Original Paper

Ophthalmic Research

Ophthalmic Res 2013;50:209–214
DOI: 10.1159/000354082

Received: May 6, 2013
Accepted: June 9, 2013
Published online: September 18, 2013

Disruption of Endogenous Purinergic Signaling Inhibits Vascular Endothelial Growth Factor- and Glutamate-Induced Osmotic Volume Regulation of Müller Glial Cells in Knockout Mice

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Key Words
Osmotic swelling · Müller cell · Vascular endothelial growth factor · Glutamate · Calcium

Abstract
Background/Aims: Osmotic swelling of Müller cells is a common phenomenon in animal models of ischemic and diabetic retinopathies. Müller cells possess a swelling-inhibitory purinergic signaling cascade which can be activated by various receptor ligands including vascular endothelial growth factor (VEGF) and glutamate. Here, we investigated whether deletion of $P2Y_1$ ($P2Y_1R$) and adenosine A_1 receptors (A_1AR), and of inositol-1,4,5-trisphosphate-receptor type 2 (IP_3R2), in mice affects the inhibitory action of VEGF and glutamate on Müller cell swelling. ***Methods:*** The cross-sectional area of Müller cell somata was recorded after a 4-min superfusion of retinal slices with a hypoosmotic solution. ***Results:*** Hypoosmolarity induced a swelling of Müller cells from $P2Y_1R^{-/-}$, $A_1AR^{-/-}$ and $IP_3R2^{-/-}$ mice, but not from wild-type mice. Swelling of wild-type Müller cells was induced by hypoosmotic solution containing barium chloride. Whereas VEGF inhibited the swelling of wild-type Müller cells, it had no swelling-inhibitory effect in cells from $A_1AR^{-/-}$ and $IP_3R2^{-/-}$ mice. Glutamate inhibited the swelling of wild-type Müller cells but not of cells from $P2Y_1R^{-/-}$, $A_1AR^{-/-}$ and $IP_3R2^{-/-}$ animals. ***Conclusion:*** The swelling-inhibitory effects of VEGF and glutamate in murine Müller cells is mediated by transactivation of $P2Y_1R$ and A_1AR, as well as by intracellular calcium signaling via activation of IP_3R2.

Introduction

The presence of retinal edema is a major cause of visual impairment in ischemic-hypoxic and inflammatory retinopathies [1]. Edema develops by vascular leakage and/or dysfunctional fluid clearance from the retinal tissue [2]. One mechanism of fluid clearance involves water transport through Müller cells [3]. By transcellular water transport, Müller cells compensate osmotic gradients within the retinal tissue and across the gliovascular interface. Intense neuronal activity is associated with a decrease in extracellular fluid osmolarity [4]. In addition, the uptake of excess neuron-derived osmolytes (potassium, sodium glutamate) by Müller cells enhances the intracellular osmotic pressure in comparison to the extracellular fluid [5]. The osmotic imbalances are exacerbated under pathological conditions that are characterized by neuro-

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nal hyperexcitation. Moreover, a decrease in blood osmolality, e.g. during hyponatremia and hypoalbuminemia, represents a major pathogenic factor of retinal edema [6].

Under ischemic-hypoxic and inflammatory conditions, the transglial water transport is disturbed, as indicated by the alteration in the osmotic swelling characteristics of Müller cells. Under normal conditions, Müller cells do not swell for 10–15 min when retinal slices or freshly isolated cells are superfused with a hypoosmotic solution [7, 8]. On the other hand, immediate swelling of Müller cells upon hypoosmotic challenge was observed in animal models of ischemic and inflammatory retinopathies including retinal ischemia reperfusion, diabetic retinopathy, retinal vein occlusion and ocular inflammation [9–12]. The change in the osmotic swelling characteristics of Müller cells suggests that the rapid water transport across Müller cell membranes is altered under pathological conditions. However, though intracellular edema of Müller cells caused by water accumulation within the cells was described to occur in animal models of ischemic and diabetic retinopathies [13, 14], a swelling of Müller cells was rarely observed. This may suggest that endogenous mechanisms are present that inhibit the osmotic swelling of Müller cells under pathological conditions.

