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# The Translocation of Signaling Molecules in Dark Adapting Mammalian Rod Photoreceptor Cells is Dependent on the Cytoskeleton

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In vertebrate rod photoreceptor cells, arrestin and the visual G-protein transducin move between the inner segment and outer segment in response to changes in light. This stimulus dependent translocation of signalling molecules is assumed to participate in long term light adaptation of photoreceptors. So far the cellular basis for the transport mechanisms underlying these intracellular movements remains largely elusive. Here we investigated the dependency of these movements on actin filaments and the microtubule cytoskeleton of photoreceptor cells. Co-cultures of mouse retina and retinal pigment epithelium were incubated with drugs stabilizing and destabilizing the cytoskeleton. The actin and microtubule cytoskeleton and the light dependent distribution of signaling molecules were subsequently analyzed by light and electron microscopy. The application of cytoskeletal drugs differentially affected the cytoskeleton in photoreceptor compartments. During dark adaptation the depolymerization of microtubules as well as actin filaments disrupted the translocation of arrestin and transducin in rod photoreceptor cells. During light adaptation only the delivery of arrestin within the outer segment was impaired after destabilization of microtubules. Movements of transducin and arrestin required intact cytoskeletal elements in dark adapting cells. However, diffusion might be sufficient for the fast molecular movements observed as cells adapt to light. These findings indicate that different molecular translocation mechanisms are responsible for the dark and light associated translocations of arrestin and transducin in rod photoreceptor cells. Cell Motil. Cytoskeleton 65: 785–800, 2008. 

**Key words: vision; arrestin; transducin; G-protein; light adaptation; molecular movements**

## INTRODUCTION

The vertebrate visual system is tuned to operate over a wide range of light intensities that spans >10 orders of magnitude [Rodieck, 1991]. In vertebrates, two types of photoreceptor cells (rods and cones) achieve that functionality range by different sensitivities and by

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adaptation mechanisms. In particular, rods have the sensitivity to respond to a single photon, but saturate at brighter light levels when cones take over light detection. Both types of photoreceptor cells are highly polarised sensory neurons which consist of morphologically and functionally specialized cell compartments (see schemes in Fig. 1). The photoreceptor cell body (perikaryon) gives rise to two polarized extensions, one proximal (axon and synaptic pedicle) and one distal (inner and outer segments). The inner segment compartment houses all cellular organelles responsible for molecular synthesis and maintenance of the cell. It is linked via a non-motile connecting cilium to the light sensitive outer segment. At the hundreds of stacked membrane disks in the outer segment all components of the visual transduction cascade are arranged. All intracellular intersegmental exchange between outer and inner segment compartments occurs through the narrow connecting cilium [Roepman and Wolfrum, 2007]. Incorporated in discs of the outer segment photoexcited rhodopsin activates the visual heterotrimeric G-protein transducin ($G_t$) mediating cGMP hydrolysis through the phosphodiesterase and closing of cGMP-gated channels in the plasma membrane of the outer segment [Burns and Arshavsky, 2005]. Closure of these cation channels leads to a drop of the circulating cationic current, resulting in the hyperpolarization of the cell membrane [Molday and Kaupp, 2000]. Termination of the light signal is characterized by rapid phosphorylation of photoactivated rhodopsin (R*) and the binding of arrestin which prevents further interaction of R* with $G_t$.

In order to prevent rod photoreceptor cells from over activating the visual signal transduction under intense light, adaptation is necessary. Light driven translocations of signalling molecules have been suggested to contribute to photoreceptor cell adaptation in vertebrates and invertebrates [Sokolov et al., 2002; Lee and Montell, 2004; Strissel et al., 2004; Calvert et al., 2006; Slepak and Hurley, 2008]. In vertebrate rod photoreceptor cells, arrestin and transducin undergo massive light dependent translocations into and out of the outer segment. This phenomenon was first noted about two decades ago [Broekhuyse et al., 1985; Philp et al., 1987; Brann and Cohen, 1987; Whelan and McGinnis, 1988] and has recently developed into an exciting and active field in photoreceptor cell biology [for review, see Calvert et al., 2006; Slepak and Hurley, 2008]. Within minutes of sufficient illumination 80% of transducin moves from the outer segment to the inner segment of rod photoreceptor cells. In contrast, the return of transducin subunits to the outer segment in the dark takes much longer [Sokolov et al., 2002; Elias et al., 2004]. Reciprocal to transducin translocations, arrestin moves during dark adaptation into the inner segment and during light adaptation into the outer segment. The adjustment of the protein composition in the outer segment may optimize the sensitivity and efficiency of photoresponses as the ambient light conditions change during the diurnal cycle [Strissel et al., 2004].

While the functional role of stimulus dependent movements of signalling molecules and the light induced trigger of the movements are starting to be decrypted [Chen, 2005; Calvert et al., 2006; Strissel et al., 2006; Lobanova et al., 2007], the molecular and cellular mechanisms governing these adaptive movements through the cytoplasm remained still elusive [Calvert et al., 2006; Slepak and Hurley, 2008]. Two principle distinct mechanisms are possible by which signalling proteins could change their cellular compartment: by diffusion or by active transport mediated by molecular motors along actin filaments or microtubules.

Recent studies suggesting molecular diffusion as the major force for driving the intersegmental exchange of arrestin and transducin [for review, see Calvert et al., 2006; Slepak and Hurley, 2008]. Nevertheless, there is liable evidence for a role of the cytoskeleton in these translocations [Marszalek et al., 2000; McGinnis et al., 2002; Giessl et al., 2004a; Nair et al., 2004; Strissel et al., 2004; Kerov et al., 2005; Nair et al., 2005a; Peterson et al., 2005]. In the present study, we investigated by cytoskeletal drug treatments of mouse retinas the role of actin filaments and microtubules in the translocations of arrestin and transducin in rod photoreceptor cells. Organotypic retina cultures containing fully differentiated photoreceptor cells were used [Reidel et al., 2006], to ensure the application of cytoskeletal drugs to retinal photoreceptor cells. Our analyses revealed that during dark adaptation actin filaments and microtubules are required for the compartmental redistribution of arrestin and transducin. Although the delivery of arrestin within the photoreceptor outer segment was slightly impaired in the absence of outer segment microtubules, the translocation of both signalling proteins was not dependent on actin filaments and microtubules during light adaptation.

## MATERIALS AND METHODS

### Animals

C57BL/6J mice and homozygote myosin VIIa null shaker-$1^{4626SB/4626SB}$ mice were maintained on a cycle of 12 h of light (200 lux) and 12 h of darkness, with food and water ad libitum [Gibson et al., 1995]. Statements of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Vision and Ophthalmic Research and the national and institutional guidelines for animal care were followed.

### Retina Culture

The elaboration of the retina culture system was previously described in [Reidel et al., 2006]. Briefly, after Proteinase K (Sigma-Aldrich, Taufkirchen, Germany) digestion retinas and the attached pigmented epithelia were dissected from postnatal day 12–14 mouse eyes under removal of sclera, ocular tissue and the hyaloid vessel. Specimens were cultured in Dulbecco's Modified Eagle's Medium with F12 supplement (DMEM-F12) and 10% fetal calf serum, L-glutamine, penicillin and streptomycin (Sigma-Aldrich) on ME 25/31 culture membranes (Whatman, Dassel, Germany) at 37°C with 5% $CO_2$.

### Light Adaptation

Retinas were cultured for at least 1 day at 12 h light (200 lux) and 12 h dark before the start of the experimental light conditioning. For light adaptation studies, cultured retinas were dark adapted for 4 h and then exposed to 200 lux of light for 30 min by light emitting diodes (LED). The white light LEDs used in this study had a broad ranging spectrum from around 400–700 nm wave length peaking at 580 nm. For dark adaptation studies, cultured retinas were exposed to light of 200 lux for 60 min before darkening. For in vivo experiments mice were adapted to light intensities of 400 lux.

