RhoA Inactivation Prevents Photoreceptor Axon Retraction in an In Vitro Model of Acute Retinal Detachment

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Purpose. An early injury response to retinal detachment is disruption of synaptic connectivity between photoreceptors and second-order neurons. Most dramatic is the retraction of rod cell axons and their terminals away from the outer synaptic layer and toward their cell bodies. This study tested whether axonal retraction in detached retina was due to the activation of the small GTPase RhoA and was preventable using RhoA antagonists.

Methods. Retinal detachments were created in in vitro preparations of porcine eyecups. RhoA activation was determined with a Rhotekin binding assay. To block axon retraction, drugs were applied to neural retinal explants either before or after detachment from the retinal pigment epithelium. Presynaptic movement was quantified by image analysis of double-labeled retinas examined with confocal microscopy.

Results. Active RhoA increases transiently after detachment followed by morphologic evidence of axonal retraction over the next 24 hours. Pretreating the retina with a RhoA antagonist, CT-04, or a Rho kinase inhibitor, Y27632, at multiple concentrations significantly inhibited axonal retraction. Reducing calcium influx through L-type calcium channels with nicardipine also blocked retraction. To create a more plausible therapeutic scenario, drug treatments were delayed and applied after retinal detachment. The Rho kinase inhibitor, but not nicardipine, significantly blocked rod axonal retraction when applied up to 6 hours after detachment.

Conclusions. Thus, RhoA and downstream Rho kinase activity constitute part of the mechanism that produces rod axonal retraction in retinal explants. Treatments that manipulate RhoA signaling may promote synaptic stability after retinal detachment. (*Invest Ophthalmol Vis Sci.* 2011;52:579–587) DOI:10.1167/iovs.10-5744

Retinal detachment leads to the loss of photoreceptors and, consequently, vision. Using a variety of animal models, the first studies on detachment-induced retinal degeneration described early disruption of photoreceptor outer segments.[1–3] Later it was reported that even before the loss of outer segments, rod photoreceptor cells respond to detachment with retraction of their axon terminals into their cell bodies.[4,5] It is now known that this retraction results in the separation of the rod-bipolar synapse and the flattening of the synaptic invagination.[6] Cone photoreceptor cells respond to detachment by rounding of their synaptic terminals, also leading to loss of invaginations in the outer plexiform layer (OPL),[5,7] and by downregulation of protein expression.[8] Connections between cone and bipolar cells, however, remain.[6,9] Similar synaptic changes have been described in detached human retina.[10–12] The early rod and cone photoreceptor responses to retinal detachment put vision at risk because of the loss of normal photoreceptor synaptic connectivity and subsequent remodeling by inner retinal neurons.[9]

Surgical reattachment has been the major treatment for patients with retinal detachment; however, successful reattachment does not restore visual function in its entirety.[13,14] Retinal sensitivity, for instance, did not recover 5 years after surgery in one study,[14] even though restoration of normal rod outer segment length has been demonstrated 5 months after reattachment in primates.[15] Synaptic changes associated with detachment are thought to be a significant contributor to these poor outcomes.[16]

This study explores the mechanism for synaptic retraction observed in rod cells after detachment in the hope that preventing this plasticity will yield improved outcomes after reattachment. We have proposed that activation of the small GTPase RhoA leads to retraction of the photoreceptor axon and presynaptic terminal toward the cell body.[17] In neurons, RhoA is involved in the regulation of cytoskeletal structure (see Refs. 18, 19 for reviews). RhoA expression and activation contribute to axonal growth cone collapse and retraction by developing neurons in response to repulsive axon guidance cues[20,21] and to inhibition of neuronal growth in response to blockers of regeneration, such as Nogo, Mag, and oligodendrocyte-myelin glycoproteins.[22–25]

In the adult central nervous system (CNS), trauma affects RhoA homeostasis. Both RhoA expression and activity have been reported to increase after spinal cord and brain injury, peripheral nerve crush, epilepsy, and perhaps stroke in animal models.[25–28] Recently, an increase in RhoA expression has been demonstrated in human traumatic brain injury.[29] In the rat retina, there is a transient increase of RhoA and Rho kinase (ROCK) protein in response to glutamate neurotoxicity.[30] Thus, in the CNS, including the retina, RhoA activation is associated with injury.

The first evidence for the involvement of RhoA activity in photoreceptor synaptic plasticity came from studies using cultured salamander rod and cone cells. Activation of RhoA with lysophosphatidic acid (LPA) inhibited neuritic process growth of all photoreceptors, whereas retraction of rod cell axons, which occurs after cell isolation, was blocked by ROCK inhibition.[17] However, activation of RhoA in these cultures could

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not be directly determined, and retraction of axons was monitored only in single cells removed from the intact retina.

To examine the role of RhoA in retinal detachment, we used an in vitro model of retinal detachment in pig developed by Khodair et al.[31,32] in which the neural retina is maintained for 24 hours in culture after being detached from the underlying retinal pigment epithelium (RPE). In this model, porcine rod photoreceptors retract their axon terminals after detachment. In addition to displaying early detachment-induced synaptic plasticity, the pig eye has several features that make it a useful model for human retinal injury: the retina is a mixed rod-cone system; the pig eye is close in size and anatomic organization to the human eye; and the rod and cone axons and axon terminals resemble human photoreceptor terminals. The use of an in vitro model has allowed the systematic examination of multiple doses of RhoA drugs, at multiple time points, both before and after retinal injury.

