Early Glial Cell Reactivity in Experimental Retinal Detachment: Effect of Suramin

Susann Uhlmann,[1] *Andreas Bringmann,*[1] *Ortrud Uckermann,*[2] *Thomas Pannicke,*[2] *Michael Weick,*[2] *Elke Ulbricht,*[2] *Iwona Goczalik,*[2] *Andreas Reichenbach,*[2] *Peter Wiedemann,*[1] *and Mike Francke*[2]

Purpose. In a rabbit model of retinal detachment, early Müller glial cell reactivity was monitored—specifically, changes in membrane features—to determine whether these changes involve an upregulation of purinergic P2 receptor–mediated responses and whether all or some of these alterations could be blocked by suramin or pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS). In addition, the immune cell reactivity (microglial cells and blood-derived immune cells) was monitored.

Methods. A local retinal detachment was induced by subretinal injection of a sodium hyaluronate solution. Three, 24, 48, and 72 hours after surgery, Müller cells were acutely isolated, and patch-clamp records of the whole-cell potassium currents were made. The presence of P2 receptor-mediated responses was determined by measuring extracellular adenosine triphosphate (ATP)–induced membrane current increases, and by recording of ATP-induced calcium responses at the vitreal surface of retinal wholemounts. The density of isolectin B_4-labeled immune cells was determined in the nerve fiber layer of retinal wholemounts.

Results. Within 24 hours of detachment, Müller cell reactivity was evident. The cells downregulated the density of their inwardly rectifying potassium currents to 60% and 47% of the control value at 48 hours and 72 hours of detachment, respectively. This downregulation was accompanied by an enhanced incidence of cells which showed calcium and current responses after ATP application (control: 14%; 24 hours of detachment: 42%; 72 hours of detachment: 80%). Müller cell hypertrophy was apparent at 48 and 72 hours of detachment. Application of suramin during surgery inhibited the downregulation of potassium currents, but not the elevated responsiveness to extracellular ATP; PPADS had no effect. Suramin also inhibited the inflammatory response that was induced by the surgical procedure and that was apparent by the increased number of immune cells.

Conclusions. Reactive responses of Müller cells occur within 24 hours of detachment. Suramin inhibits several (but not all) reactive glial alterations and therefore may represent one candidate for further investigations in the search for drugs that limit detrimental effects of immune cell activation and Müller cell gliosis during retinal detachment. (*Invest Ophthalmol Vis Sci.* 2003;44:4114–4122) DOI:10.1167/iovs.03-0183

Detachment of the neural retina from the retinal pigmented epithelium increases the distance between the choriocapillaris and the neural retina and results in a decreased energy supply of retinal cells.[1] This was suggested to cause photoreceptor cell death and subsequent retinal degeneration,[1–3] mediated, at least partially, by an impairment of glutamate recycling through Müller glial cells, and by subsequent glutamate excitotoxicity.[4,5] Retinal detachment is accompanied by an early reactivity of Müller cells,[6] the principal macroglial cells of the retina.[7] Within minutes of experimental detachment, Müller cells show increased protein phosphorylation (e.g., of fibroblast growth factor receptor 1 and of extracellular signal-regulated kinase) and increased production of transcription factors.[8] Within 1 day of detachment, Müller cells begin to proliferate, and increased expression of the intermediate filaments, glial fibrillary acidic protein (GFAP), and vimentin, is evident.[9,10] The detachment-induced proliferation of Müller cells peaks at 3 to 4 days of detachment.[9] At this time point, Müller cell hypertrophy is obvious in the retina and in the subretinal space.[9] The Müller cell reactivity may be caused (directly or indirectly) by the detachment-induced hypoxia, because ischemia has been shown to induce Müller cell proliferation,[11] and because Müller cell proliferation and hypertrophy have been shown to be reduced by oxygen supplementation during experimental detachment.[12] Retinal detachment may evolve into subretinal fibrosis and proliferative vitreoretinopathy (PVR),[13] a disease that is characterized by strong proliferation of retinal cells—Müller and microglial cells, among others—and the formation of fibrocellular membranes on the surfaces of the retina.

The gliosis of Müller cells during retinal detachment and PVR is accompanied by alterations of their membrane conductances.[14] The main membrane conductance of Müller cells from healthy retinas is provided by currents through inwardly rectifying potassium channels,[7,15–17] which also were suggested to mediate spatial buffering potassium currents, crucially involved in the extracellular potassium homeostasis in the retina.[7] Müller cells from detached retinas show a decrease in these potassium currents after 2 days,[15] whereas most Müller cells from PVR retinas display virtually no inwardly directed potassium currents; this is accompanied by a depolarization of the plasma membrane,[15–17] which must impair the electrogenic glutamate uptake.[6,7] In contrast, the activity of calcium-dependent potassium channels of big conductance (BK channels), which are necessary for the proliferation of cultured Müller cells,[18–21] was found to be enhanced in Müller cells from PVR retinas.[17] As another feature of gliotic Müller cells in PVR, probably related to proliferation, an upregulation of their

From the [1]Department of Ophthalmology, Eye Clinic, and the [2]Department of Neurophysiology, Paul Flechsig Institute of Brain Research, University of Leipzig, Leipzig, Germany.

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Corresponding author: Andreas Bringmann, Department of Ophthalmology, Eye Clinic, University of Leipzig Medical Faculty, Liebigstrasse 10-14, D-04103 Leipzig, Germany; bria@medizin.uni-leipzig.de.

responsiveness to extracellular adenosine 5′-triphosphate (ATP; acting on purinergic P2 receptors) was recently described.[20,22] It has not yet been shown whether the phenomenon is restricted to PVR or whether an increased ATP responsiveness is a more general phenomenon of Müller cell gliosis.