### Application of Cytoskeletal Drugs

Drugs were dissolved in 1% DMSO and added to the retina culture medium in following final concentrations were used: cytochalasin D (Sigma-Aldrich) and phalloidin (Sigma-Aldrich) 10 μM; thiabendazole (Fluka, Germany) 1.5 mM; taxol 40 μM; blebbistatin (Sigma) 100 μM. With regard to published data [e.g. Nair et al., 2004; Rosenblatt et al., 2004; Sawin and Snaith, 2004] and own results from preliminary empiric experiments on retina culture we generally pre-incubated the organotypic retina cultures with the drugs for 30 min prior to the light conditioning paradigm. In the case of drug treatments showing no effect on the molecular transport processes, we also extended treatment times. We extended the pre-incubation time to 120 min for blebbistatin. For treatments with taxol and phalloidin we treated the retina for up to 3 h. But the molecular translocation of transducin and arrestin was not effected. Controls were incubated with 1% DMSO under the same temporal conditions.

### Antibodies and Fluorescent Dyes

Affinity-purified polyclonal rabbit antibodies against the α-subunit ($G_t\alpha$) and the β-subunit ($G_t\beta$) of transducin were obtained from Biomol Research Laboratories, (PA) and Santa Cruz Biotechnology, respectively. Mouse antibodies directed against arrestin (MAb 3D1.2), previously characterized in [Nork et al., 1993] were applied on mouse retina slices. Monoclonal mouse antibodies against α-tubulin (clone DM 1A) and rhodamine-labelled phalloidin were purchased from Sigma-Aldrich. The monoclonal antibody against chicken gizzard actin (clone C4) has been previously characterized and successfully used in immunoelectron microscopy [Lessard, 1988; Wolfrum and Schmitt, 2000].

### Fluorescence Microscopy

Eyes of control mice were cryofixed in melting isopentane, cryosectioned and treated as previously described [Wolfrum, 1991; Reiners et al., 2003]. Cultured retinas were fixed embedded and sectioned as described in Reidel et al. [2006]. Secondary antibodies were purchased as conjugates to Alexa 488 or Alexa 568 (Molecular Probes). Sections were mounted in Mowiol 4.88 (Farbwerke Hoechst, Frankfurt, Germany), containing 2% n-propyl-gallate. No reactions were observed in control sections. Mounted retinal sections were examined with a Leica DMRP microscope. Images were obtained with a Hamamatsu ORCA ER charge-coupled device camera (Hamamatsu, Germany) and processed with Adobe Photoshop (Adobe Systems, USA).

### Immunoelectron Microscopy

Fixation, embedding and further handling of mouse retinal samples for immunoelectron microscopy were performed as previously described [Wolfrum and Schmitt, 2000]. Monoclonal antibodies directed against α-tubulin and actin were applied to ultrathin sections of mouse retina. Nanogold-labeling was silver-enhanced according to [Danscher, 1981]. After counterstaining with 2% aqueous uranyl acetate (Sigma-Aldrich), sections were analyzed in a FEI Tecnai 12 BioTwin transmission electron microscope (The Netherlands), imaged with a SCCD SIS MegaView III camera (Münster, Germany) and digital images were processed with Adobe Photoshop.

## RESULTS

### Light Dependent Translocation of Arrestin and Transducin in Organotypic Cultures of the Mouse Retina

In the first set of experiments, we validated whether explanted and cultured retinas remained in a physiological state suitable for the analysis of light driven molecular movements [Reidel et al., 2006]. For this purpose, we compared protein movements in retinas from mice to protein movements in cultured mouse retinas after complete dark or light adaptation (Fig. 1). We

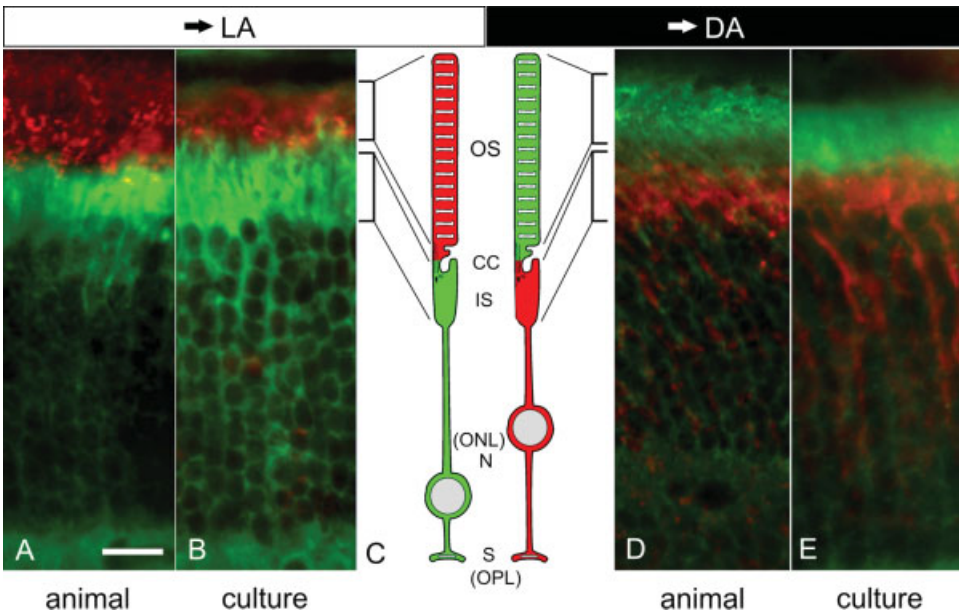


Fig. 1. Light dependent translocations of transducin and arrestin in rod photoreceptor cells in vivo and in the organotypic retina culture. Immunohistochemical localization of arrestin (red) and transducin (green) in a retina of a light adapted mouse (**A**), in a light adapted cultured mouse retina (**B**), in a retina of a dark adapted mouse (**D**) and in a dark adapted cultured mouse retina (**E**). Schemes of arrestin (red) and transducin (green) localizations in light adapted rod photoreceptor cells and in dark adapted rod photoreceptor cells (**C**). There were no differences between in situ localization and the localization of both signalling proteins in the retina culture. In the dark, arrestin was localized in the inner segment (IS) and in the perinuclear cytoplasm of photoreceptor cells and transducin was present in their outer segment (OS). After light adaptation, arrestin was localized in photoreceptor OS and transducin was found in IS, in the perinuclear cytoplasm and in the synaptic region (S) of photoreceptor cells localized in the outer plexiform layer (OPL) of the retina. Photoreceptor nuclei (N) are located in the outer nuclear layer (ONL). CC: connecting cilium; experimental conditions: → LA: dark to light adaptation; → DA: light to dark adaptation. Scale bars: 13.2 µm.