One mechanism that prevents osmotic swelling of Müller cells is the activation of an endogenous purinergic signaling cascade [15]. This cascade consists of the consecutive release of ATP and adenosine from Müller cells which activate purinergic $P2Y_1$ receptors ($P2Y_1R$) and adenosine A_1 receptors (A_1AR), respectively [8, 16, 17]. Release of ATP is induced by activation of metabotropic glutamate receptors [8, 16, 17]. Vascular endothelial growth factor (VEGF) and various other receptor ligands including neuropeptide Y (NPY) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) activate the volume-regulatory cascade upstream of glutamate release [16–19]. The final step of the cascade is the A_1AR-mediated opening of potassium and chloride channels in the Müller cell membrane; the rapid ion flux compensates the osmotic gradient across the membrane and, thus, prevents cellular swelling [15–17]. Disruption of the autocrine purinergic signaling in $P2Y_1R$- and A_1AR-deficient mice results in rapid swelling of Müller cells under hypoosmotic conditions [8, 20]. However, it is not known whether the swelling-inhibitory action of various receptor ligands besides ATP and adenosine is impaired in Müller cells of $P2Y_1R^{-/-}$ and $A_1AR^{-/-}$ mice. Therefore, we determined the effects of VEGF and glutamate on the hypoosmotic swelling of Müller cells in retinal slices from wild-type, $P2Y_1R^{-/-}$ and $A_1AR^{-/-}$ mice. Because the swelling-inhibitory effect of ATP is dependent on intracellular calcium signaling mediated by the inositol-1,4,5-trisphosphate-receptor type 2 (IP_3R2) [7, 8], we also tested the effects of VEGF and glutamate on the osmotic swelling of Müller cells in retinal slices from $IP_3R2^{-/-}$ mice.

Materials and Methods

Materials

Human recombinant VEGF-A_{165} was purchased from Chemicon (Temecula, Calif., USA). Human recombinant HB-EGF, human recombinant NPY, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (2*S*)-2-amino-2-[(1*S*, 2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), N^6-methyl-2'-deoxyadenosine-3′,5′-bisphosphate (MRS2179), and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless indicated otherwise.

Animals

All experiments were done in accordance with the European Communities Council Directive 86/609/EEC, and were approved by the local authorities. Adult (2–6 months) animals of various mouse strains were used. Mice deficient in $P2Y_1R$ [21], A_1AR and IP_3R2, and littermate wild-type controls, were validated and characterized as described [8, 22, 23]. Müller cells of control mice from different strains did not differ in their swelling characteristics (not shown). Animals were maintained with free access to water and food in an air-conditioned room on a 12-hour light-dark cycle, and were killed with carbon dioxide.

Preparation of Retinal Slices

Pieces of freshly isolated retinas (3 × 3 mm) were placed, with the photoreceptor side down, onto membrane filters (mixed cellulose ester, pore size: 0.45 μm; Schleicher & Schuell MicroScience, Dassel, Germany). Retinal slices (thickness: 1 mm) were cut from these tissues adhering to the membrane filters. The slices were transferred to recording chambers and kept submerged in extracellular solution. The chambers were mounted on the stage of an upright confocal laser scanning microscope (LSM 510 Meta; Zeiss, Oberkochen, Germany).

Osmotic Swelling Experiments

Swelling experiments were performed at room temperature (20–23°C). To determine the volume changes of Müller cell somata evoked by hypoosmotic challenge, their cell bodies in the central part of the inner nuclear layer of retinal slices were recorded. To identify cell somata, retinal slices were loaded with the vital dye MitoTracker Orange (1 μM; Molecular Probes, Eugene, Oreg., USA) for 3 min. Thereafter, the slices were continuously superfused with extracellular solution at a flow rate of 2 ml/min. Recordings were made with an Achroplan 63×/0.9 water immersion objective (Zeiss). The pinhole was set at 151 μm; the thickness of the optical section was adjusted to 1 μm. MitoTracker Orange was excited at 543 nm with a HeNe laser, and emission was recorded with a 585-nm long-pass filter. The dye-filled somata were focused at the plane of their maximal extension.