Despite this growing body of knowledge, there remain many open questions that urgently need to be answered, such as: (1) Is the enhanced ATP responsiveness an early feature of Müller cell gliosis independent of the presence of PVR; (2) is there a temporal relationship between the changes in ATP responsiveness and the loss of inward potassium currents in Müller cells; and (3) how are the Müller cell alterations correlated with the early activation of microglial cells? With respect to the last question, it is noteworthy that reactive microglial cells were shown not only to affect Müller cell functions[23] but also to play a detrimental role on their own.[24] Thus, the present study was conducted first to elucidate the time course of the early reactive changes in more detail. Furthermore, we tested whether the detachment-induced early alterations of microglia and Müller cells could be blocked by the local application of the two agents, pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS, known to block some P2Y receptor subtypes[25]) and suramin. The polysulfonated naphthylurea, suramin, has been shown to block several P2 receptor subtypes[25] and to inhibit the binding of various different cytokines, including basic fibroblast growth factor, to their cell surface receptors.[26–29] Previously, systemic application of suramin was described to decrease the severity of experimental PVR.[30] There is a great need of suitable therapeutic and/or preventive approaches in retinal detachment, PVR, and related retinal injuries involving reactive gliosis. These retinal diseases are among the main causes of blindness in the industrial countries, and there is the danger of iatrogenic induction. For instance, macular translocation surgery involves a (temporary) voluntary detachment of the retina, and detachment carries with it a risk of approximately 5% to 10% of triggering PVR.[31]

Materials and Methods

Surgical Procedure

All experiments were performed in accordance with applicable German laws and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-nine adult pigmented rabbits (2–3 kg, both sexes) were anesthetized by intramuscular injection of ketamine (50 mg/kg body weight; Ratiopharm, Ulm, Germany) and xylazine (3 mg/kg; BayerVital, Leverkusen, Germany). The pupils of the right eyes were dilated by topical application of tropicamide (1%; Ursapharm, Saarbrücken, Germany) and phenylephrine hydrochloride (5%; Ankerpharm, Rudolstadt, Germany), and 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, NV) were used to create a local retinal detachment by subretinal injection of 0.25% sodium hyaluronate (Healon; Pharmacia & Upjohn, Dübendorf, Switzerland) in saline. Nasal retinal parts (with diameters of 8–10 mm) were detached near the medullary rays. In two and three animals, respectively, PPADS (10 mM in 100 μL saline) or suramin solution (5 mM in 100 μL saline) was injected into the subretinal space (50 μL) and into the vitreous body (50 μL) just after the retina was detached. (The concentrations of the drugs mean the concentration of the injected solutions.) The left eyes had no operation and served as the control. After survival times of 3 ($n = 2$ animals), 24 ($n = 6$), 48 ($n = 10$), or 72 hours ($n = 5$), the animals were anesthetized as described, and were killed by an intravenous application of T61 (3 mL; embutramid mebezonium iodide; Hoechst, Unterschleissheim, Germany), and the eyes were excised. In addition, two further control groups ($n = 3$ animals each) were investigated: In one group, saline (100 μL) was placed over the vitreal surface of the undetached retina after vitrectomy ("saline control" group), in the other group, a small retinal hole was made after vitrectomy, and sodium hyaluronate (100 μL) was placed over the vitreal surface of the undetached retina ("sodium hyaluronate control" group). The animals of both groups were killed 48 hours after surgery.

Calcium Imaging

Wholemounts of isolated retinal pieces (3 × 3 mm) were placed in a perfusion chamber, with the vitread surface up, and were incubated for 1 hour at room temperature in extracellular solution containing two different calcium-sensitive fluorescence dyes: Fluo-4/AM (final concentration 11 μM) and Fura-Red/AM (final concentration 17 μM; Molecular Probes, Eugene, OR). The extracellular solution contained (mM) 110 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 Na_2HPO_4, 0.25 glutamine, 10 HEPES, 11 glucose, and 25 $NaHCO_3$, adjusted to pH 7.4 with tris-(hydroxy-methyl)-aminomethane (Tris-base) and bubbled with carbogen (95% O_2, 5% CO_2). After 10 minutes of washing by continuous perfusion of extracellular solution, test substances were added by fast (10 seconds) changing of the perfusate. Calcium-free extracellular solution contained 0.1 mM $CaCl_2$ and 1 mM EGTA. The nucleotides and adenosine were tested as sodium salts. ATP was purchased from Serva Electrophoresis (Heidelberg, Germany). All other substances used were from Sigma-Aldrich (Deisenhofen, Germany), unless stated otherwise. Fluorescence images were recorded using a confocal laser scanning microscope (LSM 510; Carl Zeiss Meditec, Oberkochen, Germany). The fluorescence dyes were excited at 488 nm; the emission of Fluo-4 was recorded with a band-pass filter between 505 and 550 nm, and the emission of Fura-Red was recorded with a 650 nm long-pass filter. Images were taken from the vitread surface of retinal wholemounts every 5 seconds from an area of 230 × 230 μm.

Immune Cell Reactivity

To estimate the number of microglial cells and of blood-derived immune cells in the nerve fiber layer, acutely isolated retinal wholemounts were exposed to Cy3-tagged *Griffonia simplicifolia* agglutinin (GSA) isolectin B_4 for 1 hour at room temperature, simultaneously with exposure to calcium-sensitive dyes. GSA lectin labels D-galactose residues that are expressed by both resting and activated microglial cells and by blood-derived immune cells in the rabbit retina. The Cy3 fluorescence was recorded with a 543 nm HeNe laser and a 560- to 615-nm band-pass filter. As a rough estimation of the activation–infiltration of these cells, the number of cells per unit retinal surface area (230 × 230 μm) in the nerve fiber layer of the wholemounts was counted. Cells were counted manually using the laser scanning microscope software.

Electrophysiological Recordings

Whole-cell patch-clamp recordings were performed on Müller cells acutely isolated in papain and DNase I–containing solutions, as described previously.[15] The cell suspensions were stored in serum-free modified Eagle's medium at 4°C (up to 4 hours) before use. Voltage-clamp recordings were obtained at room temperature (22–25°C) using an amplifier (EPC-7; List, Darmstadt, Germany) and computer (TIDA 5.72 software; HEKA Elektronik, Lambrecht, Germany). Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and had resistances between 3 and 5 MΩ when filled with the intracellular solution that contained (mM) 10 NaCl, 130 KCl, 3 $MgCl_2$, and 10 HEPES (pH 7.2). The series resistance (10–18 MΩ) was compensated by 30% to 50%; the signals were low-pass filtered at 3 kHz (eight-pole Bessel filter), and the sampling rate was 15 kHz. The recording chamber was continuously perfused with bath solution consisting of (mM) 110 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$ 10 HEPES, and 11 glucose with pH adjusted to 7.4 with Tris base. The test substances were added by fast (5 seconds) changing of the perfusate.

To evoke whole-cell potassium currents, de- and hyperpolarizing voltage steps of 250 ms duration, with an increment of 20 mV, were applied from a holding potential of −80 mV. To record whole-cell

currents continuously, voltage steps of 50 ms duration to +120 and to −120 mV were applied at a frequency of 2.5 Hz from a holding potential of 0 mV. Voltage steps to +120 mV were used because the mean activation threshold of BK currents at control conditions was at approximately +30 mV.[22] Therefore, each elevation of the intracellular free calcium concentration should increase the amplitude of the currents at +120 mV. A holding potential of 0 mV was chosen to minimize the activation of voltage-gated outward potassium currents and to reduce space-clamp problems during the activation of BK currents. The membrane capacitance of the cells was measured by the integral of the uncompensated capacitive artifact evoked by a hyperpolarizing voltage step from −80 to −90 mV when barium ions (1 mM) were present in the bath solution to block the predominant potassium conductance of the cells. To record the capacitive artifact, the sampling rate was 30 kHz, and the frequencies above 10 kHz were cut off. Stock solution of okadaic acid was made in dimethylsulfoxide. The vehicle itself did not affect intracellular calcium level and membrane currents.