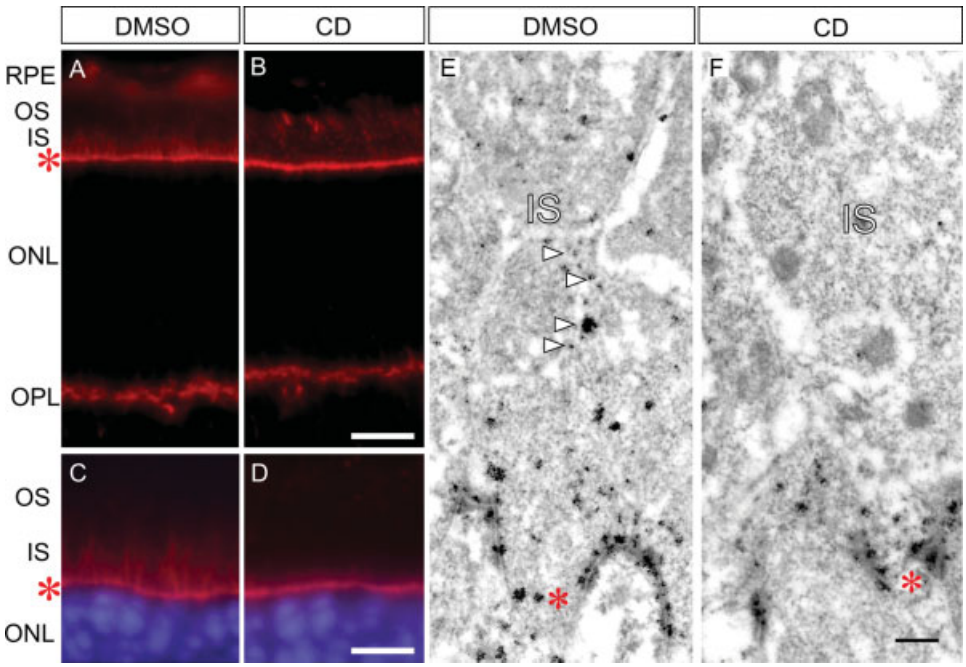


Fig. 2. Subcellular localization of actin filaments in retinal photoreceptor cells after cytochalasin D treatment. Rhodamine-phalloidin staining (red) of actin filaments in a cultured retina incubated with DMSO as a negative control (**A, C**) and in a cultured retina treated with cytochalasin D (CD) (**B, D**). After CD treatment, actin filament bundles present in photoreceptor inner segments (IS) and in microvilli fringes of Müller cells disappeared. In contrast, actin filaments localized at the cell-cell adhesions in the outer limiting membrane (asterisks) and in synapse region of the outer plexiform layer (OPL) and were not affected by CD. Note in C and D DNA was counter stained with DAPI to label photoreceptor nuclei (blue). **E** and **F:** Anti-actin immunoelectron microscopy analyses of ultrathin sections through the IS of photoreceptor cells. (E) Control incubation with DMSO. (F) Treatment with CD. Silver enhanced immunogold labelling visualized actin at cell-cell adhesions in the outer limiting membrane (asterisks) and actin filaments in photoreceptor IS (arrow heads). The actin filaments in the IS disappeared after CD treatment. OS: outer segment; N: nucleus; S: synapse. Scale bars: B: 13.2 µm; D: 7.5 µm; F: 150 nm.

did not observe differences at endpoints (>3 h) of arrestin and transducin translocations in in vivo and ex vivo adapted retinas. Immunohistochemical analysis showed that during light adaptation arrestin moved to the outer segment while transducin translocated into the inner segment under both experimental conditions (Figs. 1A–1C). After dark adaptation, both molecules redistributed as expected; arrestin to the inner segment and transducin to the outer segment (Figs. 1C–1E). These findings allowed us to use the organotypic retina culture as an ex vivo model of fully differentiated photoreceptor cells amenable to pharmacological treatments.

### Participation of Actin Filaments in Light Driven Molecular Translocations of Arrestin and Transducin

To determine whether the actin cytoskeleton contributes to the light dependent arrestin and transducin movements we treated cultured mouse retinas with cytochalasin D. Cytochalasins are membrane permeable mycotoxins, which disrupt actin filaments (F-actin) and inhibit actin polymerization leading to the inhibition of F-actin dependent processes in cells [Casella et al., 1981]. Cytochalasin was chosen over other actin depolymerizing drugs, because it has previously been shown to successfully disrupt the actin cytoskeleton in retinal cells [O'Connor and Burnside, 1981; Williams et al., 1988; Vaughan and Fisher, 1989; Hale et al., 1996; Peterson et al., 2005].

In DMSO control-treated explanted mouse retinas, F-actin labelling with rhodamine-coupled phalloidin in retinal cryosections showed a distinct staining pattern of F-actin rich structures at the synaptic region, the outer limiting membrane, the microvilli-like extensions of apical membrane of the Müller glia cells and actin filament bundles in the inner segments of the photoreceptor cells (Figs. 2A and 2C), as previously described [Woodford and Blanks, 1989]. After cytochalasin D treatment (10 μM in DMSO), the phalloidin-staining persisted in the outer plexiform layer and in the outer limiting membrane where actin filaments are present in adherens junctions between photoreceptor cells and Müller glia cells (Figs. 2B and 2D) [Williams et al., 1990]. However, the F-actin staining as previously described by Williams et al. [1990], disappeared from the apical extensions of the Müller glia cells and photoreceptor cell inner segments (Figs. 2B and 2D). The results were confirmed by analysis of anti-actin immunogold decorated filamentous structures in electron microscopy (Figs. 2E and 2F). While the actin bundles at the adherens junctions of the outer limiting membrane did withstand this drug treatment, actin staining in photoreceptor inner segments was significantly reduced. We did not collect further data on the preservation of actin filaments in the connecting cilia of photoreceptor cells after treatments with cytochalasin D in our present study. However, previous independent studies indicated that the ciliary actin filaments are depolymerised as a result of cytochalasin D treatments [Williams et al., 1988; Hale et al., 1996]. These depolymerizing effects of cytochalasin D on actin filaments in the inner segment and the ciliary region are expected to significantly impair F-actin-dependent molecular mechanisms in the inner segment and the cilium of photoreceptor cells.

Next, we investigated the influence of cytochalasin D treatments on the light dependent translocations of arrestin and transducin. Application of 10 μM cytochalasin D in DMSO to dark adapted cultured retinas prior to illumination had no visible effect on translocations of arrestin and transducin (Fig. 3). During light adaptation of explanted retinas both molecules translocated as in DMSO treated controls; arrestin moved into the outer segment and transducin into the inner segment. However, cytochalasin D treatment affected translocations of arrestin and transducin when applied to light adapted retina cultures prior to dark adaptation (Fig. 4). The present immunohistochemical analysis showed that the movements of both molecules were abolished; arrestin remained in the outer segment and transducin in the inner segment compartment during dark adaptation.

We further investigated effects of actin filament stabilization on the movements of arrestin and transducin in photoreceptor cells. For this purpose, cultured retinas were treated with the actin filament stabilization agent phalloidin prior to illumination changes. Stabilisation of actin filaments via phalloidin had no visible effect on the movements of arrestin and transducin during light or dark adaptation (data not shown). Therefore, transport mechanisms associated with polymerization cycles of actin filaments ("treadmilling") can be excluded for light driven movements of arrestin and transducin.

### Evaluation of Myosin Motors Participating in Actin Filament Dependent Molecular Movements of Arrestin and Transducin During Dark Adaptation

Non-muscle myosin II and unconventional myosin VIIa were previously demonstrated to participate in actin filament dependent motile processes in photoreceptor cells [Chaitin and Coelho, 1992; Williams and Roberts, 1992; Liu et al., 1999; Wolfrum and Schmitt, 2000]. To evaluate whether myosin II plays a role in the light driven molecular movements of arrestin and transducin we treated retinal cultures with blebbistatin, an inhibitor of conventional class II myosins [Sakamoto et al., 2005]. The immunofluorescence analysis of blebbistatin-treated