METHODS

Animals

Twenty-two eyes from 11 male and female Yorkshire pigs, 3 to 5 months old, weighing 25 to 55 kg (Animal Biotech Industries, Danbora, PA), served as donors of retinal tissue. Animals were kept on a 12-hour light/12-hour dark cycle and fed porcine chow ad libitum. Animals were euthanized at 9 AM by intravenous injection of 30 mg/kg pentobarbital sodium and 30 mL heparin (1000 USP U/mL). Eyes were removed and placed on ice in 1× Dulbecco's phosphate-buffered salt solution (D-PBS; Cellgro; Mediatech, Manassas, VA), as soon as the animal was pronounced dead. Experimental procedures and methods of euthanatization were approved by the New Jersey Medical School Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Detachment Model

The anterior segment and vitreous body were removed, and 8-mm buttons of tissue were produced using a trephine. Because all regions of retina show quantitatively similar amounts of retraction after 24 hours,[31] both the superior and the inferior retina were used. Specimens were placed in six-well dishes in Eagle's minimum essential medium (MEM, 10370-021; Invitrogen, Carlsbad, CA) supplemented with 0.2 mg/mL glutamine, 5.5 mM D-glucose, 10 μg/mL porcine insulin, 1 mM pyruvate, 0.1 mM taurine, 2.0 mM L-ascorbic acid, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B, pH 7.4, at 4°C. For detachment, the neural retina was gently teased from the underlying RPE, choroid, and sclera and overlaid by 8-μm polycarbonate membrane inserts (Fisher Scientific, Pittsburgh, PA). The time from enucleation to culture was 30 minutes to 1 hour. For the final 24-hour incubation, 10% (vol/vol) fetal bovine serum (FBS; Gibco, Grand Island, NY) was added to the medium and specimens were incubated at 37°C. Medium was aerated with a humidified mixture of 5% CO_2/95% O_2 throughout.

Pharmacologic Treatments

Retinas were bathed in fortified MEM including 10% FBS, with Y27632 (C3912; Sigma), CT-04 (Cytoskeleton, Denver, CO), or nicardipine (Sigma, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO). Pretreatments and delayed treatments are described in the Results. Control retinal preparations were obtained from the same animal as the treatment groups but were incubated in fortified MEM (plus 10% FBS) only or with 1.6% DMSO.

Fluorescence Immunohistochemistry

Specimens were fixed in 4% paraformaldehyde in 0.125 M phosphate buffer (PB; pH 7.4) overnight, embedded in 32% gelatin (275 bloom gelatin; G8–500, reconstituted in 1× D-PBS; Fisher Scientific), fixed for an additional 24 hours at 4°C, and sectioned at 100 μm with a tissue chopper. Sections were immunolabeled as previously described[31] either with a rabbit polyclonal RhoA antibody (119; Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:100) or a mouse monoclonal antibody to SV2 (gift of Kathleen Buckley, Harvard Medical School) (diluted 1:20) followed by propidium iodide (Molecular Probes, Eugene, OR) diluted 1:1000 in PBS. Control sections were processed without primary antibodies.

RhoA Activation Assay

Neural retina, either attached or detached, was placed in a dark, humidified incubator at 37°C with fortified MEM with 10% FBS. At designated time points, retina was transferred to 250 μL ice-cold lytic RIPA solution (adapted from Ren et al.[33]; 50 mM Tris base, 150 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride). For attached retinas, the underlying RPE, choroid, and sclera were removed in the RIPA buffer. Retinal lysates were centrifuged for 10 minutes at 14,000 rpm at 4°C. Supernatant was removed and snap frozen while a Bradford protein assay (Bio-Rad, Hercules, CA) was performed. Equal amounts of protein were added to 25-μg aliquots of beads bound to Rhotekin rho binding domain (Cytoskeleton). The mixture was incubated for 1 hour at 4°C, then centrifuged for 3 minutes at 5000 rpm, 4°C, washed three times with buffer (50 mM Tris (pH 7.5~7.7), 40 mM NaCl, 30 mM $MgCl_2$), resuspended in 20 to 40 μL of 2× Laemmli loading buffer (Sigma), and stored at −20°C. RhoA activation assay samples and total retinal protein were analyzed by standard Western blot analysis techniques.

Morphometric Analysis

One-micron optical sections of retinas were obtained with a laser scanning confocal microscope (LSM-510; Carl Zeiss, Oberkochen, Germany) equipped with argon and helium/neon lasers and a 40×, 1.2 numerical aperture water-immersion objective lens. Brightness and contrast were set to obtain unsaturated images. Laser power and scan rate were unchanged throughout a single experiment. Enhancements in brightness and contrast were performed (Photoshop 7.0; Adobe, Mountain View, CA) only for presentation purposes. SV2 immunolabeling was analyzed as previously described[31] (MetaMorph Image Software, version 6.1; Molecular Devices) by counting the fluorescent labeling within a rectangular area, outlining the outer nuclear layer (ONL; see Fig. 1C in Ref. 31 and Fig. 3). Measurements are reported as pixels per micrometer of ONL length. Data were collected from two to four sections per specimen examining at least three different areas of each section and three optical levels for each area. Statistical analysis was performed with statistical analysis software (Sigma Stat, version 3; Systat, Chicago, IL) using one-way analysis of variance, with Student-Newman-Keuls' post hoc test for all pair-wise multiple comparisons. Data are expressed as mean ± SEM.