Data Analysis

The electrophysiological data were not leak subtracted and were not corrected for liquid junction potentials, because these did not exceed 3 mV. The amplitude of the steady state whole-cell currents were measured at the end of 250-ms voltage steps. To obtain current densities, the amplitudes were divided by the cell membrane capacitance. In continuous recordings, current amplitudes were measured at the end of the 50-ms voltage steps. As a rough estimate of the resting membrane potentials, the zero-current potentials of the steady state current-voltage curves were determined.

To evaluate the calcium responses, the fluorescence ratio $F_{\text{Fluo-4}}/F_{\text{Fura-Red}}$ was calculated. Subsequently, the values were normalized to the prestimulus baseline value by calculation of the ratio F/F_0, where F_0 represents the mean baseline fluorescence ratio that was calculated using the mean of the last 10 values before agonist application. The incidence of responding Müller cells was estimated using a procedure recently described in detail.[32] As shown in that study, at the vitreal surface of the rabbit retina (i.e., in the nerve fiber/ganglion cell layers), only Müller cells take up sufficient amounts of calcium fluorescence dyes. Therefore, stimulus-induced calcium responses are restricted to the Müller cells. After subtraction of the area occupied by neurons and nerve fibers, the area that was filled by Müller cell end feet was obtained. Because the sizes of individual Müller cell end feet within a retinal piece do not vary considerably,[33] the percentage of end foot areas that showed calcium responses was taken as an estimate of the incidence of responding Müller cells. Statistical analysis was performed on computer (Prism; GraphPad Software, San Diego, CA). Significance was determined by Student's *t*-test for two groups and by ANOVA, followed by comparisons for multiple groups, respectively. Data are expressed as the mean ± SEM; *n* represents the number of retinal wholemounts (calcium imaging) and the number of isolated cells investigated (electrophysiological recordings).

Results

Ophthalmoscopic Observations

Before enucleation, all eyes were subjected to indirect ophthalmoscopic examination. The left eyes of the animals, used as the control, revealed normal views of the fundus in all cases. In the surgically altered right eyes, bullous retinal detachments were found in the nasal part of the eye, below the medullary rays. Ophthalmoscopically, no signs of PVR were detected. All eyes had a clear cornea after surgery. One eye showed a slight opacity of the posterior capsula of the lens due to mechanical lens irritation during vitrectomy. No intravitreal or subretinal bleeding occurred. The medullary rays and the retinal vasculature showed no abnormalities. Between control saline and suramin/PPADS application, no differences were found ophthalmoscopically.

Plasma Membrane Properties of Isolated Müller Cells

Electrophysiological whole-cell recordings were used to reveal the time course of the alterations in plasma membrane features of Müller cells in detached retinas, particularly in the early stages (3–48 hours), which had not yet been studied.[7,14–17] Examples of whole-cell records of typical cells from a control retina and from a retina detached for 48 hours are shown in Figure 1A. The plasma membrane conductance of Müller cells from control retinas is predominated by inwardly rectifying potassium currents[7] that cause large inward currents (downwardly depicted) in response to hyperpolarizing voltage steps, and smaller outward currents (upwardly depicted) during membrane depolarization. The current density–voltage relations of the whole-cell currents (Fig. 1C) reveal a weak inward rectification of the potassium currents near the reversal potential of the whole-cell currents (at approximately −80 mV), as previously described, to be typical for rabbit Müller cells[34] and for currents mediated by the Kir4.1 channel,[35] the predominant Kir channel subtype expressed by Müller cells.[36] In cells from retinas detached for 48 hours, the amplitude of the potassium currents varied considerably (Fig. 1A). However, as shown in Figure 1B, the mean densities of inward potassium currents decreased in dependence on the duration of the retinal detachment (at 24 hours, to 84.6% ± 4.8%, $P < 0.05$; at 48 hours, to 59.8% ± 5.6%, $P < 0.01$; and at 72 hours, to 46.7% ± 3.7% of the controls, $P < 0.01$). A slight depolarization of the plasma membrane was observed after 48 hours of detachment (−80.4 ± 1.4 mV vs. −83.0 ± 0.7 mV in control cells, $P < 0.05$); the depolarization was slightly stronger at 72 hours (−79.4 ± 1.4 mV; $P < 0.05$).

Müller cell hypertrophy was previously observed in detached retinas after 3 days.[10] To reveal possible earlier changes, the membrane capacitance of acutely isolated Müller cells was measured electrophysiologically. The mean membrane capacitance did not change up to 24 hours after detachment but increased significantly at 48 and 72 hours (Fig. 1D).

ATP-Evoked BK Current Responses in Isolated Müller Cells

An increase of the intracellular free calcium concentration causes an opening of calcium-dependent BK channels in Müller cells.[18,34] In whole-cell records, the activation of BK channels is reflected in amplitude increases of the potassium currents at positive membrane potentials.[22] An example of such an activation of BK currents in one cell is shown in Figure 2A, left. Extracellular application of the BK channel opener phloretin evoked an increase of the amplitude of the currents at +120 mV. Simultaneous application of the BK channel inhibitors, iberiotoxin (as shown previously)[22] or tetraethylammonium chloride (Fig. 2A) reversibly blocked the action of phloretin, indicating that the amplitude increase of the whole-cell currents, at +120 mV predominantly, reflects an activation of BK channels.