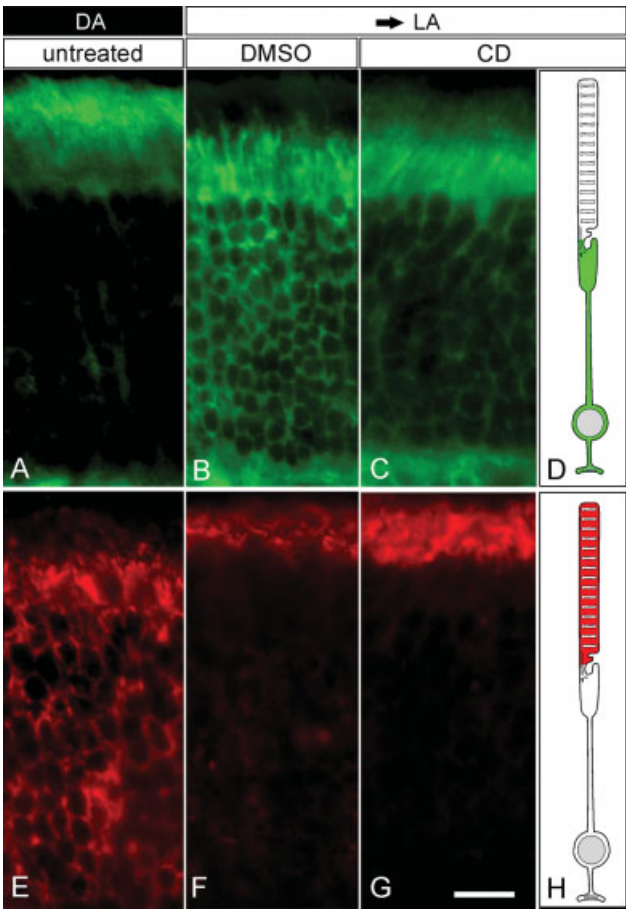


Fig. 3. Localization of transducin and arrestin in photoreceptor cells of dark to light adapted retina cultures, treated with cytochalasin D for destabilization of actin filaments. Immunohistochemical localization of transducin (green) in retinas before (**A**) and after light adaptation incubated with DMSO (**B**) and in a retina after light adaptation treated with cytochalasin D (CD) (**C**). Scheme of transducin localization (green) in a photoreceptor cell corresponding to C (**D**). Immunohistochemical localization of arrestin (red) in retinas before (**E**) and after light adaptation incubated with DMSO (**F**), and after light adaptation treated with CD (**G**). Scheme of arrestin localization (red) in a photoreceptor cell corresponding to G (**H**). Destabilization of actin filaments by CD did not interfere with the translocation of arrestin and transducin between outer and inner segments of photoreceptor cells during light adaptation in cultured retinas. DA: dark adapted, → LA: dark to light adapted. Scale bar: 13.2 μm.

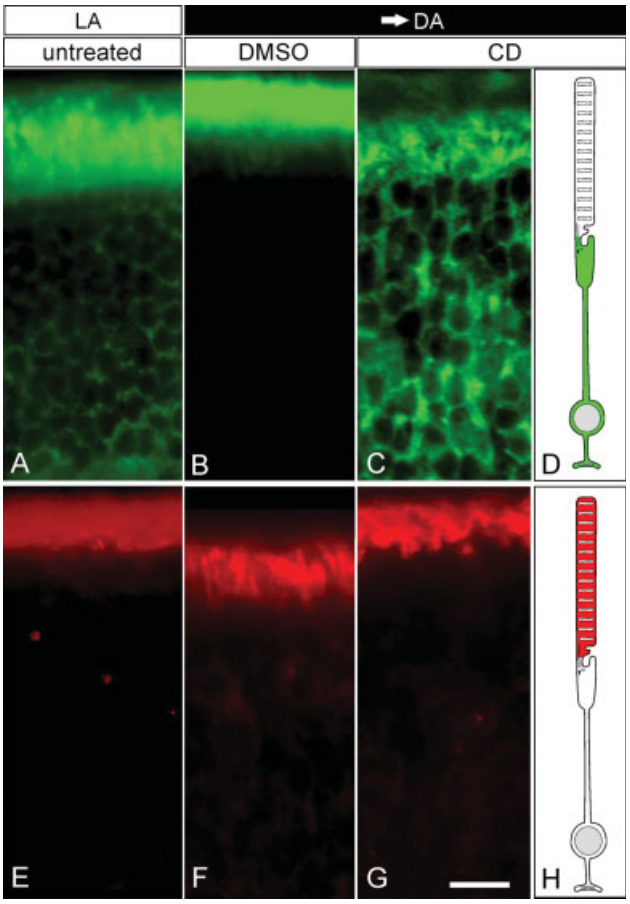


Fig. 4. Localization of transducin and arrestin in photoreceptor cells of light to dark adapted retina cultures, treated with cytochalasin D for destabilization of actin filaments. Immunohistochemical localization of transducin (green) in retinas before (**A**) and after dark adaptation incubated with DMSO (**B**) and in a retina after dark adaptation treated with cytochalasin D (CD) (**C**). Scheme of transducin localization (green) in a photoreceptor cell corresponding to C (**D**). Immunohistochemical localization of arrestin (red) in retinas before (**E**) and after dark adaptation incubated with DMSO (**F**), and after dark adaptation treated with CD (**G**). Scheme of arrestin localization (red) in a photoreceptor cell corresponding to G (**H**). CD destabilization of actin filaments interfered with translocations of arrestin and transducin between outer and inner segments of photoreceptor cells during dark adaptation. LA: light adapted, → DA: light to dark adapted. Scale bar: 13.2 μm.

retinas did not show any differences to DMSO-incubated controls during dark adaptation (data not shown).

To address whether myosin VIIa participates in light dependent movements of arrestin and transducin we analyzed these translocations in myosin VIIa deficient shaker-1 ($sh1^{-/-}$) mice [Gibson et al., 1995]. We did not observe any differences between $sh1^{-/-}$ mice and wild type or heterozygote $sh1^{-/+}$ littermates in any light dependent movement of arrestin or transducin (supplement materials Fig. S1). In conclusion, these findings indicated that neither unconventional myosin II nor myosin VIIa participate in the actin dependent translocations of arrestin and transducin during dark adaptation of rod photoreceptor cells.

## Participation of Microtubules in Light Driven Molecular Translocations of Arrestin and Transducin

To determine whether microtubules contribute to the light dependent arrestin and transducin movements we treated explanted mouse retinas with thiabendazole. Thiabendazole is a benzimidazole derivate which is

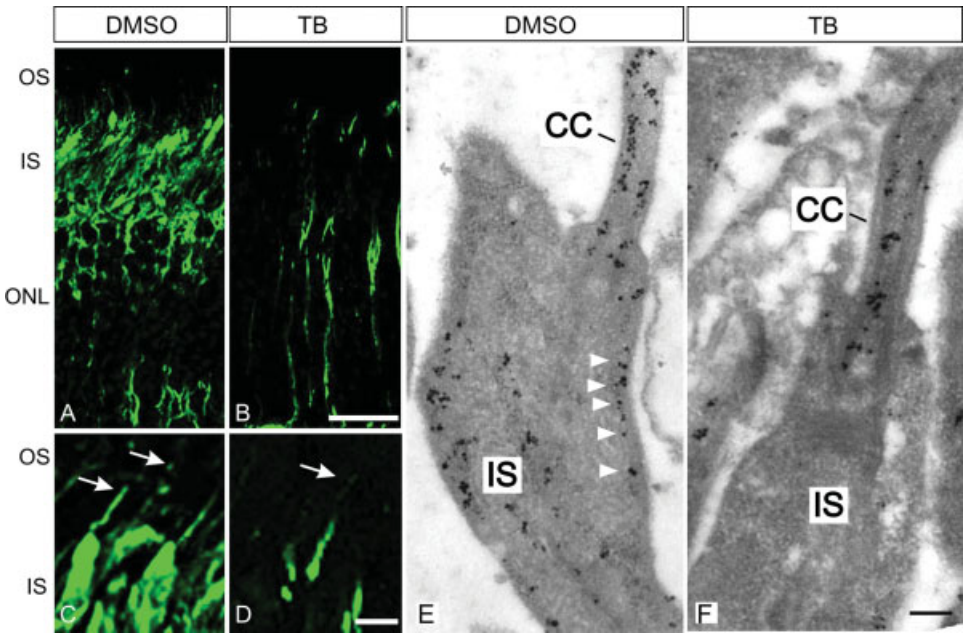


Fig. 5. Subcellular localization of α-tubulin in photoreceptor cell compartments of cultured mouse retinas. Immunohistochemical localization of α-tubulin (green) in cultured control mouse retina treated with DMSO (**A**) and cultured retina treated with thiabendazole (TB) (**B**). Tubulin staining is visible almost over the entirety of the photoreceptor cytoplasm, in the synaptic region, around cell nuclei, in inner segments and as fading fibers in outer segments. Staining of α-tubulin in the apical part of photoreceptor cells of control mouse retina at higher magnification (**C**) and in the apical part of photoreceptor cells of TB treated retina at higher magnification (**D**). The staining of tubulin in microtubules in the inner and outer segments is largely reduced, due to microtubules depolymerizing affect of TB. While also the axonemal microtubules in outer segments were destabilized, staining of microtubules in the connecting cilium (arrows) persisted. Electron micrograph of an ultrathin section through photoreceptor cells of cultured mouse retina treated with DMSO (**E**) and cultured mouse retina treated with TB. The silver enhanced immunogold labelling of α-tubulin is visible in the inner segment (arrow heads) and the connecting cilium of photoreceptor cells of cultured control mouse retina. In contrast, silver enhanced immunogold labelling of tubulin in thiabendazole treated mouse retinas shows gold decoration in the connecting cilium only. OS: outer segment, CC: connecting cilium, IS: inner segment, ONL: outer nuclear layer and OPL: outer plexiform layer. Scale bars: B: 13.2 μm; D: 7.5 μm; F: 0.2 nm. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