A gravity-fed system with multiple reservoirs was used to perfuse the recording chamber continuously with extracellular solution; test substances were applied by changing the perfusate. The bathing solution in the recording chamber was totally changed

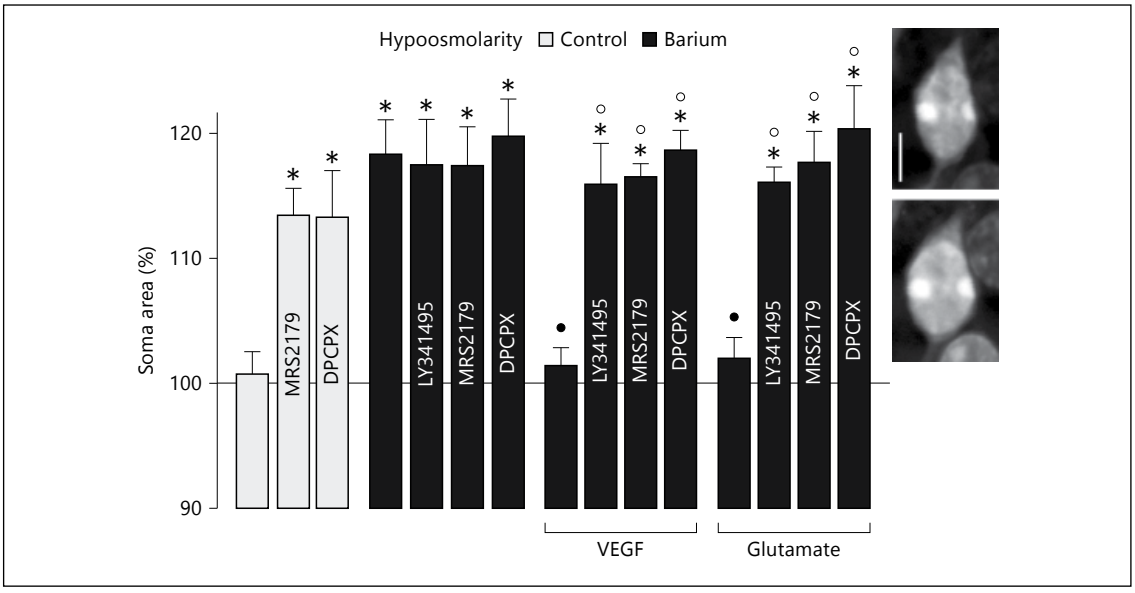

Fig. 1. Receptor-mediated inhibition of osmotic swelling of Müller cell somata in retinal slices from wild-type mice. The cross-sectional area of the somata was measured after a 4-min superfusion of the slices with a hypoosmotic solution (60% of control osmolarity) in the absence (control) and presence of barium chloride (1 mM), and is expressed in percent of the soma size measured before osmotic challenge (100%). In the absence of barium, the selective $P2Y_1$ antagonist MRS2179 (30 μM) and the adenosine A_1 receptor antagonist DPCPX (100 nM) induced swelling of Müller cell somata. The barium-induced swelling was prevented by VEGF (10 ng/ml) and glutamate (1 mM), respectively. The swelling-inhibitory effects of VEGF and glutamate were abrogated in the presence of the antagonist of group II metabotropic glutamate receptors LY341495 (100 μM), MRS2179 (30 μM) and DPCPX (100 nM), respectively. The images display original records of a dye-filled Müller cell soma obtained before (above) and during (below) superfusion of the hypoosmotic solution in the presence of barium. Bar = 5 μm. Each bar represents values obtained in 5–14 cells. * $p < 0.05$: significant swelling induction; ● $p < 0.05$: significant swelling inhibition; ○ $p < 0.05$: significant inhibition of the agonist effect.

within 1 min. The extracellular solution consisted of (in mM) 136 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 11 glucose, adjusted to pH 7.4 with Tris. The hypoosmotic solution (60% of control osmolarity) was made up by adding distilled water. Barium chloride (1 mM) was preincubated for 10 min in extracellular solution before it was applied within the hypoosmotic solution. Blocking substances were preincubated for 15–45 min, and agonists were applied simultaneously with the hypoosmotic solution.

Statistics

To determine the extent of the swelling of Müller cell somata, the cross-sectional area of the cell bodies was measured off-line using the image analysis software of the laser scanning microscope. For each test, 5–15 cells from 2–3 animals were measured. Data are expressed as means ± SEM. Statistical analysis was made using Prism (GraphPad Software, San Diego, Calif., USA). Significance was determined by Mann-Whitney U test, and was accepted at $p < 0.05$.

