Published in final edited form as:

Neurosci Lett. 2009 June 26; 457(2): 85–88. doi:10.1016/j.neulet.2009.04.001.

Calcium responses mediated by type 2 IP₃-receptors are required for osmotic volume regulation of retinal glial cells in mice

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Abstract

Prevention of osmotic swelling of retinal glial (Müller) cells is required to avoid detrimental decreases in the extracellular space volume during intense neuronal activity. Here, we show that glial cells in slices of the wildtype mouse retina maintain the volume of their somata constant up to ~4 minutes of perfusion with a hypoosmolar solution. However, calcium chelation with BAPTA/AM induced a rapid swelling of glial cell bodies. In glial cells of retinas from inositol-1,4,5-trisphosphate-receptor type 2-deficient (*IP₃R2*^{-/-}) mice, hypotonic conditions caused swelling of the cell bodies without delay. Exogenous ATP (acting at P2Y₁ receptors) prevented the swelling of glial cells in retinal slices from wildtype but not from *IP₃R2*^{-/-} mice. Müller cells from *IP₃R2*^{-/-} mice displayed a strongly reduced amplitude of the ATP-evoked calcium responses as compared to cells from wildtype mice. It is concluded that endogenous calcium signaling mediated by IP₃R2 is required for the osmotic volume regulation of retinal glial cells.

Keywords

osmotic swelling; glial cell; retina; IP₃R2; ATP

In the sensory retina, intercellular calcium signaling within the network of glial cells can be triggered by various stimuli such as electrical or mechanical stimulation and focal application of neurotransmitters [11]. These calcium waves were suggested to underlie an extraneuronal long-range signaling system that may control neuronal activity [12]. Glial calcium waves are associated with a modulation of the firing rate of neighboring neurons, leading to enhancement or depression of the light-evoked spike activity of retinal ganglion cells [12]. The propagation of the waves from astrocytes to Müller glial cells, and between Müller cells, depends upon the release and extracellular diffusion of adenosine 5'-triphosphate (ATP), activation of purinergic P2Y receptors, and release of calcium from internal stores [10,11].

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Intense glutamatergic neuronal activity in the retina is associated with a volume increase of neuronal cell structures [16] and a decrease in the osmolarity of the extracellular space (ECS) fluid [5]. The reduction of the extracellular osmolarity leads to an osmotic gradient that favors water flux from the extracellular to the intracellular space. In respect to retinal glial cells, the osmotic imbalance was suggested to be intensified by the uptake of neuron-derived osmolytes such as potassium and sodium-glutamate [2,3] which may cause an increase in the intracellular osmotic pressure [1]. To avoid deleterious activity-dependent decreases in the perisynaptic space that may result in neuronal hyperexcitability [4,6], glial cells should avoid cellular swelling, or should even decrease their volume, when neurons display an activity-dependent swelling, and the extracellular osmolarity decreases. It has been shown in preparations of the rat retina that pharmacological activation of purinergic P2Y receptors by ATP prevents the swelling of glial cells under hypoosmotic conditions [18,19]. However, the intracellular signaling cascade which is involved in the regulation of glial cell volume is incompletely understood. It is unclear, for example, whether intracellular calcium signaling is required for the prevention of osmotic glial cell swelling. Therefore, we investigated the swelling of the glial cell bodies in freshly isolated slices of the mouse retina in response to acute perfusion of a hypoosmolar solution. By using retinal tissues from inositol-1,4,5-trisphosphate (IP₃)-receptor type 2 (IP₃R2)-deficient mice [7], we identified the type of calcium stores which is implicated in the regulation of glial cell volume.

All experiments were done in accordance with the European Communities Council Directive 86/609/EEC, and were approved by the local authorities. Adult (2–4 months) homozygous mice and littermate wildtype (IP₃R2^{-/-} IP₃R2^{+/+}) controls (both in a 129Sv/Black Swiss background) were used. Offspring from intercrosses were genotyped by PCR analysis as described [7]. Animals were sacrificed with carbon dioxide. Pieces of freshly isolated retinas (3 × 3 mm) were placed, with the photoreceptor side down, onto membrane filters (mixed cellulose ester, 0.45 µm pore size; 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 by a metal grid with nylon threads. The chambers were mounted on the stage of an upright confocal laser scanning microscope (LSM 510 Meta; Zeiss, Oberkochen, Germany).