commonly applied as a cytoskeletal drug to disturb microtubule-dependent cellular processes [Wallin et al., 1988; Albertini, 1990; Martin et al., 1997; Pisano et al., 2000; Peterson et al., 2005; Maerker et al., 2008]. In the cell, most microtubules are highly dynamic and rapidly turnover. Thiabendazole inhibits the de novo formation of microtubules by means of binding to β-tubulin which interferes with the assembly of α/β-tubulin heterodimers [Robinson et al., 2004]. First we investigated if treatments of retina cultures with thiabendazole could efficiently destabilize microtubules in photoreceptor cells. After treatment with thiabendazole, the microtubule staining pattern by indirect anti-α-tubulin immunofluorescence, was altered throughout the photoreceptor cells. Microtubules present in the inner segment and in the axonemal microtubules in the outer segment disappeared in the thiabendazole treated specimens (Figs. 5A–5D). In contrast, the microtubules localized in the connecting cilium persisted. Once again our immunoelectron microscopy confirmed the light microscopic analyses. Lines of anti-tubulin staining by silver enhanced nanogold™ which represent microtubules in the inner segment were no longer detectable after thiabendazole treatment (Figs. 5E and 5F), while the microtubules of the connecting cilium were not affected by the thiabendazole treatment (Figs. 5E and 5F). These latter ciliary microtubules are known to be stabilized by post-translational tubulin modifications, e.g. acetylation [Schulze et al., 1987; Pagh-Roehl et al., 1991], and have previously been shown to be resistant to destabilizing agents [Sale et al., 1988].

Application of 1.5 mM thiabendazole to dark adapted cultured retinas prior to illumination had no visible effect on the translocation of transducin (Fig. 6), but led to an incomplete translocation of arrestin into the photoreceptor outer segment during light adaptation. Immunohistochemistry revealed that in the absence of inner and outer segment microtubules, transducin translocated unaffected into the inner segment during light adaptation (Figs. 6A–6D). After 3 h of light adaptation, arrestin only reached the basal part of the outer segment, with significant amounts remaining in the apical inner

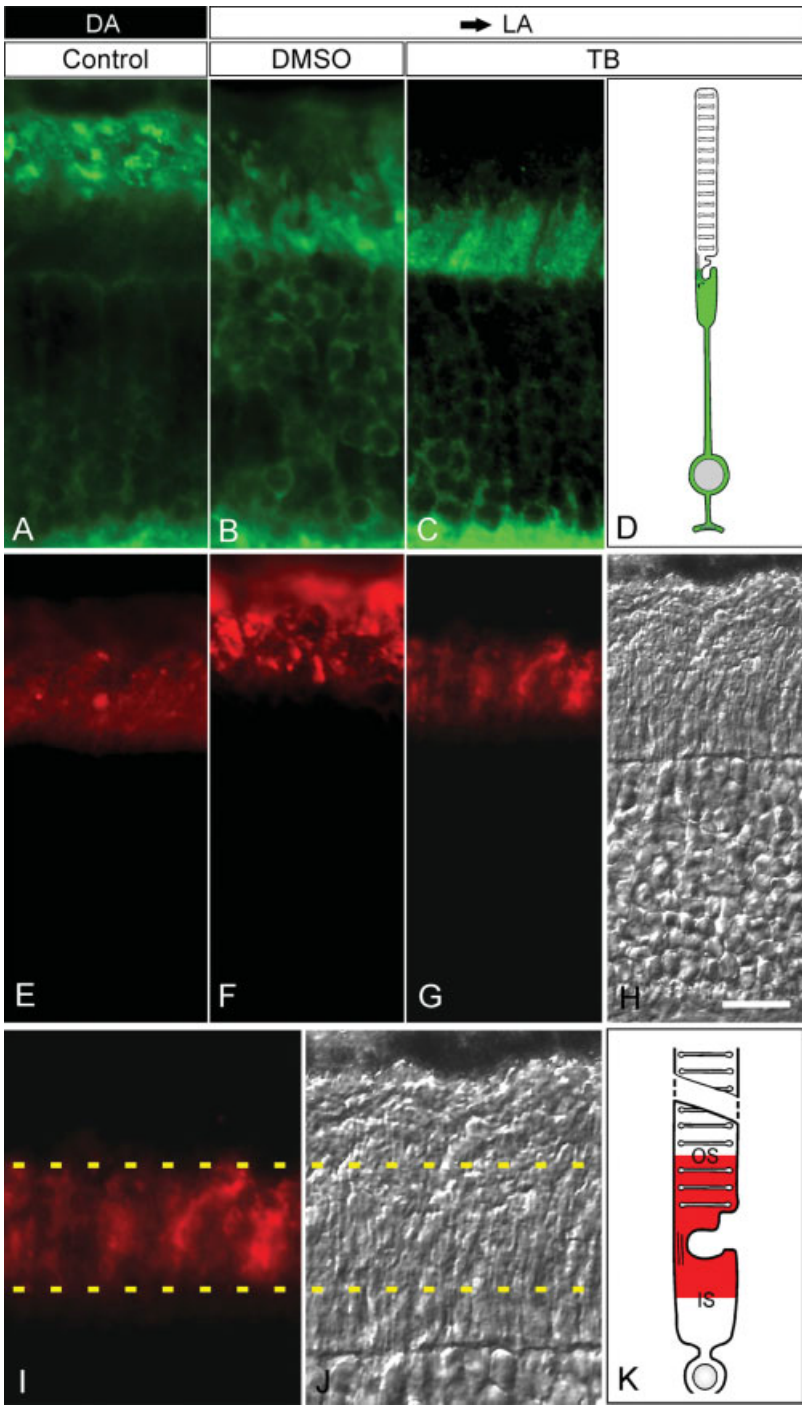


Fig. 6. Localization of transducin and arrestin in photoreceptor cells of dark to light adapted retina cultures, treated with thiabendazole for destabilization of microtubules. Immunohistochemical localization of transducin (green) retinas before (**A**) and after light adaptation incubated with DMSO (**B**) and in a retina after light adaptation treated with thiabendazole (TB) (**C**). Scheme of transducin localization (green) in a photoreceptor cell corresponding to C (**D**). Immunohistochemical localization of arrestin (red) in retinas before (**E**) and after light adaptation incubated with DMSO (**F**), and after light adaptation treated with TB (**G**). Differential interference contrast light micrograph of G (**H**). 1.5 times magnification of upper part in G (**I**) and H (**J**). Scheme of arrestin localization (red) in the intersection between inner segment (IS) and the outer segment (OS) of a photoreceptor cell corresponding to I and J (**K**). Destabilization of microtubules by TB lead to an incomplete translocation of arrestin between apical inner segment and the base of the outer segment, but did not interfere with transducin movement to the inner segments of photoreceptor cells during light adaptation in cultured retinas. DA: dark adapted, → LA: dark to light adapted, TB: thiabendazole. Scale bar: 13.2 μm.

segment (Figs. 6E–6K). This incomplete movement of arrestin was already observed after 30 min of light adaptation and was still present after prolonged translocation light exposures of e.g. 4 h (data not shown). During dark adaptation translocations of arrestin and transducin were fully affected by thiabendazole treatments (Fig. 7). Movements of both molecules were completely abolished; arrestin remained in the outer segment and transducin stayed in the inner segment compartment.