Results

The swelling of Müller cell somata was investigated by superfusion of freshly isolated retinal slices with a hypoosmotic solution (60% of control osmolarity). As shown in figure 1, hypoosmotic exposure for 4 min did not evoke a significant swelling of glial cell bodies in retinal slices from wild-type mice. However, coadministration of potassium channel-blocking barium ions with the hypoosmotic solution induced significant ($p < 0.05$) swelling of Müller cell somata. Hypoosmotic exposure of retinal slices also induced a significant ($p < 0.05$) swelling of Müller cell somata in the presence of the selective $P2Y_1R$ antagonist MRS2179 and the A_1AR antagonist DPCPX, respectively (fig. 1). The data suggest that swelling of Müller cells under hypoosmotic conditions is normally prevented by autocrine activation of $P2Y_1$ and A_1 receptors.

It has been shown that VEGF and glutamate activate the volume-regulatory purinergic signaling cascade in Müller cells [8, 16, 17]. As recently described [18], VEGF and glutamate prevented the barium-induced hypoosmotic swelling of Müller cells (fig. 1). The swelling-inhibitory effects of the receptor ligands were abrogated in the presence of the antagonist of group II metabotropic glutamate receptors LY341495, the selective $P2Y_1R$ antagonist MRS2179 and the A_1AR antagonist DPCPX, respectively (fig. 1) [18].

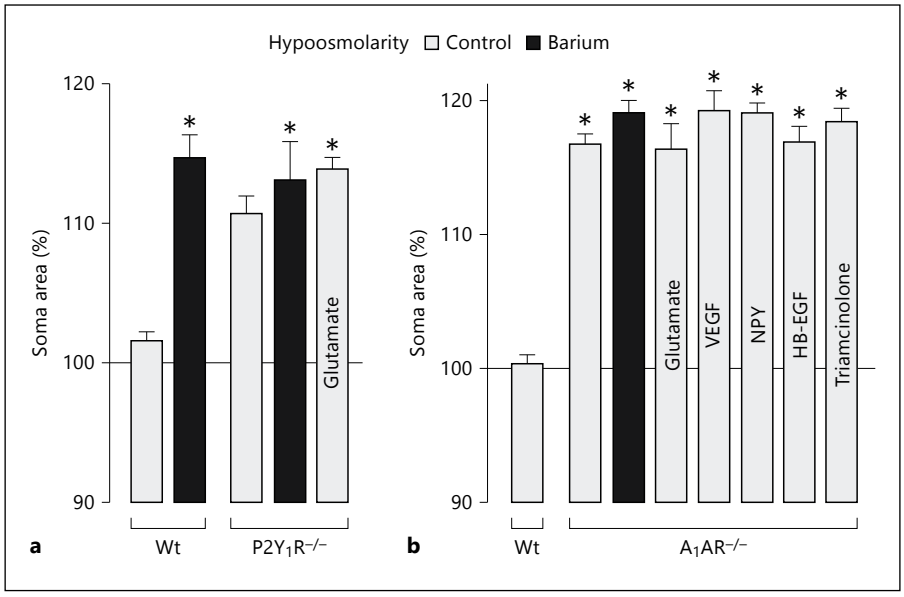

Fig. 2. Various receptor ligands do not inhibit the osmotic swelling of Müller cell somata in retinal slices from $P2Y_1R^{-/-}$ (**a**) and $A_1AR^{-/-}$ mice (**b**). *Wt* = Wild-type. The cross-sectional area of the somata was measured after a 4-min superfusion of the slices with the hypoosmotic solution in the absence (control) and presence of barium chloride (1 mM), and is expressed in percent of the soma size measured before osmotic challenge (100%). As comparison, the cross-sectional area of Müller cell somata in retinal slices from wild-type mice was recorded. The following receptor ligands were tested: glutamate (1 mM), VEGF (10 ng/ml), NPY (20 nM) and HB-EGF (20 ng/ml). In addition, triamcinolone acetonide (50 μM) was tested. Each bar represents values obtained in 5–13 cells. * $p < 0.05$: significant swelling induction.

The data suggest that the swelling-inhibitory effect of VEGF is mediated by induction of the release of glutamate, ATP and adenosine, and transactivation of metabotropic glutamate receptors $P2Y_1R$, and A_1AR, as previously shown in Müller cells of mice and rats [17, 18].