Extracellular application of ATP should enhance the amplitude of the currents elicited at +120 mV through a P2 receptor-mediated intracellular free calcium increase. In a subpopulation of cells investigated, extracellular ATP evoked current increases at +120 mV (Fig. 2A, middle). The current increases were transient; the ATP-evoked currents inactivated within 1 to 2 minutes, even in the presence of the agonist. These current increases were mediated by activation of metabotropic P2Y receptors rather than ionotropic P2X receptors, because similar responses were observed after application of uridine

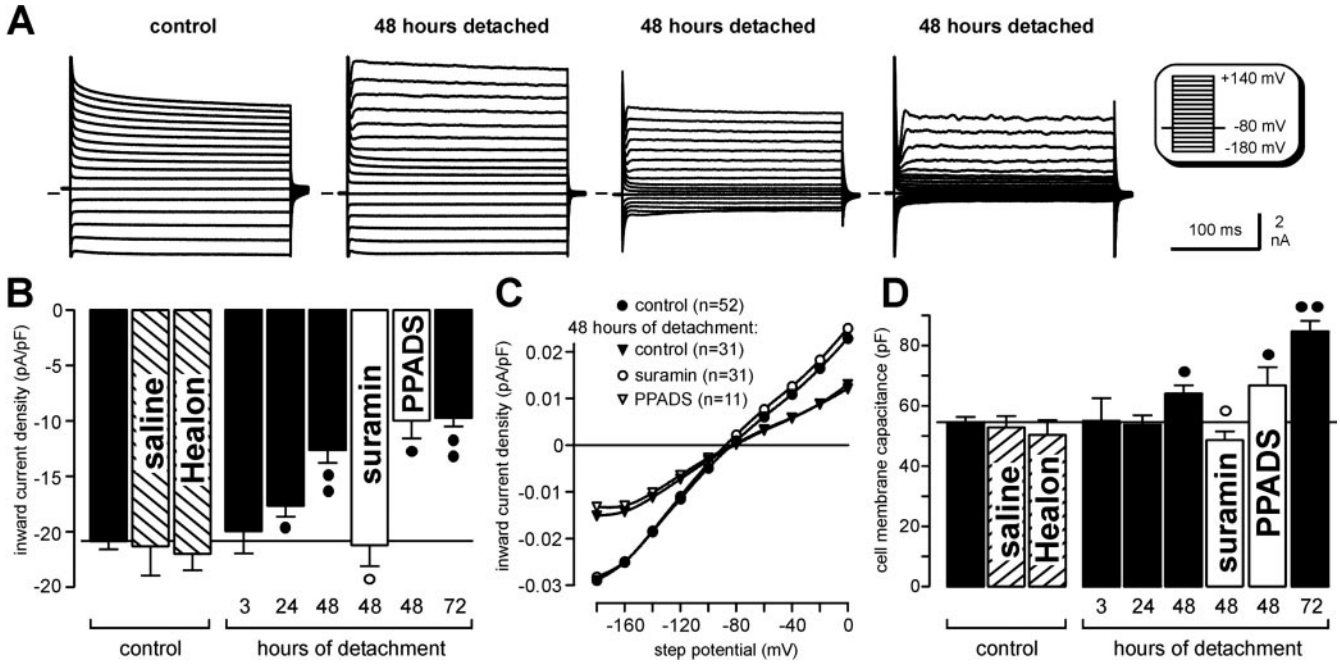

FIGURE 1. Müller cells in detached retinas reduced their potassium currents in dependence on the duration of the detachment. (**A**) Whole-cell current traces of four cells derived from a control retina and from a retina detached for 48 hours. Particularly, the inwardly directed currents (downwardly depicted) were reduced in a subpopulation of cells from the detached retina. Voltage steps were applied from a holding potential of −80 mV to increasing de- and hyperpolarizing potentials between −180 and +140 mV (250 ms, 20-mV increments). *Small bars*, *left*: zero-current levels. (**B**) Mean ± SEM density of the inward currents depending on the duration of detachment. The currents were measured between the voltage steps to −100 and −160 mV. (**C**) Mean current density–voltage relationships of the whole-cell currents of Müller cells, which were derived from control and 48-hour detached retinas, respectively. The steady state currents were measured at the end of 250-ms voltage steps. (**D**) Mean ± SEM cell membrane capacitance of the cells. Suramin (5 mM in 100 μL saline) or PPADS (10 mM in 100 μL saline) was injected into the subretinal space, and the vitreous body near the detached retina at the time of the surgery. The saline and sodium hyaluronate (Healon; Pharmacia & Upjohn) controls were measured at 48 hours after application of 100 μL of saline or sodium hyaluronate to the vitreal surface of undetached retinas. Cell number investigated: control, 116; saline control, 24; sodium hyaluronate control, 26; 3-hour detached retina, 14; 24-hour, 75; 48-hour, 51; 48-hour+suramin, 34; 48-hour+PPADS, 11; 72-hour, 58. Significant difference versus control: $\bullet P < 0.05$; $\bullet\bullet P < 0.01$. Significant effect of the blocker between cells from the same stage: $\circ P < 0.05$.

triphosphate (UTP; Fig. 2A, right trace) and because no current increases were observed at negative membrane potentials (−120 mV) that would require the opening of nonspecific cation channels of P2X receptors.

After experimental retinal detachment, there occurred a gradual increase of the incidence of isolated Müller cells that responded to extracellular ATP with a transient increase of their BK currents (Fig. 2B). Whereas approximately 10% of the investigated cells from control retinas showed ATP-evoked BK current responses, after 24 hours of detachment ~52% of the investigated cells were responsive and after 48 hours the incidence further increased to ~63%. As shown previously,[22] the absence of ATP responses in nonresponsive cells was not caused by a lack of BK channel expression in these cells (for an example, see Fig. 2A, left). Apparently, the availability of functional P2Y receptors was increased in Müller cells from retinas that were detached for 24 hours and more. When we evaluated our results obtained in nonresponsive and responsive Müller cells separately, it was apparent that cells from 24- and 48-hour detached retinas, which showed ATP-evoked BK current responses, displayed a stronger reduction of their Kir currents ($P < 0.05$; Fig. 2C).

ATP-Evoked Single-Channel Responses

In the whole-cell records shown in Figure 2A, the BK currents were activated at positive potentials. However, it is known from previous studies of human[17,37] and rabbit [34] Müller cells that BK channels may be activated at the native resting membrane potential. Very probably, the strong positive shift of the BK channel activation observed in whole-cell records reflects the absence of essential cytoplasmic components in the pipette solution that coactivate BK channels, such as activators of the protein kinase A.[38] To determine whether ATP application increases the BK channel activity at the native resting membrane potential, cell-attached records were made on cells from 48-hour detached retinas.

In cell-attached records, potassium currents through single large-conductance potassium channels into Müller cells were measurable (Fig. 3A). The channels displayed a mean slope conductance of 133.7 ± 4.5 pS ($n = 10$ membrane patches; Fig. 3B). At the resting membrane potential, the channels were active with a relatively low open probability (0.016 ± 0.010). The open probability increased on depolarization of the membrane, by applying negative potentials to the pipette electrode (Fig. 3C). Extracellular application of ATP (100 μM) increased the open probability from 0.020 ± 0.016 before agonist application to 0.132 ± 0.055 at peak activation ($n = 4$ membrane patches; Fig. 3D). It is concluded that P2Y receptor activation transiently increases the open probability of single large-conductance potassium channels (very likely representing BK channels) at the resting membrane potential of Müller cells.