RESULTS

RhoA Localization

We have previously shown in salamander retina that RhoA is present in the inner and outer retina, including photoreceptors.[17] RhoA immunolabeling in pig retina has a similar distribution (Fig. 1A). RhoA was present in all retinal cell types. Labeling was in the ganglion cell layer, inner plexiform layer (IPL), and inner nuclear layer (INL). In the photoreceptor layers, RhoA was found predominantly in the outer plexiform layer (OPL; Fig. 1A, arrows) and inner segments of the photoreceptors (IS; Fig. 1A). However, labeling was also seen in the photoreceptor outer segments; this immunolabel was specific because there was no label with the secondary antibody in a negative control (Supplementary Fig. S1, http://www.iovs.org/

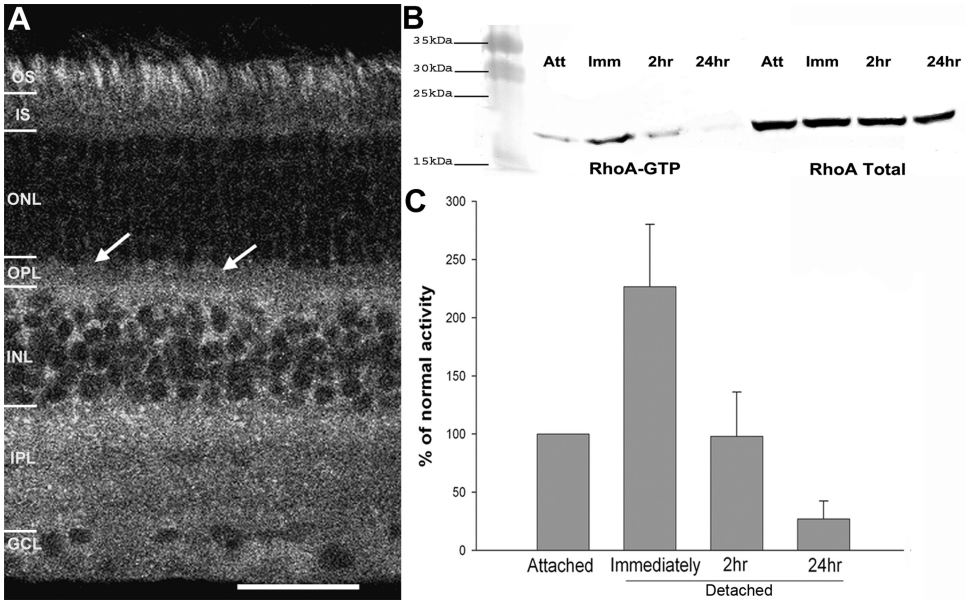

FIGURE 1. RhoA protein is found throughout the pig neural retina and is activated with detachment. (**A**) Confocal section (1 μm) immunolabeled for RhoA. RhoA label is lowest in the ONL, where the cell bodies of the photoreceptors are located, but is increased in the OPL (*arrows*), where the photoreceptor terminals lie and in the inner and outer segments of the photoreceptors (IS, OS). It is also present in the inner retina. GCL, ganglion cell layer. Scale bar, 20 μm. (**B**) Western blot from a RhoA activation assay (*left*) and from total retinal protein (*right*) from one animal. Total amount of RhoA protein (active and inactive) does not change with detachment. Att, attached retina; Imm, immediately; 2 hours and 24 hours after detachment. (**C**) Active RhoA was normalized to the attached retinas. Data for detached retinas came from retinal lysates collected immediately, 2 hours, or 24 hours after detachment. Immediately after detachment, there was more than a 200% increase in active RhoA that decreased thereafter. $n = 3$ animals.

lookup/suppl/doi:10.1167/iovs.10-5744/-/DCSupplemental). Thus, RhoA was found throughout the porcine retina.

RhoA Activation

To address the hypothesis that detachment activates RhoA resulting in photoreceptor synaptic retraction, a RhoA activation assay (adapted from Ren et al.[33]) was performed on attached and detached retina. The assay is based on the binding of the active guanosine triphosphate-bound form of RhoA to the downstream effector, Rhotekin; the inactive form, RhoA, bound to guanosine diphosphate, is unable to interact with Rhotekin.[34]

Assays were performed on 8-mm buttons of pig retina that were either apposed to the underlying RPE and choroidal and scleral layers or detached for approximately 1 minute, 2 hours, or 24 hours. Total and active RhoA was determined by Western blot analysis (Fig. 1B). Total RhoA remained constant before and after detachment; however, the proportion of active RhoA more than doubled immediately after detachment (Fig. 1C). Active RhoA dropped below attached levels 24 hours after detachment. Thus, retinal detachment led to a rapid, transient rise in active RhoA.

Morphologic Analysis of Retraction

To assess structural changes in synapses due to detachment, retinas were double labeled for nuclei and synaptic vesicles using propidium iodide (PI) and an antibody against synaptic vesicle protein 2 (SV2), respectively (Fig. 2). In normal retina, photoreceptor terminals were heavily labeled for SV2 in the OPL, where they make synaptic connections with bipolar and horizontal cells (Fig. 2, Imm). However, with increasing time after detachment, the distribution of the SV2 immunolabel changed. The amount of SV2 immunolabel that moves into the PI-labeled ONL was used to determine the extent of retraction of rod axon terminals. In attached retinas or immediately after detachment, very low levels of SV2 label were found in the ONL (~5.6 pixels/length of ONL; see Methods). In confirmation of a previous report on rod cell retraction in pig retina,[31] increased SV2 labeling was first evident in the ONL 2 hours after detachment, and immunolabel was prominent in the ONL 24 hours after detachment (Fig. 2; at 24 hours, an average of ~19–30 pixels of SV2 label/length of ONL).

As previously discussed, retraction is a property of rod cell spherules and axons; cone cell terminals may change shape but do not retract into the ONL.[5] For porcine cone terminals, a change in shape was evident 2 hours after detachment (Fig. 2, 2 hours), but some cone terminals could still be identified in the OPL even 24 hours after detachment.