To determine whether disruption of the purinergic signaling cascade results in impaired swelling-inhibitory action of VEGF and glutamate in Müller cells, we tested the compounds in retinal slices from $P2Y_1R^{-/-}$ and $A_1AR^{-/-}$ mice. As previously shown [8], Müller cells of $P2Y_1R^{-/-}$ and $A_1AR^{-/-}$ mice displayed hypoosmotic swelling under control conditions, which was not observed in cells from wild-type mice (fig. 2a, b). Though glutamate inhibited the osmotic swelling of Müller cells from wild-type mice (fig. 1), it had no swelling-inhibitory effect in cells from $P2Y_1R^{-/-}$ (fig. 2a) and $A_1AR^{-/-}$ mice (fig. 2b). Likewise, VEGF did not inhibit the osmotic swelling of Müller cells from $A_1AR^{-/-}$ mice (fig. 2b). In addition, NPY and HB-EGF had no effects on the swelling of Müller cells from $A_1AR^{-/-}$ mice (fig. 2b); both compounds were previously shown to inhibit the osmotic swelling of Müller cells by activation of the volume-regulatory purinergic signaling cascade [16, 19]. The glucocorticoid triamcinolone acetonide, which is clinically used for the rapid resolution of retinal edema, had been shown to prevent the hypoosmotic swelling of Müller cells by stimulation of the transporter-mediated release of endogenous adenosine and subsequent activation of A_1AR [24]. We found that triamcinolone did not inhibit the hypoosmotic swelling of Müller cells from $A_1AR^{-/-}$ mice (fig. 2b). The data confirm the suggestion that the inhibitory effect of triamcinolone on the cytotoxic swelling of Müller cells is mediated by activation of endogenous adenosine signaling.

It has been demonstrated that the swelling-inhibitory effect of ATP (but not adenosine) in murine Müller cells is dependent on IP_3R2-mediated intracellular calcium signaling [7, 8]. As previously shown [7, 8], deletion of IP_3R2 in mice resulted in swelling of Müller cell somata under hypoosmotic conditions (fig. 3), which was not observed in cells of wild-type mice (fig. 1). Adenosine, but not ATP, inhibited the osmotic swelling of Müller cells from $IP_3R2^{-/-}$ mice (fig. 3). We found that glutamate and VEGF failed to inhibit the osmotic swelling of Müller cells from $IP_3R2^{-/-}$ animals (fig. 3). The data suggest that intracellular calcium signaling mediated by IP_3R2 is involved in mediating the swelling-inhibitory effects of VEGF and glutamate in murine Müller cells.

Discussion

It has previously been shown that various receptor ligands including glutamate, VEGF, NPY and HB-EGF inhibit the barium-induced hypoosmotic swelling of Müller cells in control retinas, as well as the osmotic swelling of Müller cells in retinas injured by retinal ischemia-reperfusion and experimental diabetes, for example [16–19]. The inhibition of osmotic swelling induced by receptor ligands is mediated by activation of a volume-regulatory purinergic signaling cascade which includes the auto-