ATP-Evoked Calcium Responses in Retinal Wholemounts

It has been shown recently that, in wholemounts of rabbit retinas, predominantly Müller cell end feet are labeled with the calcium-sensitive dyes Fluo-4 and Fura-Red, whereas neuronal

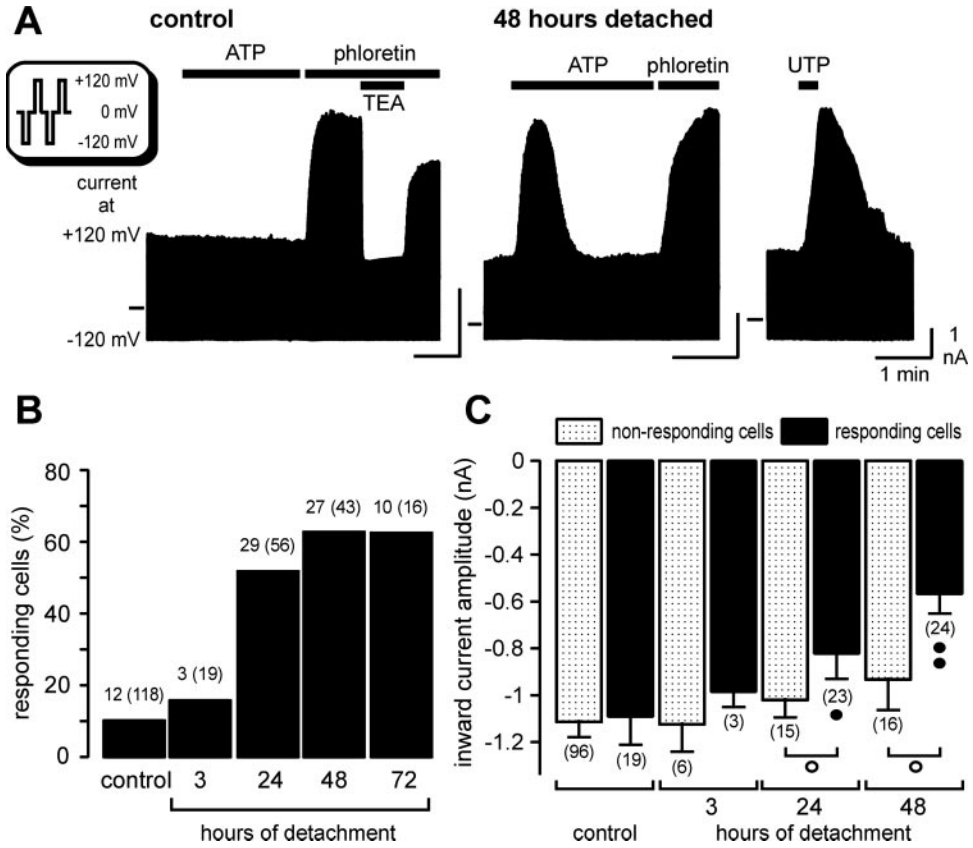

Figure 2. Extracellular ATP evoked transient increases of BK currents predominantly in cells from detached retinas. Nucleotides were tested at a concentration of 100 μM. (**A**) *Left*: example of whole-cell currents recorded in one cell from a control retina. Although extracellular ATP did not increase the currents at +120 mV, the BK channel opener phloretin (200 μM) evoked an increase of these currents that was reversibly blocked by tetraethylammonium ions (1 mM). In two other cells from a 48-hour detached retina, extracellular ATP (*middle*) or UTP (*right*) transiently increased the currents at +120 mV. *Small bars*, *left*: zero-current levels. (**B**) Percentage of cells that responded to extracellular ATP with a transient BK current increase. (**C**) Dependence of the amplitude of inward potassium currents on the duration of detachment. The data are arranged in two groups: cells that showed an ATP-evoked BK current response, and cells that did not show such a response. The currents were measured between voltage steps to −100 and to −160 mV. (**B**, **C**) The number of cells investigated at each time point appears in *parentheses*. Significant difference versus the respective control: $\bullet P < 0.05$; $\bullet\bullet P < 0.01$; significant difference between cells from the same stage: $\circ P < 0.05$.

cell bodies remained largely devoid of the dyes.[32] Therefore, agonist-evoked intracellular calcium responses can be recorded selectively in Müller cell end feet.[32] Extracellular application of ATP (200 μM) evoked transient increases of the intracellular free calcium in some Müller cell end feet of control retinas (Fig. 4A). The ATP (200 μM)-evoked calcium responses were partially inhibited by the P2 receptor antagonist PPADS (100 μM; to 73.9% ± 5.1%, $n = 3$, $P < 0.05$), and they persisted in calcium-free extracellular solution (peak amplitude 100.6% ± 17.7% compared with the control value, $n = 3$; not significant; Fig. 5A). This suggests that the responses were mainly mediated by activation of P2Y receptors, coupled to a release of calcium from intracellular stores. Adenosine 5′-diphosphate (ADP), a selective agonist for $P2Y_1$ receptors, and uridine 5′-triphosphate (UTP), a selective agonist for $P2Y_2$ and $P2Y_4$ receptors, evoked responses that were similar in amplitudes and durations to those obtained using ATP, whereas uridine 5′-diphosphate (UDP) or adenosine had no effect (Figs. 5B, 5C), suggesting that an activation of multiple P2Y receptor subtypes was involved in the ATP-evoked calcium responses of Müller cell end feet. The absence of current increases at negative membrane potentials (−120 mV) during ATP exposure (Fig. 2A) suggests that P2X receptors were not activated under the experimental conditions used.

Detachment-Induced Increase of ATP-Evoked Calcium Responses

The ATP-evoked calcium responses of Müller cell end feet in retinal wholemounts were used to determine the incidence of Müller cells that express functional P2Y receptors, in dependence on the duration of retinal detachment. The detachment caused a significant increase of the incidence of Müller cells that showed calcium responses on application of ATP (Fig. 4B). In control retinas, 14.4% ± 3.7% of the end feet showed ATP-evoked calcium responses ($n = 34$; Fig. 4C). In retinas detached for 24 hours, the incidence of ATP-responsive cells was significantly elevated to 41.9% ± 8.0% ($n = 8$, $P < 0.05$), a further increase was observed at 48 hours (55.0% ± 9.8%; $n = 12$, $P < 0.01$) and 72 hours (79.8% ± 7.5%; $n = 11$, $P < 0.01$). The data indicate that an upregulation of calcium responsiveness to ATP occurs in Müller cells within 24 hours of experimental retinal detachment. In contrast, application of saline or sodium hyaluronate to the vitreal surface of unde-

