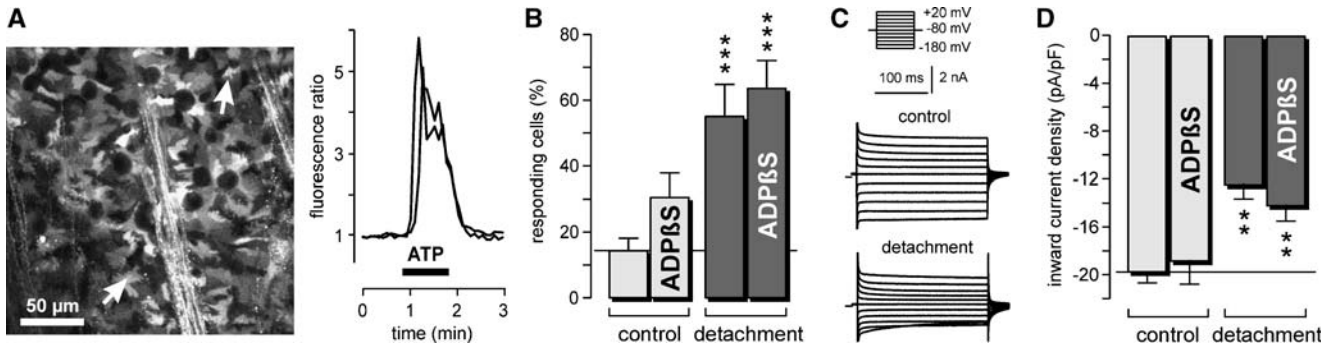
Figure 3. ADPβS had no effect on two physiologic parameters of Müller cell gliosis. (A) Example of a calcium imaging record in two endfeet of Müller cells in a 48 h-detached retina. ATP (200 μM) evokes a transient calcium response. (B) ADPβS did not alter the detachment-induced increase of the incidence of Müller cells that respond to extracellular application of ATP (200 μM) with a transient elevation of their intracellular free calcium concentration. The incidence is expressed as percentage of the total Müller cell number investigated (100%). Numbers of investigated wholemounts in parenthesis. (C) Examples of whole-cell patch-clamp records in a Müller cell from a control retina and in a cell from a 48 h-detached retina. (D) ADPβS did not alter the detachment-induced decrease of inwardly rectifying potassium currents. The currents were measured between the voltage steps to -100 mV and to -160 mV; the holding potential was -80 mV. The bars represent values obtained in 9 to 34 wholemounts and 15 to 70 cells, respectively. ** $P < 0.01$, *** $P < 0.001$.

when compared to untreated control eyes ($30.6 \pm 7.4\%$). The results indicate that ADPβS application did not change the ATP-induced calcium responsiveness of Müller cells.

Another hallmark of Müller cell gliosis is the down-regulation of Kir currents [3]. The density of Kir currents was measured by using whole-cell patch-clamp recordings (Figure 3C). Müller cells from retinas that were detached for 48 h displayed significant smaller inward currents than Müller cells from untreated control retinas; in the mean, the Kir current density decreased to $62.9 \pm 7.0\%$ ($P < 0.01$) (Figure 2D). Application of ADPβS did not alter the density of the Kir currents in Müller cells isolated from control or detached retinas.

Discussion

The results indicate that application of ADPβS, a non-hydrolyzable ADP analogue acting on various P2Y receptor subtypes (P2Y$_1$, P2Y$_{12}$, P2Y$_{13}$), evokes microglia activation in control retinas, as indicated by the morphological alterations of the cells, facilitates the activation of microglial cells in detached retinas, but exerts no effects on the degree of Müller cell gliosis. The effects of ADPβS on microglial cells were, at least in part, mediated by activation of P2Y$_1$ receptors, as indicated by the reversing effects of a selective blocker, MRS-2179. However, it cannot be ruled out that, in addition to P2Y$_1$ receptors, also the other ADPβS-sensitive receptor subtypes are involved in mediating the effects of ADPβS.

The present data suggest that Müller cell gliosis is induced relatively independently from microglial cell activation in the detached retina. The reason for this difference in the ADPβS effect is unclear; most likely, microglial cells and Müller cells express different subtypes of P2Y receptors, with the presence of P2Y$_1$ receptors on microglial cells and the absence of this receptor subtype on Müller cells. However, it has been shown that Müller cells of the

rat express P2Y$_1$ receptors [11], and ADP when extracellularly applied to wholemounts of the rabbit retina evokes intracellular calcium responses which are similar in shape and amplitude as ATP-evoked responses [4], suggesting that also rabbit Müller cells may express the P2Y$_1$ receptor subtype. Another explanation for the different ADPβS effect on microglial and Müller cells may be that different P2 receptor subtypes are intracellularly coupled to different cellular effects, as previously shown for cultured retinal microglial cells [7]. Therefore, it may be possible that P2Y$_1$ receptor activation may affect some aspects of Müller cell gliosis, e.g., the release of neuroptrophic factors, but not the markers investigated in the present study (Kir current decrease and increased responsiveness to ATP). This question needs further investigations.

Both retinal detachment and stimulation of P2Y$_1$ receptors by ADPβS in control retinas caused similar morphological alterations of microglial cells, resulting in smaller cell somata and retraction of cell processes. Moreover, ADPβS accelerated the detachment-induced reduction of the number of primary cell processes, suggesting that during retinal detachment, the morphological alterations of the microglial cells may be, at least in part, mediated by activation of endogenous P2Y$_1$ receptors. Stimulation of P2Y$_1$ receptors by ADPβS resulted in a dramatic decrease of the microglial cell density in detached retinas and had no effect on the cell number in control retinas (Figure 1B). The reason for this different effects of ADPβS in control and detached retinas is unclear. ADPβS did not stimulate the migration of the cells into inner retinal layers, whereas an effect of ADPβS on microglial cell apoptosis cannot be ruled out. In this context it is noteworthy that in cultured microglial cells, the capability of the cells to respond to P2 receptor activation depend on the activation state of the cells [12]. Retinal detachment induces photoreceptor deconstruction and increases the distance between the choriocapillaris and the neural retina which results in a decreased oxygen supply of retinal cells [13]; this hypoxic condition may cause release of bioactive molecules

such as growth factors from the detached retina. Indeed, within minutes of experimental detachment in cats, the retinal fibroblast growth factor (FGF) receptor-1 has been observed to be phosphorylated, indicating a retinal release of FGF [14].

In summary, retinal microglial cells *in situ* express functional $P2Y_1$ receptors, the stimulation of which accelerates the morphological transformation of microglia. It is assumed that the cellular effects of $P2Y_1$ receptor stimulation partially differs in dependence on the degree of co-activation by other factors intraretinally released during injury, possibly resulting in enhanced cell death. The results suggest that ADPβS is a relatively specific inhibitor of microglial cell proliferation in the injured retina, without apparent effects on macroglial cell activation. Therefore, ADPβS may be used in future experiments to investigate possible neurotoxic effects of activated microglia in the injured retina.

Acknowledgements

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