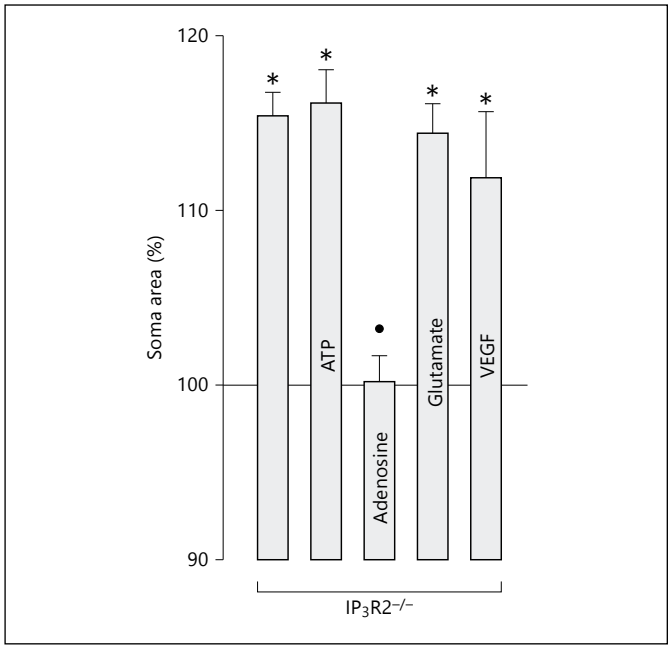

Fig. 3. The osmotic swelling of Müller cell somata in retinal slices of $IP_3R2^{-/-}$ mice is inhibited by adenosine (10 μM), but not by ATP (200 μM), glutamate (1 mM) and VEGF (10 ng/ml), respectively. The cross-sectional area of the somata was measured after a 4-min superfusion of the slices with the hypoosmotic solution, and is expressed in percent of the soma size measured before osmotic challenge (100%). Each bar represents values obtained in 6–15 cells. * $p < 0.05$: significant swelling induction; • $p < 0.05$: significant swelling inhibition.

crine release of ATP and adenosine and the activation of $P2Y_1R$ and A_1AR [16–19]. In the present study we show that disruption of this endogenous signaling cascade by deletion of $P2Y_1R$ or A_1AR in mice results in abrogation of the swelling-inhibitory effects of VEGF and glutamate (fig. 2a, b). In addition, we found that NPY and HB-EGF, as well as triamcinolone acetonide, did not inhibit the hypoosmotic swelling of Müller cells from $A_1AR^{-/-}$ mice (fig. 2b). Furthermore, we show that the swelling-inhibitory effects of glutamate and VEGF require intracellular calcium signaling mediated by IP_3R2 (fig. 3).

The functional consequences of the disrupted glial volume regulation in retinas of $P2Y_1R^{-/-}$, $A_1AR^{-/-}$ and $IP_3R2^{-/-}$ mice remain to be determined in future experiments. It has been suggested that neuron-derived signaling molecules such as glutamate regulate Müller cell volume in relation to the momentary neuronal activity [16, 17]. Regulation of the Müller cell volume by inhibition of cellular swelling is required because intense neuronal activity in the retina is associated with a decrease in extracellular fluid osmolarity [4], a swelling of retinal neurons [17, 25] and a shrinkage of the extracellular space [4, 25]; unregulated shrinkage of the extracellular space will cause neuronal hyperexcitation [26].

Disturbance of the transglial water transport was suggested to represent one cause of retinal edema [3]. Intracellular edema of Müller cells was observed in animal models of ischemic and diabetic retinopathies [13, 14]. However, a swelling of Müller cells was rarely observed in retinopathies, suggesting that endogenous mechanisms are present that inhibit osmotic swelling of Müller cells under pathological conditions in situ. Such a mechanism, which inhibits Müller cell swelling despite intracellular edema, may be the activation of the endogenous swelling-inhibitory purinergic signaling cascade by receptor ligands such as VEGF, NPY and HB-EGF. It has been shown that, in addition to VEGF [12], the expression of NPY and HB-EGF is elevated in the ischemic retina [19, 27]. Moreover, the P2Y-mediated calcium responsiveness of Müller cells is increased in the ischemic retina [15]. Thus, it is conceivable that VEGF, NPY and HB-EGF may inhibit Müller cell swelling under conditions of increased osmotic imbalances in situ.

In the present study we show that deletion of A_1AR results in abrogation of the swelling-inhibitory effects of glutamate and VEGF (fig. 2b). We also found that triamcinolone acetonide was ineffective in preventing the swelling of Müller cells in $A_1AR^{-/-}$ mice (fig. 2b). It has been suggested that the A_1AR-induced opening of ion channels may facilitate the retinal fluid clearance through Müller cells [15]. Because A_1AR mediates the swelling-inhibitory effects of various different receptor ligands, as well as of triamcinolone [24], agonists of A_1AR may represent potential agents for the rapid treatment of retinal edema. Further research is required to elucidate the functional significance of the purinergic signaling cascade in the facilitation of transglial fluid transport in edema.

Acknowledgments

We thank H. Kettenmann (Berlin) for the supply of $A_1AR^{-/-}$ mice, and P. Illes and U. Krügel (Leipzig) for the supply of $P2Y_1R^{-/-}$ mice. This work was supported by grants from the Deutsche Forschungsgemeinschaft (GRK 1097/1 to A.R.; PA615/2-1 to T.P.) and NHLBI (P01 HL080101 to J.C.).

Disclosure Statement

Each author certifies that he/she has nothing to disclose about commercial sponsorship and no conflicts of interest that may be relevant to the submitted work.

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