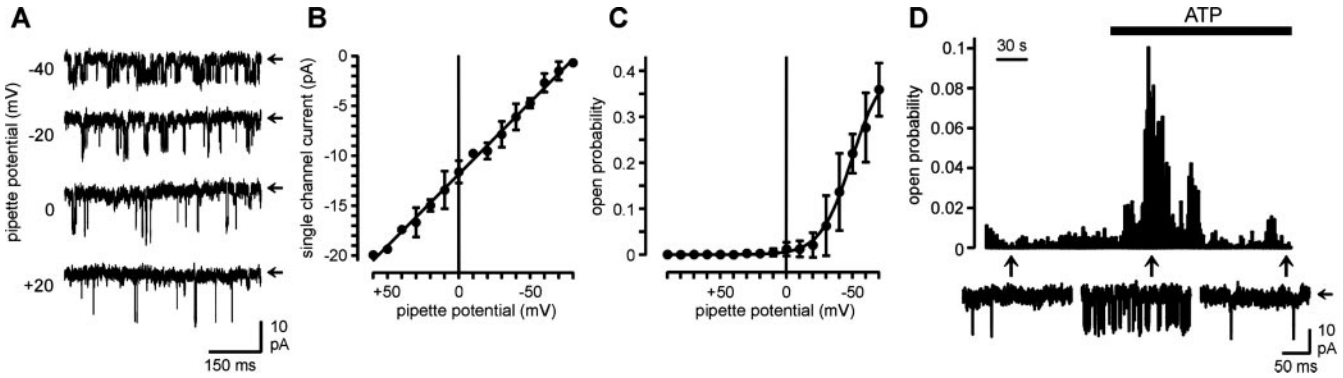

FIGURE 3. The open probability of BK channels in cell-attached membrane patches of rabbit Müller cells from 48-hour detached retinas increased after extracellular application of ATP. (**A**) Example of recordings of single-channel activity at different pipette potentials in a patch that apparently contained one functional channel. A pipette potential of 0 mV reflects the native resting membrane potential of the cell; a negative pipette potential means a plasma membrane depolarization, a positive pipette potential a hyperpolarization. *Arrows*: closed state levels. (**B**) The mean current–voltage relation of the channel currents revealed a slope conductance of 133.7 ± 4.5 pS. (**C**) The mean dependence of the open probability on the pipette potential shows that the channel was activated when the membrane was depolarized (at negative pipette potentials). The data in (**B**) and (**C**) were obtained in 10 patches from different cells. (**D**) Time-dependent change of the channel's open probability (calculated at 1-second intervals) in one cell at a pipette potential of 0 mV. Extracellular application of ATP (100 μM) transiently increased the open probability. *Bottom*: examples of channel records at three time points.

tached retinas did not significantly alter the incidence of responding cells when measured at 48 hours after surgery (Fig. 4C).

Immune Cell Activation

Experimental detachment caused an increase of the number of GSA lectin-stained cells in the nerve fiber layer of retinal wholemounts (Fig. 6B versus 6A). However, similar increases of the immune cell number were observed in the saline and sodium hyaluronate control groups (Fig. 6C), suggesting that the surgical procedure itself induced immune cell activation.

Effects of Suramin and PPADS

To approach an understanding of the mechanisms involved in the induction of reactive glial cell alterations as well as to search for potential therapeutic and preventive agents, the P2 receptor blockers PPADS (10 mM in 100 μL saline) or suramin (5 mM in 100 μL saline) were applied to both sides of several detached retinas, at the time of surgery. Neither of the blockers depressed the increased calcium responsiveness of Müller cells to ATP (200 μM) when the responses were recorded at 48 hours of detachment (Fig. 4C). However, suramin significantly ($P < 0.05$) inhibited the reduction of the potassium current density at 48 hours of detachment (Fig. 1B) and inhibited the slight increase of the cell membrane capacitance that was apparent at 48 hours of detachment (Fig. 1D). Finally, suramin application prevented the increase of the density of GSA lectin-stained immune cells in the nerve fiber layer (Fig. 6C). PPADS application had no effect on all parameters tested.

DISCUSSION

The results of the present study provide new information about the early reactive alterations of Müller glial cells after retinal detachment: (1) An increased ATP responsiveness, mediated by several subtypes of P2Y receptors, occurred in Müller cells within a few hours after experimental detachment, independent of the occurrence of PVR; (2) the increased ATP responsiveness was accompanied by a decrease in the inward potassium currents (Fig. 7); (3) when compared with results in long-term studies,[14–17] the decrease in inward potassium currents reached their maximum within 72 hours of detachment and remained unchanged thereafter, at least for 3 weeks of detachment; (4) the Müller cell hypertrophy, indicated by an increased membrane capacitance, began comparatively late (after >2 days) and increased further; and finally, (5) application of suramin (but not of PPADS) at the time of detachment inhibited the decrease of inward potassium currents but failed to reverse the elevated ATP responsiveness of Müller cells.

The present data suggest that vitreal manipulation during surgery induces an inflammatory response of the retina, as indicated by the increased number of immune cells in the nerve fiber layer. In contrast, Müller glial cells were not significantly activated by the surgical procedure, as indicated by the lack of alterations of the membrane current amplitude, of the cell membrane capacitance, and of the incidence of cells that responded to extracellular ATP. A transient surgery-induced microglia activation has been shown in other experimental models (e.g., in retinas of sham-operation control eyes of rats in experimental glaucoma studies).[39]

The present results indicate that, within hours of detachment, Müller cells begin to downregulate their potassium currents and to increase the responsiveness of their purinergic P2Y receptors. The data close a hitherto-existing gap in accumulating evidence[15,20,22,32,34,40,41] for the general rule that in Müller cells, the expression levels of potassium inward currents and ATP responsiveness are inversely correlated during development and retinal disease (Fig. 7). Because at a similar time course during experimental detachment, Müller cells show transient proliferative activity,[9] it can be assumed that the altered physiological features of Müller cells may support, besides other functions, their proliferation. Recent findings that ATP enhances precursor cell proliferation in the embryonic chick retina[42–44] suggest that P2 receptor-induced signaling may be generally involved in retinal glial cell proliferation and (de-) differentiation, both in ontogenetic development and during retinal injury.[20,21,45] Whether there are causal relationships between both features of dedifferentiated Müller cells (low expression level of potassium currents and high ATP responsiveness) and detachment-induced glial cell proliferation remains to be investigated in future experiments. The present result that the decrease in the potassium currents was inhibited by suramin but not the enhancement of the ATP responsiveness would favor a more correlative appearance of both features in reactive Müller cells.