We further investigated the effects of microtubule stabilization on the light driven movements of arrestin and transducin in photoreceptor cells. For this purpose cultured retinas were treated with taxol in DMSO prior to illumination changes. Taxol stabilization of microtubules for 30 min and even after prolonged drug exposure

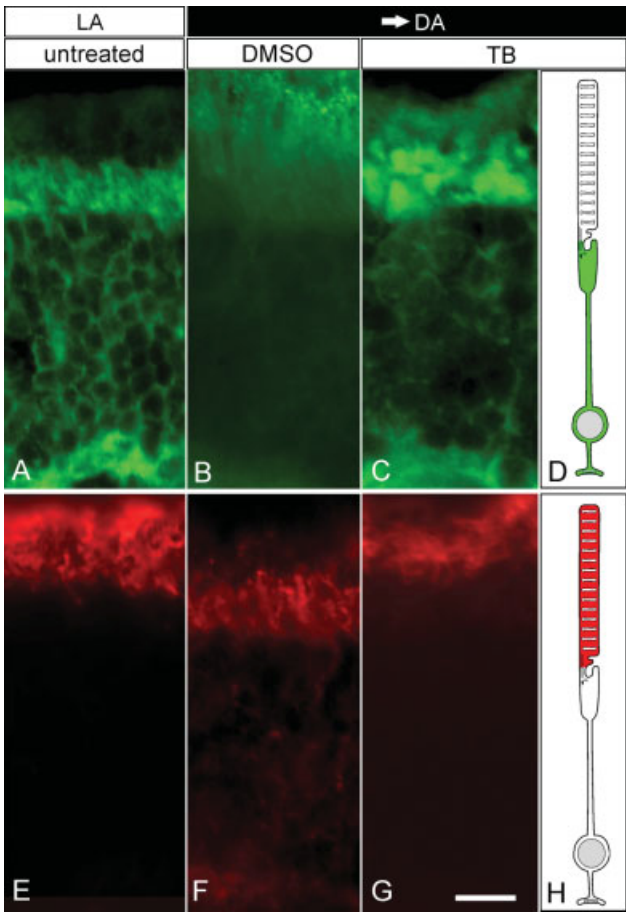


Fig. 7. Localization of transducin and arrestin in photoreceptor cells of light to dark adapted retina cultures, treated with thiabendazole for destabilization of microtubules.Immunohistochemical localization of transducin (green) in retinas before (**A**) and after dark adaptation incubated with DMSO (**B**) and in a retina after dark adaptation treated with thiabendazole (TB) (**C**). Scheme of transducin localization (green) in a photoreceptor cell corresponding to C (**D**). Immunohistochemical localization of arrestin (red) in retinas before (**E**) and after dark adaptation incubated with DMSO (**F**), and after dark adaptation treated with TB (**G**). Scheme of arrestin localization (red) in a photoreceptor cell corresponding to **G** (**H**). Destabilization of microtubules by TB interfered with translocations of arrestin and transducin between outer and inner segments of photoreceptor cells during dark adaptation in cultured retinas. LA: dark adapted, → DA: light to dark adapted. Scale bar: 13.2 μm.

up to 2 and 3 h prior to light condition changing had no visible effect on arrestin and transducin movements during light adaptation and dark adaptation (data not shown).

## DISCUSSION

The molecular mechanisms governing stimulus-dependent molecular movements into and out of signal transduction compartments are the focus of current research [for review, see Calvert et al., 2006; Slepak and Hurley, 2008]. Nevertheless, the cellular mechanisms underlying these phenomena still remain elusive [Strissel et al., 2004; Lobanova et al., 2007]. Here, we investigated the dependency of the light driven redistribution of arrestin and the visual G-protein transducin between subcellular compartments of mammalian photoreceptor cells on the actin filaments and the microtubule cytoskeleton. For this purpose, we utilized the accessibility of the organotypic retina culture, which we had previously introduced [Reidel et al., 2006] for the application of cytoskeletal drugs such as cytochalasin D or thiabendazole, respectively. Both cytoskeletal drugs are commonly used for analyses of cellular processes dependent on actin filaments or on microtubules, respectively [Bershadsky and Vasiliev, 1988]. The results of our drug treatments strongly support an involvement of both actin filaments and microtubules in the light dependent bidirectional movements of arrestin and transducin between inner and outer segments (Fig. 8). During dark adaptation the translocations of arrestin and transducin were blocked by treatments with cytochalasin D or thiabendazole, respectively. These results indicated the dependency of these processes on the actin filament and the microtubule cytoskeleton. In contrast, during light adaptation only the translocation of arrestin from the inner to the outer segment was impaired by disruption of microtubules, while the translocation of transducin was not affected. These findings indicate that principal different mechanisms govern the molecular movements during light and dark adaptation which is further supported by differences in kinetics [Sokolov et al., 2002; Elias et al., 2004; Strissel et al., 2006].

Present results obtained in mouse organotypic cultures partially confirm data on the role of cytoskeletal elements in light driven protein translocations achieved in *Xenopus* tadpoles [Peterson et al., 2005]. In both studies, results revealed that, arrestin translocation from the outer to the inner segment of photoreceptor cells required intact microtubules during dark adaptation. The reciprocal movement of arrestin from inner to outer segments also was slightly impaired in both models after treatments with thiabendazole. Furthermore, actin filament destabilization completely abolished the translocation of transducin in photoreceptor cells during dark adaptation in both organisms. These functional parallels in both vertebrate species with large phylogenetic distance indicate that the role of the cytoskeleton in the molecular mechanisms of translocations of arrestin and transducin during dark adaptation is evolutionary conserved within the vertebrate phylum.

However, our comparison also revealed differences between the present study and the data published for *Xenopus* tadpoles [Peterson et al., 2005]. While in *Xenopus* tadpoles treated with thiabendazole, the transducin