Effect of Blocking the RhoA Pathway on Photoreceptor Axon Retraction

Two different treatments were used to block RhoA signaling after detachment. CT-04 is a cell-permeable form of C3 transferase from *Clostridium botulinum*, which inactivates RhoA through ADP ribosylation, interfering with the ability of RhoA to bind its downstream effectors. Y27632 inhibits ROCK, one of the downstream effectors of active RhoA, which we have shown in salamander photoreceptor cells to be active in axonal retraction of rod cells.

CT-04, RhoA Inhibitor

To ensure penetration of CT-04 into photoreceptor layers, 8-mm buttons of retinas detached or attached to RPE and underlying layers were preincubated with CT-04 for 4 hours at 4°C in serum-free medium. Activation assays showed that incubation with 5 μg/mL, but not 0.5 μg/mL, reduced the levels

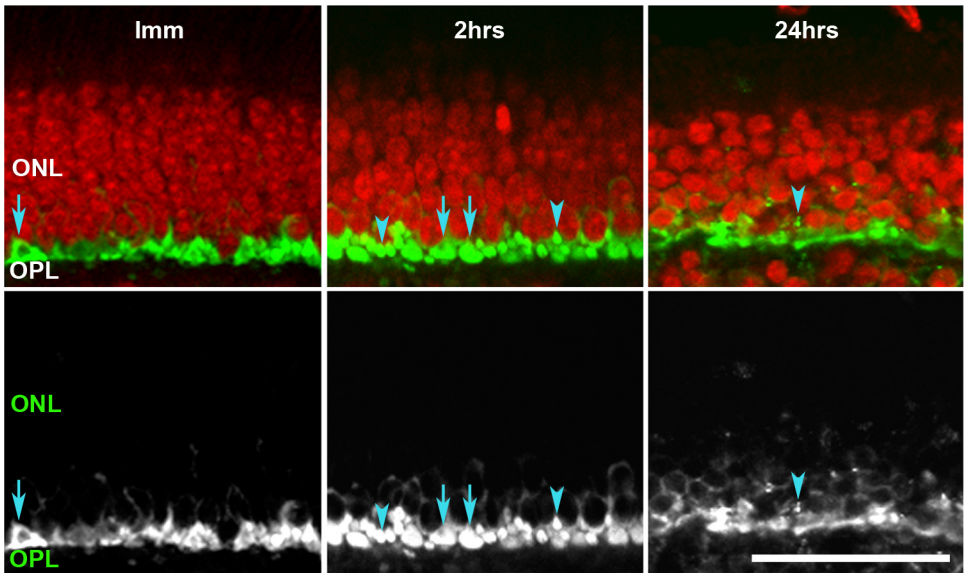

Figure 2. Localization of SV2 label changes with detachment. The ONL is defined with PI staining (*red*); SV2 labeling (*green*) highlights the OPL. The *green* (SV2) channel for each time point is shown monochromatically. Retinas 2 and 24 hours after detachment show increased SV2 label in the OPL compared with retinas examined immediately (Imm) after detachment. This movement of SV2 labeling into the ONL indicates retraction of rod axon terminals. In retina that has been detached for 2 hours, there is rounding of rod and cone terminals. After 24 hours, the terminals of photoreceptors are not easily distinguishable. 1 μm confocal sections. *Arrows*: cone cell terminals. *Arrowheads*: rod cell terminals. Scale bar, 20 μm.

of active RhoA (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5744/-/DCSupplemental); therefore the higher concentration was used. After the initial 4-hour preincubation with CT-04, all attached retinas were detached. Retinas were then either immediately fixed at the 4-hour time point or were maintained in medium containing 10% FBS and CT-04 for an additional 24 hours at 37°C. Control retinas followed the same procedures but excluded CT-04.

At the end of the 4-hour preincubation period, treated and untreated retinas had 6 to 9 pixels of label/length of ONL (n = 2 animals), an amount of immunolabel similar to that of attached retinas fixed immediately after dissection. Control retinas that were detached at the end of the 4-hour preincubation period and were maintained for 24 hours in vitro had an average of 18.5 ± 0.9 pixels of SV2 label/length of ONL. In contrast, treated retinas had an average of 4.7 ± 0.37 pixels of SV2 label/length of ONL (Figs. 3A, 3B). Control retinas that were detached during the preincubation period showed an average of 27.3 ± 2.2 pixels of immunolabel/length of ONL after 24 hours at 37°C (n = 2 animals). Treated retinas that had been detached during preincubation had an average of 5.9 ± 0.54 pixels of SV2 label/length of ONL (n = 2 animals). Thus, there was a significant reduction of SV2 label in the ONL with the CT-04 treatment in both cases, whether retinas were detached or attached during the 4-hour preincubation period.

Y27632, ROCK Inhibitor

Active RhoA interacts with ROCK, a serine threonine kinase, which phosphorylates the myosin binding site (MSB) of myosin light chain (MLC) phosphatase, MLC itself, LIM kinase, and CRMP.[35–42] Y27632 is an inhibitor of ROCK. The 8-mm buttons of retina attached to underlying RPE, choroid, and sclera were preincubated with 1, 10, or 100 μM Y27632 for 1 hour at 4°C. After preincubation, retinas were detached and incubated in medium containing 10% FBS with Y27632 for the next 24 hours at 37°C and then fixed (Figs. 4A, 4B). SV2 immunolabel in the ONL of untreated, control retinas was 33.1 ± 3.3 pixels/length of ONL. Retinas treated with Y27632 showed a significant reduction in the amount of SV2 label in the ONL at all doses compared with control (Fig. 4C). The reductions were linear with increasing Y27632 concentration. Inhibiting ROCK activity, therefore, effectively blocked retraction.