The swelling experiments were performed at room temperature (20–23°C). To monitor volume changes of retinal glial cells in response to hypotonic challenge, the somata of Müller glial cells in the inner nuclear layer of retinal slices were focussed. The slices were loaded with the vital dye Mitotracker Orange (10 µM; Molecular Probes, Eugene, OR); it has been shown that this dye stains selectively the somata of Müller glial cells in the inner nuclear layer of retinal tissues [17]. After an incubation time of three minutes, the slices were continuously perfused with extracellular solution at a flow rate of 2 ml/min, and recordings were made with an Achromplan 63x/0.9 water immersion objective. The pinhole was set at 172 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 560 nm long-pass filter. In the course of the experiments, the Mitotracker Orange-stained somata of Müller glial cells were recorded at the plane of their maximal extension. To assure that the maximum soma areas were precisely measured, the focal plane was continuously adjusted in the course of the experiments.

A gravity-fed system with multiple reservoirs was used to perfuse the recording chamber continuously with extracellular solution; test substances were applied by rapidly changing the perfusate. The bathing solution in the recording chamber was totally changed within ~2 minutes. The extracellular solution consisted of (in mM) 136 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 11 glucose, adjusted to pH 7.4 with Tris. The hypoosmolar solution (60% of control osmolarity) was made up by adding distilled water. Barium chloride (1 mM)

was preincubated for 10 minutes. Blocking substances were preincubated for 15 to 45 minutes, and agonists were applied simultaneously with the hypoosmolar solution.

Calcium imaging in the endfeet of retinal glial (Müller) cells was carried out in retinal wholemounts with the calcium-sensitive fluorescence dye Fluo-4/AM. Freshly isolated retinal tissues were incubated for one hour at room temperature in extracellular solution containing Fluo-4/AM (11 μ M; Molecular Probes). Wholemounts (3×3 mm) of the tissues were placed in a perfusion chamber and continuously perfused with extracellular solution for 10 minutes; thereafter, ATP was added by rapid changing the perfusate. Fluo-4 was excited at 488 nm; emission was recorded with a 505 nm long pass filter.

For immunohistochemical staining, isolated retinas were fixed in 4% paraformaldehyde for two hours. After several washing steps in buffered saline, the tissues were embedded in saline containing 3% agarose (w/v), and 70- μ m thick slices were cut by using a vibratome. The slices were incubated in 5% normal goat serum plus 0.3% Triton X-100 in saline for two hours at room temperature and, subsequently, in a mixture of primary antibodies overnight at 4°C. After washing in 1% bovine serum albumin, secondary antibodies were applied for two hours at room temperature. Control slices were stained without primary antibodies; no unspecific labelling was observed following incubation with secondary antibodies alone (not shown). Images were taken by using the LSM 510 Meta (Zeiss). The following antibodies were used: rabbit anti-Kir4.1 (1:200; Sigma-Aldrich, Taufkirchen, Germany), mouse anti-glial fibrillary acidic protein (GFAP; 1:200; G-A-5 clone, Sigma), mouse anti-cellular retinaldehyde-binding protein (CRALBP; 1:1000; Acris), Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova), and Cy2-coupled goat anti-mouse IgG (1:400; Dianova).

Statistical analysis was made by using the Prism program (Graphpad Software, San Diego, CA); significance was determined by the non-parametric Mann-Whitney *U* test. To determine the extent of the swelling of glial cell somata, the cross-sectional area of the glial cell bodies was measured off-line using the image analysis software of the laser scanning microscope.

The swelling of the somata of Müller glial cells was investigated by perfusion of freshly isolated retinal slices with a hypoosmolar solution (containing 60% of control osmolarity). As shown in Figure 1A, hypotonic exposure for 4 minutes did not evoke a significant swelling of glial cell bodies in retinal slices from wildtype mice. The increase in the size of glial cell bodies was significantly ($P<0.001$) stronger and faster when barium chloride (1 mM) was co-applied with the hypoosmolar solution (Fig. 1A). Barium ions are known to block the principal conductance of retinal glial cells mediated by potassium channels [9,15]. To reveal whether intracellular calcium signaling is required for the regulation of glial cell volume under hypoosmotic conditions, we applied the cell-permeable calcium chelator bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA/AM). As shown in Figure 1B, calcium chelation was associated with a significant ($P<0.001$) swelling of glial somata under hypotonic conditions even in the absence of barium. The amplitude of the swelling in the presence of BAPTA/AM was similar to the amplitude of the barium-evoked swelling, and no additive effect was observed when BAPTA/AM and barium were co-applied. These data suggest that endogenous calcium signaling is required to prevent osmotic swelling of retinal glial cells.