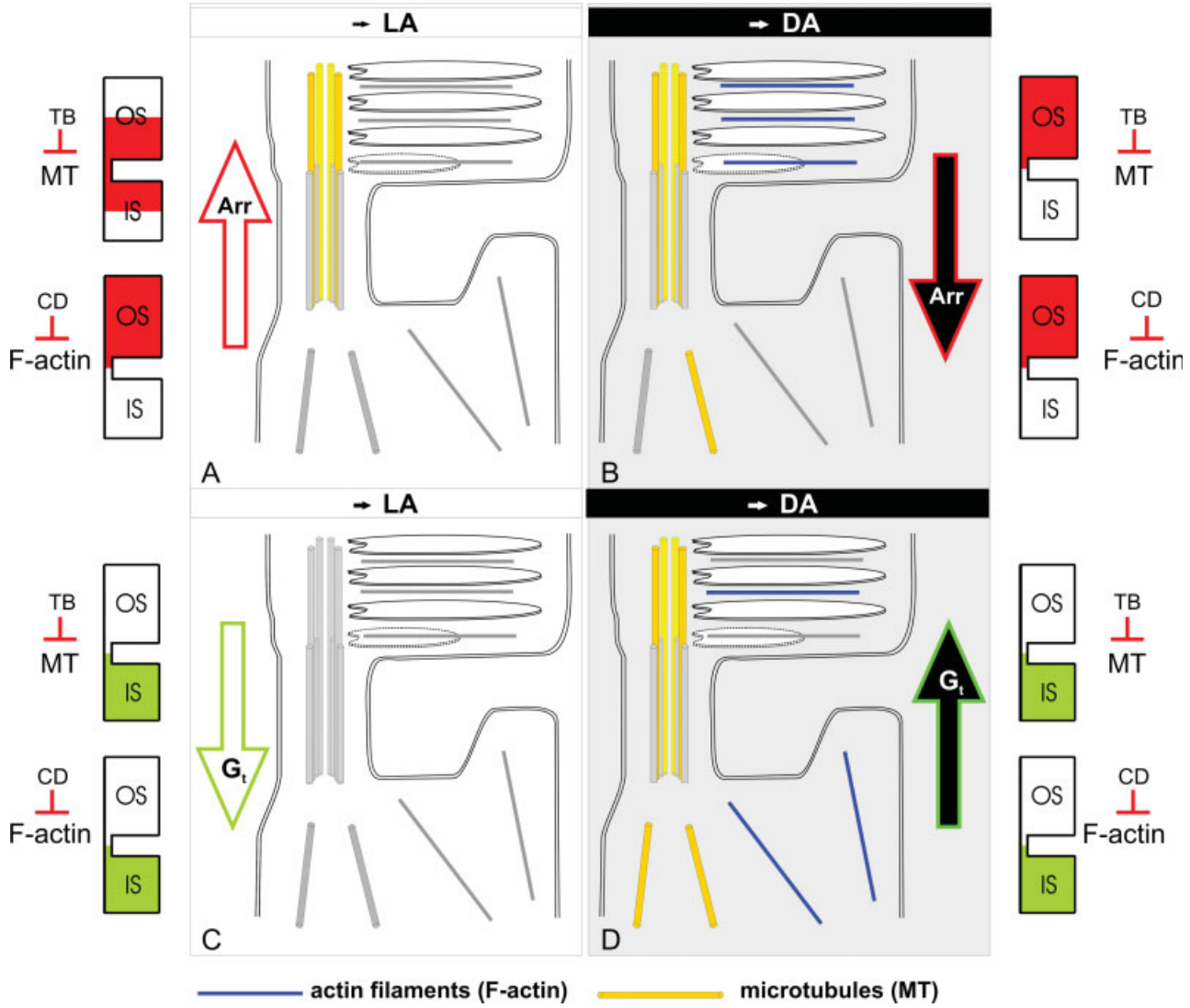


Fig. 8. Schematic illustration of the role of cytoskeletal elements during light driven adaptive movements of arrestin and transducin in rod photoreceptor cells. Schematic illustration of the distribution of arrestin (red) and transducin (green) in light adapted (**A, C**) and dark adapted (**B, D**) adapted photoreceptor cells after cytoskeletal drug treatments (small lateral schemes) and of cytoskeletal elements in photoreceptor cells (large scheme). Cytoskeletal elements essential for molecular translocations are colorized: actin filaments (F-actin) in blue and microtubules (MT) in yellow. The translocation of transducin during light adaptation is independent from actin filaments and from microtubules (C). In the absence of microtubules the movement of arrestin during light adaptation to the outer segment is incomplete (A). In contrast, translocations of arrestin and transducin associated with the dark adaptation are fully dependent on actin filaments and microtubules (B, D). Arrestin (red): Arr; transducin (green): $G_t$; dark to light adaptation: → LA; light to dark adaptation: → DA; outer segment: OS; inner segment: IS; cytochalasin D treatment: CD; thiabendazole treatment: TB.

movement to the inner segment during light adaptation was slowed, in mouse photoreceptor cells, the microtubule disruption did not affect transducin movement in this direction. In contrary, in mouse photoreceptors the translocation of transducin to the outer segment during dark adaptation was dependent on microtubules. Furthermore, in photoreceptors of organotypic mouse retina cultures disruption of actin filaments affected the arrestin movement to the inner segment during dark adaptation, which was not the case in *Xenopus* tadpoles.

Although these discrepancies between the two experimental approaches could be based on differences in the cellular structure of mammalian and amphibian photoreceptor cells or principle differences in the molecular basis of protein movements between both systems, they are most likely due to differences in the application of cytoskeletal drugs [Peterson et al., 2005]. Peterson et al. [2005] already pointed out that their systemic application of cytoskeletal drugs to the entire organism may cause non-specific effects. In the present study on organotypic

retina cultures of the mouse, all drugs were applied directly to the medium of retinal explants and non-specific effects on other tissues were therefore excluded. Furthermore, the accessibility of the uncovered photoreceptor cells in the organotypic retina culture supports the tissue penetration of the drugs, which leads to a more direct effectiveness of the applied cytoskeletal drug.

### The Role of Cytoskeletal Elements in Light Dependent Movements of Arrestin and Transducin

Actin filaments and microtubules have multiple cellular functions and participate in almost all basic cellular processes. Besides their eponymous skeletal function stabilizing the shape of cells and their subcompartments, cytoskeletal polymers can serve as targets and scaffolds for binding of proteins in protein networks. A further major function of microtubules and actin filaments is the supply of tracks for the bi-directional intracellular molecular transport and cargo delivery by molecular motors. In any case, maintenance and function of cytoskeletal elements with or without the association with motor proteins is energy dependent. All known molecular motors use ATP for force generation [Schliwa and Woelke, 2003]. In addition, polymerization and maintenance of actin filaments and microtubules are energy dependent [e.g. Carlier, 1998; Howard and Hayman, 2007]. A GTP-tubulin cap and an ATP-G-actin complex, respectively, support microtubule and actin filament polymerization and prevent polymer destabilization and depolymerization. Therefore, any participation of the cytoskeleton in the adaptive movements of arrestin and transducin is in contrast to the findings obtained by the Slepak lab [Nair et al., 2005a; Rosenzweig et al., 2007; Slepak and Hurley, 2008]. Based on ex vivo ATP and GTP depletion experiments using mouse eyecups Nair and colleagues [2005a] suggested that the translocation of transducin from inner segment to outer segment does not require energy and complete energy-independence of the adaptive arrestin redistribution [Nair et al., 2005a; Rosenzweig et al., 2007]. Nevertheless, the authors claim that arrestin binding to microtubules is involved in the retention of arrestin in the photoreceptor inner segment [Nair et al., 2005a; Slepak and Hurley, 2008]. However, as indicated above GTP depletion should affect microtubules [Howard and Hayman, 2007] and therefore, arrestin's abidance in the inner segment of dark adapted rod photoreceptor cells. Present results demonstrate that microtubules are indeed necessary during dark adaptation of rods.

In vertebrate photoreceptor cells, analyses of intracellular transport processes indicated that transport machineries differ between the inner and the outer segment compartments, and the intersegment exchange through the connecting cilium [Roepman and Wolfrum, 2007]. Therefore, disruptions of cytoskeletal elements should differentially affect these transport processes.

The present cytoskeletal drug treatments revealed that not all actin filaments and microtubules are affected in photoreceptor cells of cultured mouse retinas. Epifluorescence and electron microscopy analyses demonstrated that actin filaments at cell-cell adhesions in the outer limiting membrane of the retina and the axonemal microtubules of the connecting cilium are not altered by cytoskeletal drug treatments. These findings confirm previous studies. Actin filaments present in cell adherenses and desmosomes are commonly protected from depolymerisation by a specific set of actin-associated proteins [Balzar et al., 1998]. In cilia, posttranslational modifications stabilize axonemal microtubules and protect them from disruption [Pagh-Roehl et al., 1991; Arikawa and Williams, 1993].

In the present study, light adaptive movements of transducin from the outer to the inner segment were not altered by any cytoskeletal drug treatment. This indicate that the sets of actin filaments and microtubules present in the inner and outer segment of the rod cells, which were not protected from disruption, are certainly not involved in the translocation of transducin to the inner segment. Thus, our results favour the molecular diffusion as the driving force for the movement of transducin out of the outer segment [Calvert et al., 2006; Slepak and Hurley, 2008]. However, based on our present data we can not exclude that the drug-protected axonemal microtubules or proteins interacting with these microtubules, e.g. centrin isoforms [Giessl et al., 2004b, 2006; Trojan et al., 2008a,b] participate in the translocation of transducin to the outer segment. Since, destabilization of actin filaments or microtubules in rod photoreceptor cells completely abolished the movements of transducin from the inner to the outer segment during long term dark adaptation, actin filaments and the microtubule cytoskeleton play certainly a role in these translocation processes. Furthermore, present data demonstrate that microtubules also facilitate the translocation of arrestin into the outer segment during light adaptation.

### Participation of Microtubules and Actin Filaments in the Transport of Transducin Through the Inner Segment During Dark Adaptation

Our data revealed a dependency of the transducin translocation from the inner segment to the outer segment on both microtubules and actin filaments in rod photoreceptor cells. Molecular transport towards the outer segment was previously intensely studied only for newly synthesized rod opsin [reviewed in Sung and Tai, 2000; Papermaster, 2002]. These studies indicate that the

transport of opsin containing vesicles from the Golgi apparatus to the apical membrane of the inner segment occurs along microtubules and is mediated by the minus end-directed molecular motor cytoplasmatic dynein [Tai et al., 1999]. The identification of transducin subunits in a proteomic analysis of opsin transport carriers [Morel et al., 2000] indicates an association of the transport processes for opsin and transducin. Both signalling molecules may use at least the same transport path, microtubules, as tracks through the photoreceptor cell inner segment. This hypothesis is certainly supported by our present results which demonstrate that destruction of inner segment microtubules inhibits transducin movements to the outer segment.