Effect of Blocking Calcium Channels on Photoreceptor Axon Retraction

In cardiac myocytes, RhoA can increase calcium influx through L-type channels.[43] L-type Ca^{2+} channels are present at the rod photoreceptor axon terminals.[44] Nicardipine, an L-type Ca^{2+} channel blocker,[45,46] has been successfully used to block retraction of axon terminals of isolated salamander rod photoreceptors.[44] Therefore, nicardipine was applied to detached porcine retina. Attached retinas were pretreated with 10 or 100 μM nicardipine for 1 hour at 4°C before being detached and transferred to medium containing 10% FBS and nicardipine. Retinas were maintained at 37°C for 24 hours before fixation. Control retinas showed an average of 19.1 ± 0.90 pixels of SV2 label/length of ONL. Both 10 and 100 μM nicardipine showed a significant reduction of SV2 immunolabel in the ONL. However, 100 μM nicardipine might have had some confounding effect because 10 μM nicardipine was more effective at reducing retraction (Fig. 5).

In summary, CT-04, Y27632, and nicardipine all caused a significant decrease in the movement of SV2 label into the ONL associated with retraction of rod cell axons during detachment. Morphologic examination suggested that the retinas were healthy after all treatments. There was no evidence of photoreceptor degeneration or apoptosis during the 24-hour experimental period. Some disorganization in photoreceptor layers, seen as a small decrease in the number of cell layers, was observed, which could indicate loss of photoreceptors; however, loosening of the packing of PI-labeled nuclei was also seen, suggesting instead that there is lateral spreading of the retina during culture. The apparent lack of photoreceptor cell death was consistent with previous work on this in vitro model of pig retinal detachment, which successfully maintained pig retina for up to 48 hours.[32]

Effect of Delaying Treatment on Photoreceptor Axon Retraction

The results using retinas pretreated with drug before detachment provide "proof of principle" for the effectiveness of targeting the RhoA signaling pathway to prevent rod axon retraction. However, pretreatment is not a viable therapeutic protocol. Thus, drug treatment 3 to 6 hours after detachment was examined. Y27632 and nicardipine were tested for the delayed treatments because RhoA activation was shown to

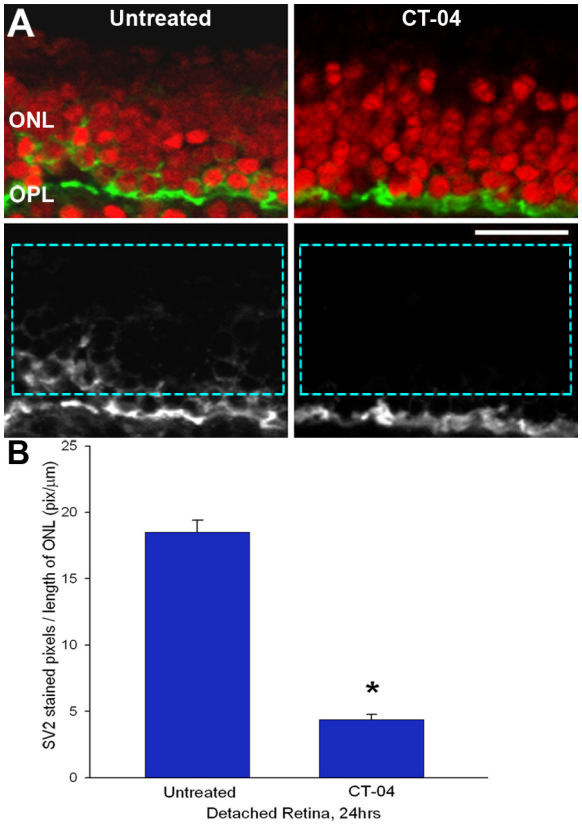

FIGURE 3. C3 transferase (CT-04, 5 μg/mL) reduced the movement of SV2 labeling in detached retinas. Retinas were pretreated with CT-04 for 4 hours at 4°C before detachment. (**A**) OPL and ONL 24 hours after detachment. PI staining (*red*) and SV2 immunolabel (*green*). SV2 label is also shown monochromatically (*bottom*; *dotted outlines* refer to areas analyzed for SV2 immunolabel). *Left*: in untreated retina, SV2 immunolabel is present in the ONL, indicating retraction of axon terminals into the cell body 24 hours after detachment. *Right*: retina treated with CT-04 shows dramatically less SV2 label in the ONL after detachment. Confocal sections (1 μm). Scale bar, 10 μm. (**B**) CT-04 treatment reduced the number of SV2-labeled pixels in the ONL. $^*P \leq 0.001$; $n = 3$ animals.

increase immediately after detachment in this in vitro animal model.

Retinas were detached, incubated in medium containing 10% FBS at 37°C for 3 or 6 hours, and then administered either 100 μM Y27632, 10 μM nicardipine, or Y27632 plus nicardipine. Control and delay-treated retinas were maintained at 37°C for 24 hours, from initial detachment, before fixation (Fig. 6A). Control retinas showed an average of 18.9 ± 1.7 pixels of SV2 label/length of ONL. Results for treatment delayed by 3 or 6 hours were similar (Figs. 6B, 6C). Application of 10 μM nicardipine was not effective in preventing axon retraction. However, either Y27632 alone or Y2732 plus nicardipine showed a significant reduction of SV2 immunolabel in the ONL. These results suggest that the stimulatory effect of calcium influx on axon retraction occurs early, whereas ROCK activity increases more slowly; its delayed inhibition, therefore, successfully reduced rod photoreceptor synaptic plasticity.