It has been shown that activation of P2Y receptors by ATP prevents the osmotic swelling of glial cells in the rat retina [18,19]. Exogenous ATP prevented the glial swelling also in the mouse retina (Fig. 1B). Apparently, the inhibitory effect of ATP on glial cell swelling was mediated by activation of P2Y₁ receptors, as indicated by the effect of the selective blocker *N*⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) (Fig. 1C). To reveal whether the P2Y₁ receptor-mediated swelling inhibition requires intracellular calcium signaling, we tested

ATP in the presence of BAPTA/AM. As shown in Figure 1B, calcium chelation fully abrogated the swelling-inhibitory action of ATP. These data suggest that the effect of ATP is mediated by activation of P2Y₁ receptors coupled to intracellular calcium signaling.

To reveal whether a release of calcium from IP₃-gated intracellular stores is implicated in the action of ATP, we investigated the osmotic swelling characteristics of glial cells in retinal slices from *IP₃R2*^{-/-} mice. These cells displayed a rapid swelling of their somata under hypoosmotic conditions even without application of barium or calcium chelators (Fig. 1D), further supporting the conclusion that endogenous calcium signaling is required to prevent osmotic swelling. In retinal slices from *IP₃R2*^{-/-} animals, ATP did not prevent the swelling of glial cells (Fig. 1D). This suggests that the swelling-inhibitory effect of ATP is mediated by P2Y₁ receptors which are coupled to IP₃R2-gated calcium stores.

Using fluorometric calcium imaging in retinal wholemounts from wildtype and *IP₃R2*^{-/-} mice, we investigated whether ATP evokes cytosolic calcium responses in the endfeet of retinal glial cells. ATP evoked cytosolic calcium responses in the glial endfeet from both animal strains investigated. The peak amplitude of the calcium response was significantly ($P < 0.001$) smaller in cells from *IP₃R2*^{-/-} mice than that of the response in cells from wildtype mice (Fig. 1E). These data suggest that the ATP-evoked calcium response in retinal glial (Müller) cells is mediated by more than one mechanism; however, most of the cytosolic calcium is released from IP₃R2-gated internal stores.

In retinal slices from wildtype mice, potassium channel-blocking barium ions induce glial cell swelling under hypoosmotic conditions (Fig. 1B). It is known from rat models of various retinopathies that retinal ischemia-reperfusion and inflammation, for example, are associated with an upregulation of GFAP in Müller glial cells, a redistribution of the Kir4.1 protein, and an induction of osmotic Müller cell swelling [13,14]. We found that retinal glial cells of *IP₃R2*^{-/-} mice display hypoosmotic swelling under control conditions (Fig. 1D). Therefore, we studied by using immunohistochemical staining of retinal slices whether the distribution of GFAP and Kir4.1 proteins is altered in retinas of *IP₃R2*^{-/-} mice. We found no differences in the distribution of both proteins in retinal slices derived from wildtype and *IP₃R2*^{-/-} animals (Fig. 2), suggesting that deletion of IP₃R2 does not result in retinal gliosis in these mice.

Summarizing our results, two conclusions can be drawn in respect to the question how the prevention of osmotic glial cell swelling is achieved to avoid a detrimental decrease in the ECS volume. First, in cells from wildtype mice the efficient volume homeostasis (Fig. 1A) is mediated by endogenous calcium signaling, as it is impaired in the presence of a calcium chelator (Fig. 1B). The swelling of cells in tissues from *IP₃R2*^{-/-} mice (Fig. 1D) suggests that a release of calcium from IP₃R2-gated internal stores (rather than from other sources) is crucial for endogenous calcium signaling and thus for the swelling-inhibitory action of ATP mediated by activation of P2Y₁ receptors in wildtype mice. Second, we show that glial cells of *IP₃R2*^{-/-} animals lack such an efficient, ATP-mediated volume homeostasis (Fig. 1D) although small calcium rises can be induced by application of ATP (Fig. 1E). The absence of retinal gliosis and degeneration in these animals (Fig. 2) suggests that alternative mechanisms of (less efficient but still sufficient) glial volume homeostasis, and/or a low level of neuronal activity, may compensate for the deficit - and maintain retinal integrity - in these mice.