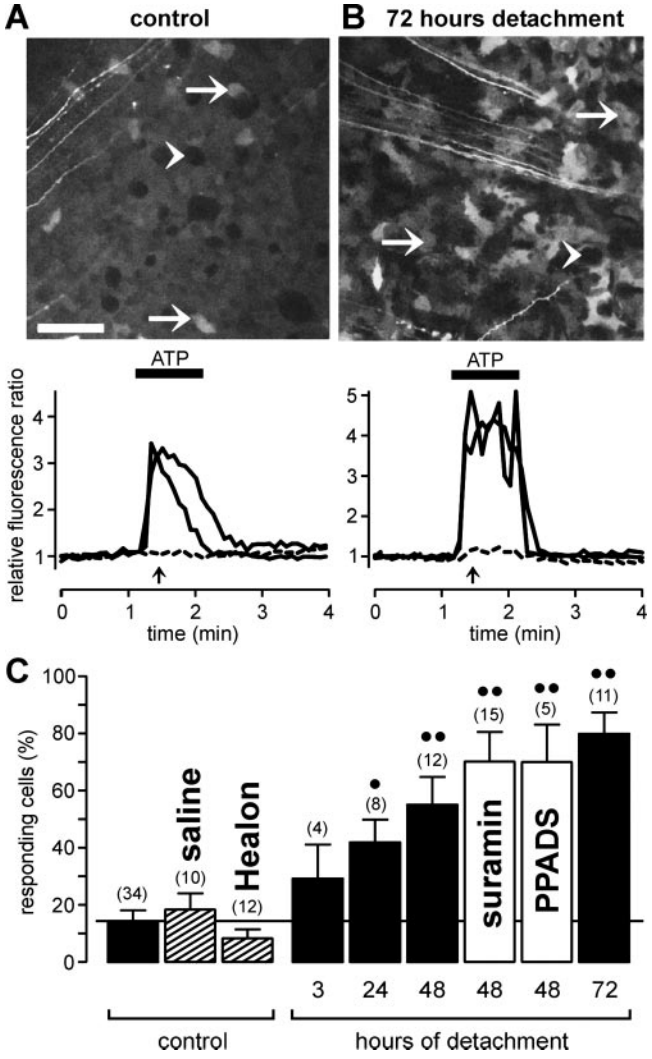

FIGURE 4. Experimental retinal detachment caused upregulation of calcium responses on extracellular ATP application in Müller cell end feet of retinal wholemounts. Confocal images were recorded by laser scanning microscopy at the vitread surface of retinal wholemounts (i.e., in the nerve fiber and ganglion cell layers. The images show neuronal somata (*dark circular shape*), dye-filled axon bundles, and end feet of Müller cells. (**A**) In a control retina, only a few end feet responded to application of ATP with a transient increase of the intracellular free calcium. (**B**) In a retina detached for 72 hours, significantly more end feet showed a calcium response on ATP. The images in (**A**) and (**B**) were recorded at the peak calcium responses (*arrows below traces*). Scale bar, 50 μm. *Traces*: time-dependent changes of the fluorescence emission at selected regions indicated in (**A**) and (**B**) by *arrows* (Müller cell end feet that showed calcium responses, *solid lines*) and *arrowheads* (neuronal somata that did not respond, *broken lines*), respectively. (**C**) Mean (±SEM) number of Müller cell end feet that showed calcium responses to ATP (200 μM), in dependence on the duration of the detachment. The number of responding end feet is expressed as a percentage of all end feet investigated (100%). The number of wholemounts investigated at each time point appears in *parentheses*. The saline and sodium hyaluronate (Healon; Pharmacia & Upjohn) controls were measured at 48 hours after application of 100 μL saline or sodium hyaluronate to the vitreal surface of undetached retinas. PPADS (10 mM in 100 μL saline) or suramin (5 mM in 100 μL saline) were applied to the subretinal space and the vitreous body near the detached retina at the time of surgery. Significant differences versus control: $\bullet P < 0.05$ $\bullet\bullet P < 0.01$.

The assumption that an autocrine–paracrine signaling loop, involving ATP release and P2 receptor activation,[46] may be involved in the intracellular signaling cascades leading to the decrease of potassium inward currents is also not supported by the results of our experiments using PPADS. The P2 receptor blocker PPADS was ineffective to inhibit the decrease of potassium currents (Fig. 1B), as well as cellular hypertrophy (Fig. 1D). However, this conclusion should be drawn with caution, as PPADS inhibited the P2Y receptor-mediated calcium responses by only ~25% (Fig. 5A), and as PPADS was shown to inhibit not all subtypes of P2Y receptors.[25] An alternative explanation of the inhibiting effects of suramin may be provided by its ability to block the binding of various different cytokines to their cell surface receptors.[26–29] This implies that the injury-induced alterations of Müller cells and immune cells may be caused by presently undetermined cytokines. Candidate molecules for such cytokines may be the fibroblast growth

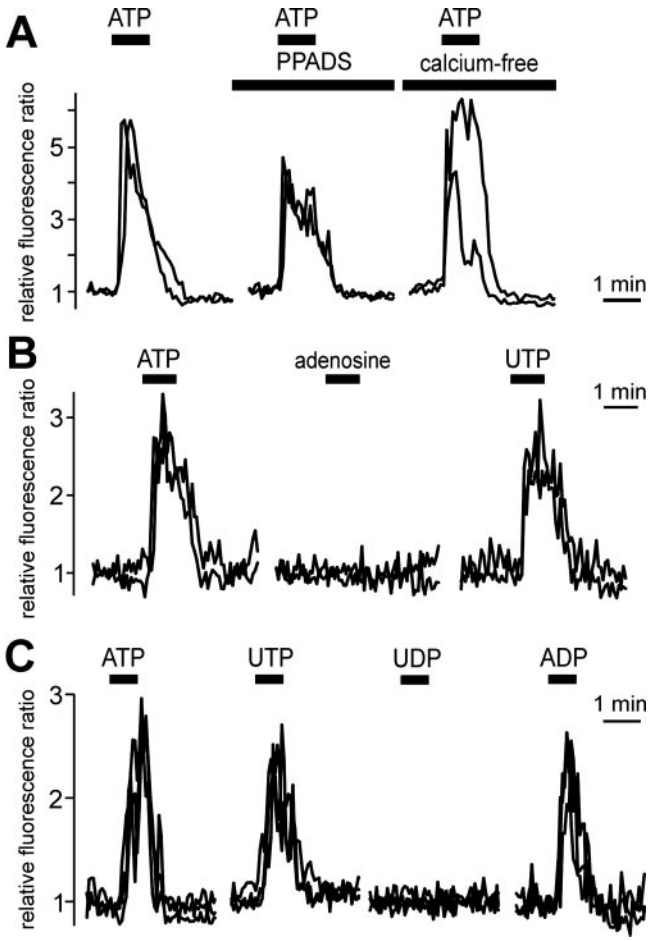

FIGURE 5. The ATP (200 μM)-evoked calcium responses in Müller cell end feet were mainly mediated by activation of metabotropic P2Y receptors. (**A**) The P2 receptor blocker PPADS (100 μM) decreased the ATP-evoked calcium response, and the response remained largely unaffected in extracellular calcium-free conditions. The blocker and the calcium-free solution, respectively, were perfused for 7 minutes before the second and third ATP applications. (**B**) Whereas UTP (200 μM) evoked similar calcium responses as ATP, the P1 receptor agonist adenosine (200 μM) had no effect on the intracellular free calcium of Müller cell end feet. (**C**) The P2 receptor agonists ADP and UTP evoked similar responses as ATP, whereas UDP had no effect. The substances were applied at 10 μM. Examples of fluorescence records of two and three end feet, respectively, in a control retina (**C**) and in retinas that were detached for 48 hours (**A**, **B**).