DISCUSSION

Structural change at the photoreceptor synapse occurs as an acute and destructive reaction to retinal detachment. Changes are most dramatic for the rod cells, which pull their axon terminals back toward their cell bodies. Electron microscopy has definitively demonstrated that retraction results in disruption of the rod-bipolar connection.[6] Unfortunately, reattachment does not reliably regenerate this synapse.[7,10] Thus, preserving the first synapse in the visual pathway by preventing the disconnection of rod terminals from bipolar dendrites is highly desirable and may prevent visual loss associated with detachment even after successful reattachment.

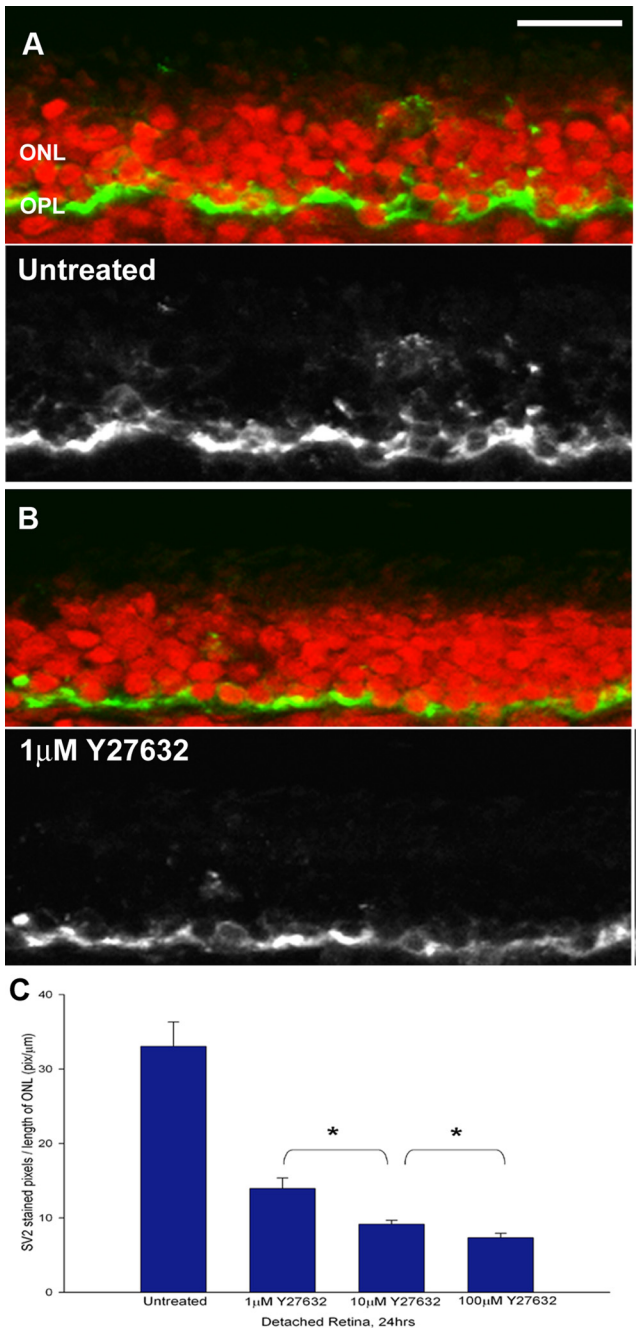

FIGURE 4. Y27632 treatment reduced SV2 immunolabel in the ONL. Retinas were maintained with or without Y27632 for 1 hour at 4°C before detachment. (**A**) In untreated retina, SV2 labeling is present in the ONL 24 hours after detachment. (**B**) Treatment with 1 μM Y27632 reduced the amount of SV2 labeling in the ONL. *Red*: PI staining; *green*: SV2 immunolabel. Monochromatic panels, *green* channel. 1 μm confocal sections. Scale bar, 10 μm. (**C**) Y27632 treatment significantly reduced SV2 in the ONL. The highest concentration, 100 μM, had the largest effect. $^*P \leq 0.001$; $n = 3$ animals.

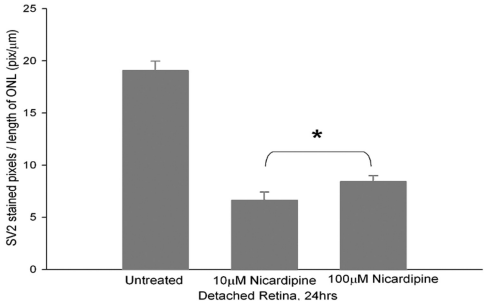

FIGURE 5. Nicardipine treatment reduced SV2 in the ONL. Quantitative analysis shows significant reduction of SV2 labeling with either 10 or 100 μM in comparison with untreated retinas. The 10-μM dose had a significantly larger effect than the 100-μM dose. $^*P \leq 0.001$; $n = 3$ animals.