The precise mechanism of the calcium-dependent cell volume regulation remains to be determined in future experiments. It can be assumed that the ATP-evoked calcium response results (directly or indirectly) to an opening of (barium-insensitive) ion channels; an efflux of ions compensates the osmotic gradient across the glial membrane and, thus, prevents cellular swelling under hypoosmotic conditions. A similar mechanism has been described in retinal glial cells of the rat. Here, pharmacological activation of adenosine receptors was shown to

inhibit osmotic swelling; this effect was mediated by the opening of two pore-domain potassium channels and chloride channels [20].

Finally it appears noteworthy that the propagation of long-range calcium waves in retinal glial cells has been shown to be mediated by the release of ATP and activation of P2Y receptors [10,11] but that the functional significance of these calcium waves is still unclear. It has been shown that calcium waves are associated with alterations in neuronal activity [12], and that glial calcium signaling is probably involved in the mediation of neurovascular coupling [8]. The present data which show that the swelling-inhibitory effect of ATP is mediated by intracellular calcium signaling may support the assumption that calcium waves also transmit cell volume-regulatory signals over long distances across the network of glial cells.

Acknowledgments

We are grateful for the support by the Deutsche Forschungsgemeinschaft (RE 849/12-1, RE 849/10-2, GRK 1097/1, to A.R.), the Faculty of Medicine of the University of Leipzig (984000-137, to S.L.; NBL Formel.1-133, to A.W.), and NHLBI (P01 HL080101, to J.C.).

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Fig. 1.

Intracellular calcium signaling is required to prevent the osmotic swelling of glial cell bodies in slices of the mouse retina. **(A)** Perfusion of retinal slices from wildtype mice with a hypoosmolar solution (containing 60% of control osmolarity) in the absence (control) and presence of barium chloride (1 mM) resulted in a time-dependent increase in the cross-sectional area of glial cell somata recorded in the inner nuclear layer. The bar diagram (*right*) displays the mean ($\pm$ SEM) soma areas which were measured after a 4-minutes perfusion with a hypoosmolar solution, expressed in percent of the soma size recorded before hypoosmotic challenge (100%). Note that perfusion of retinal slices with the hypoosmolar solution for 4 minutes did not significantly increase the size of glial somata. However, hypoosmotic swelling of glial somata was evoked in the presence of barium. The images display original records of a dye-filled glial soma obtained before (left) and during (right) perfusion with the barium-containing hypoosmolar solution. Bar, 5 μ m. **(B)** Effect of the cell-permeable calcium chelator

BAPTA/AM (100 μ M) on the swelling of glial cell somata in retinal slices from wildtype mice. BAPTA/AM was tested in the absence (control) and presence of barium chloride and in the presence of ATP (200 μ M), respectively. (C) The swelling-inhibitory effect of ATP (200 μ M) was prevented in the presence of the selective inhibitor of P2Y₁ receptors, MRS2179 (30 μ M). (D) ATP (200 μ M) did not prevent the osmotic swelling of glial cell somata in retinal slices from *IP₃R2*^{-/-} mice. (E) Mean ($\pm$ SEM) peak amplitude of the cytosolic calcium response evoked by ATP (50 μ M) in the endfeet of Müller glial cells. The responses were recorded in retinal wholemounts of wildtype and *IP₃R2*^{-/-} mice. The bar diagrams display data obtained in 6–29 (A–D) and 31–60 cells (E), respectively. Significant differences vs. control: ** P <0.01; *** P <0.001. Significant swelling-inhibitory effects: ●●● P <0.001. Significant inhibition of the agonist effects: ○○ P <0.01; ○○○ P <0.001. ▼ P <0.001.

Fig. 2.

Immunohistochemical localization of GFAP and Kir4.1 proteins in retinal slices from wildtype and $IP_3R2^{-/-}$ mice. GFAP labeling was restricted to retinal astrocytes which are localized to the nerve fiber (NFL) and ganglion cell layers (GCL) of the retina, but was absent from Müller glial cells. The Kir4.1 protein was enriched in both limiting membranes (arrowheads) and around the blood vessels (arrows). The co-labeling of Kir4.1 and the glial cell marker CRALBP (yellow merge signal) indicates that Kir4.1 protein is localized largely to Müller cells. Cell nuclei were stained with Hoechst 33258 (blue). INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRS, photoreceptor segments. Bars, 20 μ m.