factors that it has been suggested are released into the retina within minutes after experimental detachment.[8]

Independent of its site of action, the present study suggests that application of suramin may be helpful in preventing some detrimental features of Müller cell gliosis during detachment, such as downregulation of potassium channels and cellular hypertrophy (as measured by cell membrane capacitance in isolated cells). It has already been mentioned that the loss of inward potassium channel activity must impair both retinal buffering of excess extracellular potassium ions and glial uptake (and thus, recycling) of glutamate, which together will aggravate excitotoxic neuronal cell death.[6,14–17,41] The importance of preventing Müller cell hypertrophy is supported by the observation that the subretinal fibrosis, caused by the growth of Müller cell processes through the outer limiting membrane, inhibits the regeneration of the outer photoreceptor segments during detachment.[47] Furthermore, the blocking effect of suramin on immune cell activation (Fig. 6C) may diminish detrimental effects of activated microglia on neuronal cell survival.[24] Earlier it had been shown that systemic application of suramin reduced the severity of experimental PVR.[30] However, further experiments are necessary to determine whether suramin also exerts beneficial effects in animals with vascularized retinas and whether detrimental side effects appear after prolonged application of the drug.

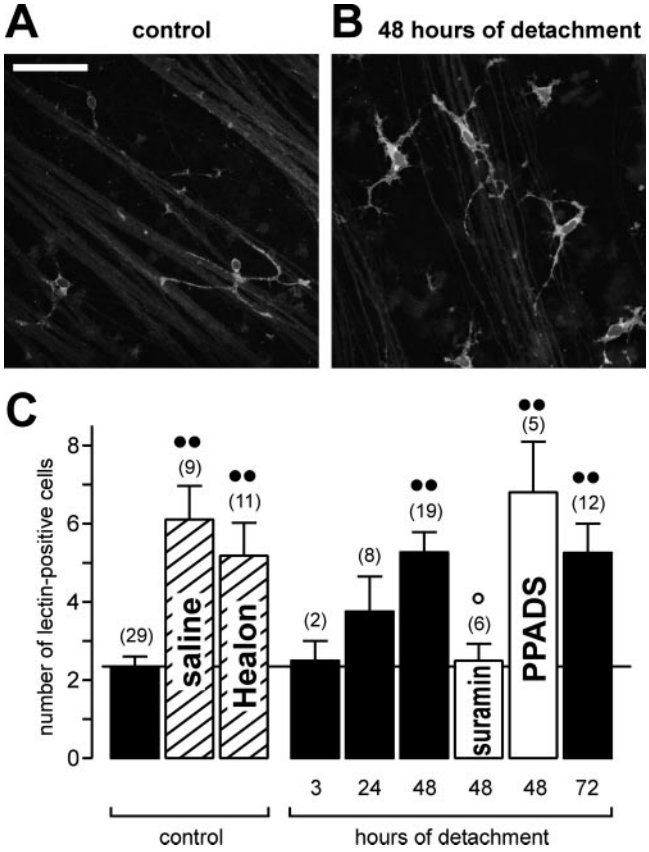

FIGURE 6. Number of GSA lectin–positive cells in the nerve fiber layer of rabbit retinas. (**A**) Image record of the vitreal surface (i.e., view into the nerve fiber layer) of a wholemount from a control eye. Scale bar, 50 μm. (**B**) The nerve fiber layer of a 48-hour detached retina displayed a higher density of GSA lectin–stained cells. Elongated structures are nerve fibers weakly stained with the calcium-sensitive dyes. (**C**) Mean ± SEM of GSA lectin–positive cells counted in such images (230 × 230 μm). Number of wholemounts investigated at each time point appears in *parentheses*. The saline and sodium hyaluronate (Healon; Pharmacia & Upjohn) controls were measured at 48 hours after application of 100 μL saline or sodium hyaluronate to the vitreal surface of undetached retinas. PPADS (10 mM in 100 μL saline) or suramin (5 mM in 100 μL saline) were applied into the subretinal space and the vitreous body near the detached retina at the time of surgery. Significant difference versus control: $\bullet\bullet P < 0.01$. Significant effect of the blocker: $\circ P < 0.05$.

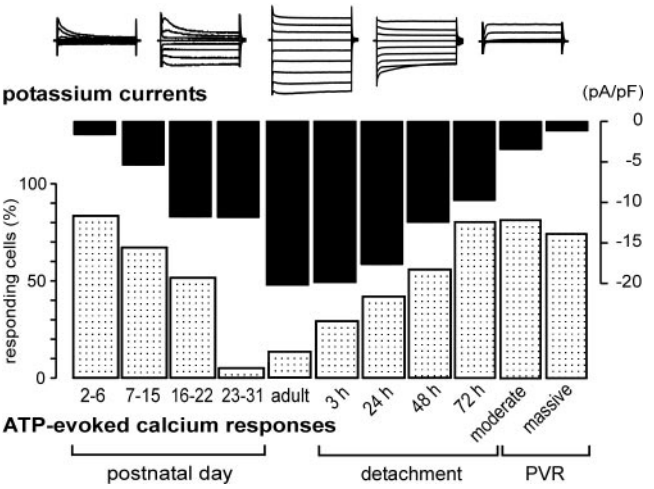

FIGURE 7. In rabbit Müller cells, the responsiveness to extracellular ATP was inversely related to the amplitude of the inwardly directed potassium currents. *Top*: alterations of the mean density of the whole-cell potassium currents (measured between the voltage steps to −100 and to −160 mV) dependent on postnatal age and retinal injury (experimental retinal detachment and PVR, respectively).[15,34] *Bottom*: alterations of the incidence of Müller cells that showed a calcium response to ATP (200 μM), dependent on postnatal age and retinal injury, respectively.[22,32]

Acknowledgments

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