The in vitro model of porcine retinal detachment used in the present study mimics the morphologic changes, rounding, and rod axon retraction, seen in the rod and cone cell terminals of in vivo animal models and in human retinal detachment.[9,11,12] Isolated porcine retina also shows the characteristic increase in glial fibrillary acidic protein (GFAP; unpublished data, J.-F. Wang, E. Townes-Anderson, 2010) seen after in vivo detachment.[47]

We have demonstrated that detachment of porcine neural retina from underlying RPE in vitro leads to RhoA activation. Treating detached retina with the RhoA and ROCK inhibitors C3 transferase and Y27632, respectively, had an inhibitory effect on axon terminal retraction from the OPL. Using nicardipine, an L-type Ca^{2+} channel blocker, also significantly reduced rod cell axon retraction. Thus, RhoA and calcium are most likely involved in the retraction of mammalian rod axons and terminals after detachment. Calcium blockage and ROCK inhibition have also been shown to reduce rod axon retraction in isolated salamander photoreceptors.[17,44]

It is possible that RhoA and Ca^{2+} both affect myosin light chain (MLC) and, thus, actomyosin contraction (Fig. 7). ROCK phosphorylates MLC and the myosin-binding site (MBS) of myosin phosphatase, thereby increasing levels of phosphorylated MLC and inducing actomyosin contraction and presumably retraction (see Ref. 19 for review). Influx of calcium has been shown to bind and activate calmodulin, which in turn activates MLC kinase (see Ref. 48 for review). MLC kinase phosphorylates MLC, leading to retraction. Thus, both ROCK and Ca^{2+} can cause an increase in MLC phosphorylation. However, if one looks at the percentage decrease of SV2 labeling in the ONL, it appears that blocking Ca^{2+} channels with nicardipine is less effective than blocking RhoA-ROCK activity (65% reduction of staining in the ONL with nicardipine vs. 75% and 79% reduction with CT-04 and Y27632, respectively). In addition, delayed treatment indicated that the timing of ROCK and Ca^{2+} signaling is different, with calcium signaling more quickly stimulating actomyosin contraction than ROCK. It is possible that retinal detachment, which produces a spreading depression, opens calcium channels immediately after detachment,[49] accounting for the rapid timeline of Ca^{2+} signaling.

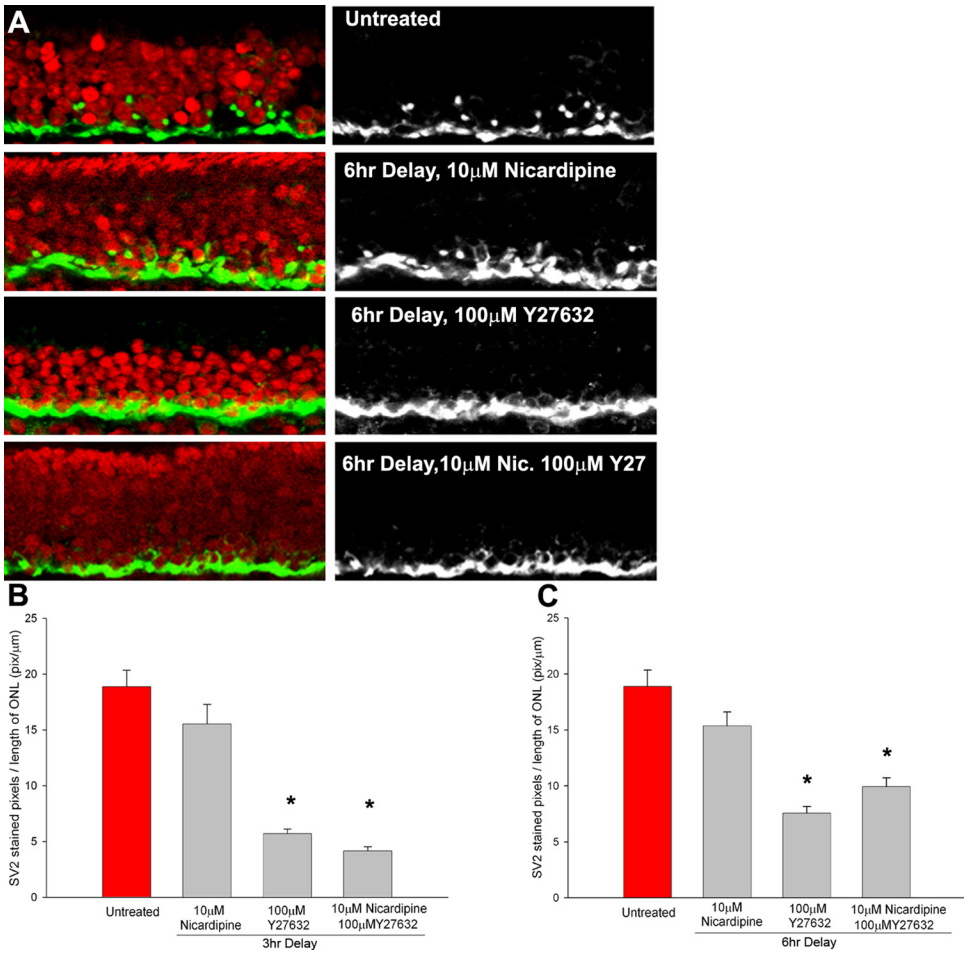

FIGURE 6. Delayed treatment with Y27632 significantly reduced the level of SV2 in the ONL. (**A**) SV2 labeling observed in the ONL 24 hours after detachment (untreated) is reduced in retinas treated with 100 μM Y27632 and Y27632 + nicardipine 6 hours after detachment. *Red*: PI; *green*: SV2 immunolabel. Monochromatic panels are for the *green* channel. (**B**) Quantitative analysis of retinas that received treatment 3 hours after detachment. Y27632 and Y27632 + nicardipine, but not nicardipine alone, significantly reduced SV2 levels in the ONL compared with untreated detached retinas. (**C**) Y27632 and Y27632 + nicardipine significantly reduced SV2 levels in the ONL even after 6 hours of delayed treatment. The addition of nicardipine to Y27632 did not make a statistical difference to the effect at either 3 or 6 hours. $^*P \leq 0.001$; $n = 3$ animals.