There are strong molecular indices for an important role of actin filaments in the molecular delivery of opsin to the outer segment [Deretic et al., 2004, 2006]. Actin filament-associated processes may participate in the molecular "handover" mechanism between the inner segment transport and the further transport pathway through the connecting cilium to the outer segment of the photoreceptor cell [Papermaster, 2002; Roepman and Wolfrum, 2007]. If analogue mechanisms exist for the delivery of transducin to the connecting cilium, depolymerization of actin filaments should interfere with translocation of transducin to the outer segment, exactly what we observed in the present study after cytochalsin D treatment. We assume the participation of microtubules and actin filaments at the transport process of transducin through the inner segment of rod photoreceptor cells. Nevertheless, since the transducin delivery through the inner segment to the base of connecting cilium is already impaired; a further role of microtubules in the transport through the cilium and the outer segment compartment can not be excluded.

### Microtubules and Actin Filaments are Necessary Cytoskeletal Elements for the Translocation of Arrestin out of the Outer Segment During Dark Adaptation

Cytoskeletal drug treatments confirm that during dark adaptation the translocation of arrestin out of the photoreceptor outer segment is fully dependent on microtubules and actin filaments [Peterson et al., 2005; present results]. In previous in vitro studies, direct binding of arrestin to microtubules has been demonstrated, particularly in biochemical fractions of dark adapted retinas [Krispel et al., 2003; Nair et al., 2004; Hanson et al., 2006]. Based on these binding properties, it has been proposed that microtubules form a binding scaffold for arrestin which sequestrate arrestin diffused into the inner segment during dark adaptation of rod photoreceptor cells [Nair et al., 2004, 2005b; Slepak and Hurley, 2008]. Our results on arrestin translocation during dark adaptation obtained after depolymerization of microtubules are in agreement with this hypothesis. The disruption of the microtubule scaffold for arrestin binding should drastically reduce the affinity for arrestin in the inner segment and should interfere with arrestin movements out of the outer segment. Nevertheless, we can also not exclude that the microtubules in other parts of photoreceptor cells do not contribute to this translocation.

Our results also indicate a role of actin filaments in the photoreceptor outer segment during dark adaptive movements of arrestin from the outer segment towards the inner segment. Actin filament associated trafficking of arrestin has been demonstrated for the invertebrate visual arrestin (Arr2) in the rhabdomeric photoreceptor cells of *Drosophila* [Lee and Montell, 2004]. Although the vertebrate photoreceptor outer segment compartments originate from cilia containing a characteristic microtubule cytoskeleton, also actin filaments were found in the connecting cilium and at the place of disk neogenesis at the outer segment basis [Chaitin and Bok, 1986; Arikawa and Williams, 1989; Williams et al., 1992; Wolfrum and Schmitt, 2000]. Further an association of actin filaments with the outer segment disks was also indicated [Korschen et al., 1999; Kajimura et al., 2000]. These actin filaments may support myosin motor movements or simply function as structural components, e.g. by spacing the cytoplasmic cleft between the outer segment membranous disks. Such filamentous spacers were recently demonstrated by cryoelectron tomography of mammalian rod outer segments [Nickell et al., 2007]. The present study demonstrated that both myosins, namely non-muscle myosin II and myosin VIIa, which are known to be associated with actin filaments in the outer segment basis [Chaitin and Coelho, 1992; Williams et al., 1992] or connecting cilium, [Liu et al., 1997; Wolfrum and Schmitt, 2000] respectively, are not involved in adaptive movements of arrestin. In *Drosophila* photoreceptors, the class III myosin NINAC is required in arrestin trafficking [Lee and Montell, 2004]. However, the vertebrate homologues of NINAC, myosin IIIA/B are absent from the outer segment of vertebrate photoreceptors [Dose et al., 2003] and therefore, can not be involved in arrestin translocation. Nevertheless, there are 15 additional classes of myosin motors [Sellers, 2000] which could participate in arrestin transport out of the outer segment. On the other hand, it is conceivably that a disruption of actin filament cytoskeleton may cause occlusion of the narrow trafficking paths, for instance between adjacent disk membrane.

### Microtubules Support the Light Adaptive Delivery of Arrestin to the Outer Segment

Previous results in *Xenopus* tadpoles [Peterson et al., 2005] and our present analysis of mouse photore-

ceptor cells showed that movements of arrestin molecules into the outer segment during light adaptation are dependent on microtubules. In both studies, the disruption of microtubules caused an incomplete arrestin backfill of the outer segment; arrestin did not reach more than the proximal third of the outer segment. These results indicate that in the absence of microtubules arrestin does move out of the inner segment, but is not delivered along the entire outer segment. A reduced molecular transport through the connecting cilium may be a possible cause. It has been suggested that the transport of arrestin through the connecting cilium is mediated by the microtubule-associated molecular motor kinesin-II, [Marszalek et al., 2000] a heterotrimeric kinesin which is a component of intraflagellar transport (IFT) complexes in prototypic cilia and also present in photoreceptor cells [Rosenbaum and Witman, 2002; Roepman and Wolfrum, 2007]. However, present immunocytochemistry and immunoelectron microscopy revealed that the thiabendazole treatment does not affect the microtubules of the connecting cilium, but disrupted the so-called "axonemal" microtubules [Kaplan et al., 1987; Liu et al., 2004; Roepman and Wolfrum, 2007] which continue from the connecting cilium and project through the cytoplasmic compartment of the photoreceptor outer segment. Previous studies did not exclude kinesin-II mediated intraflagellar transport along the axonemal microtubules of the photoreceptor outer segment [Roepman and Wolfrum, 2007], but recent data indicated that a second kinesin motor, the homodimeric kinesin family protein Kif17 may take over the translocation along the microtubules in the axenemal part of photoreceptor cilia [Insinna et al., 2008]. The disruption of this set of microtubules should lead to the inhibition of the associated molecular transport which should consequently result in an impaired cargo transport as seen for the light induced arrestin movements in the absence of axonemal microtubules. These findings support the notion that the apical translocation of arrestin along the outer segment is probably governed by microtubule associated kinesin-II activity. However, we can not rule out other roles of outer segment microtubules which are impaired in their absence. It is possible that these microtubules are part of the structural architecture of the outer segment cytoplasm to keep the cytoplasm space open for molecular diffusion.

In conclusion, our results further support that the two-way traffic of arrestin and transducin from and into the outer segment are dependent on different mechanisms. While translocations of transducin and arrestin related to light adaptation are seemingly based on molecular diffusion, microtubules and actin filaments are required for their oppositional movements during dark adaptation. Our hypothesis favours following three alternative functions of these cytoskeletal elements involved in these movements: (i) they may provide the tracks for active transport mechanisms mediated by molecular motors (e.g. microtubules and cytoplasmic dynein in transducin translocation during dark adaptation), or (ii) may participate in the regulation of processes related to the cargo handover between inner segment and ciliary transport systems (e.g. actin filaments in arrestin translocation during dark adaptation) and (iii) as scaffolding may also "keep open" the cytoplasmic space for molecular diffusion (e.g. actin filaments and microtubules localized in the outer segment). In contrast to previous studies [Peterson et al., 2005] describing the role of cytoskeletal elements in translocations in amphibian photoreceptor cells, our mammalian data suggests that the dependency on these elements is to a lesser extend a matter of direction of movements, but more likely a matter of the physiological condition. That means that cytoskeletal elements are necessary for compartmentalization of arrestin and transducin during the dark adaptation of the photoreceptor cell, but are less important for the fast redistribution of these signalling molecules during light adaptation. These findings further correlate with the major differences in the kinetics of dark and light dependent translocations [Sokolov et al., 2002; Elias et al., 2004; Strissel et al., 2006].

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