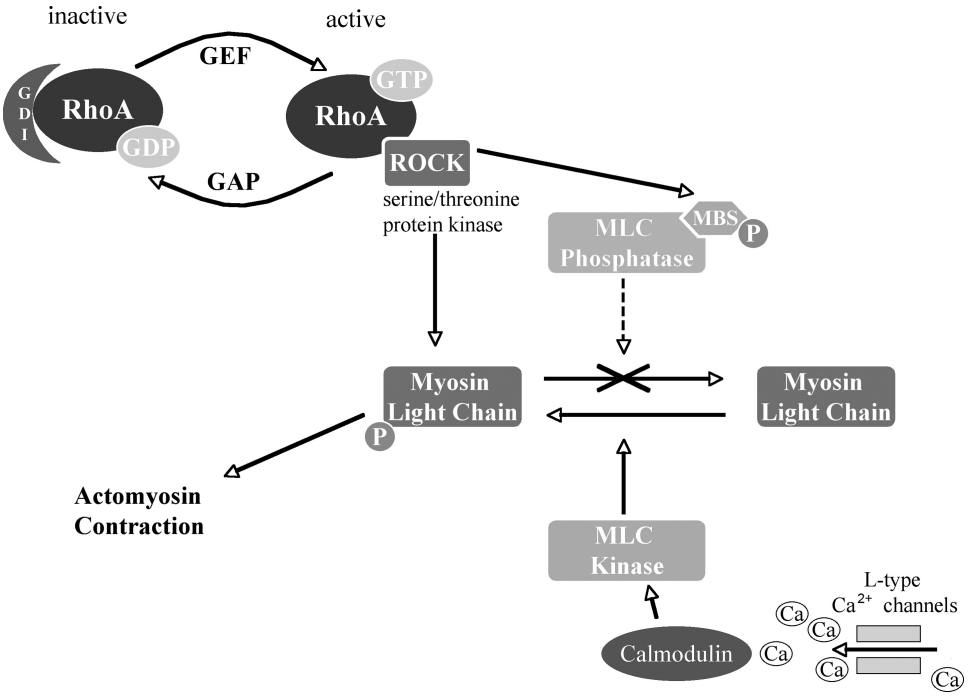

FIGURE 7. Diagram of the suggested signaling pathways involved in rod cell axonal retraction. GDI, guanine nucleotide dissociation inhibitor; GEF, guanine exchange factor; GAP, GTPase-activating protein.

Previous research on porcine retina showed that retraction due to detachment could also be reduced and modulated by the application of cAMP analogues or forskolin, which stimulates adenylyl cyclase.[32] Although cAMP has been shown to increase Ca^{2+} influx in some neurons, in rod photoreceptors it is thought to reduce L-type channel activity.[50] Additionally, cAMP, through PKA, has been shown to phosphorylate RhoA at serine 188.[51] This phosphorylation blocks the ability of RhoA to bind its downstream effector, ROCK. Interestingly, this phosphorylation has been shown to interfere specifically with the interaction between RhoA and ROCK but not with other RhoA downstream effectors such as mDia and protein kinase N.[52] Thus, cAMP may reduce retraction by both reducing L-type channel activity and blocking the RhoA-ROCK interaction.

Sakai et al.[53] have shown that one of the causes of loss of outer segments and synaptic connectivity after detachment is hypoxia. These changes can be largely prevented with exposure to O_2 levels above normal room level immediately after detachment. Hyperoxia (O_2 70% of chamber air) produced a radical improvement in retinal morphology and reduced retraction of photoreceptor terminals. How might O_2 rescue photoreceptors and stop retraction? In pig pulmonary artery endothelial cells, RhoA activity has been linked to oxygen levels.[54] Endothelial cells responded to low and high levels, respectively, of O_2 by activating and inactivating RhoA. Low levels of O_2 led to a reduction of Rac1 activity but an increase of RhoA activity. When O_2 was increased, Rac1 activity increased and RhoA activity was inhibited. This may explain how increasing O_2 reduces axon retraction.

We observed a transient rise in RhoA activation immediately after detachment and a drop in activation after 2 and 24 hours. Retraction begins within minutes to hours after detachment[31] but is not maximal until approximately 24 hours after detachment. There appears to be a lag between RhoA activation and completion of retraction. Previous work in mouse neuroblasts and embryonic fibroblasts found that RhoA activation, stimulated by LPA, also occurs rapidly, within minutes after stimulation, and drops quickly.[55,56] In contrast, the activation of ROCK does not follow immediately after RhoA activation, and downstream myosin II motor activity is prolonged.[56] Shifting time lines as the cascade moves toward MLC phosphorylation may explain why the peak of retraction occurs as much as 24 hours after RhoA activation.

The apparent slow development of activity in the RhoA-ROCK pathway leading to retraction may allow for effective treatment of synaptic remodeling by rod, and perhaps also cone, photoreceptors in detached retinas and suggests that the RhoA pathway is a reasonable target for drug development. To our knowledge, this is one of the first demonstrations that delayed treatment can prevent a detachment-induced photoreceptor change, specifically rod terminal retraction. Most previous studies have used protocols in which therapies were applied concomitantly with or as much as 2 weeks ahead of the detachment.[53,57] Delayed treatment is clearly more clinically applicable. There are several drugs that target the RhoA pathway, some already approved for use in clinical trials for other CNS injuries.[58–61] These approved drugs could be tested in animal models of retinal detachment. Further, increased understanding of the RhoA pathway may yield additional therapeutics for application to retinal injury and degeneration.

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