Published in final edited form as:

Adv Exp Med Biol. 2014 ; 801: 91–96. doi:10.1007/978-1-4614-3209-8_12.

Lack of Effect of Microfilament or Microtubule Cytoskeleton-Disrupting Agents on Restriction of Externalized Phosphatidylserine to Rod Photoreceptor Outer Segment Tips

Linda Ruggiero, Silvia C. Finnemann

Department of Biological Sciences, Center for Cancer, Genetic Diseases, and Gene Regulation, Fordham University, 441 East Fordham Road, Larkin Hall, Bronx, 10458 NY, USA

Abstract

In the mammalian retina, life-long renewal of rod photoreceptor outer segments involves circadian shedding of distal outer segment tips and their prompt phagocytosis by the adjacent retinal pigment epithelium (RPE) every morning after light onset. Failure of this process causes retinal dystrophy in animal models and its decline likely contributes to retinal aging and some forms of degeneration of the human retina. We previously found that surface exposure of the membrane phospholipid phosphatidylserine (PS) is restricted to outer segment tips with discrete boundaries in mouse retina and that both frequency and length of tips exposing PS peak after light onset. Here, we sought to test mechanisms photoreceptors use to restrict PS specifically to their outer segment tips. To this end, we tested whether nocodazole or cytochalasin D, perturbing microtubule or F-actin microfilament cytoskeleton, respectively, affect localization of externalized PS at outer segment tips. Fluorescence imaging of PS exposed by rods in freshly dissected, live mouse retina showed normal PS demarcation of outer segment tips regardless of drug treatment. These results suggest that the mechanism that restricts externalized PS to rod tips is independent of F-actin and microtubule cytoskeletal systems.

Keywords

Actin; Cytoskeleton; Microtubules; Outer segment; Phosphatidylserine; Photoreceptor; Retina; Shedding

12.1 Introduction

In the mammalian retina, life-long renewal of light-sensitive rod photoreceptor outer segments (POS) involves circadian shedding of distal POS tips and their subsequent phagocytosis by the adjacent retinal pigment epithelium (RPE) every morning after light onset [1]. We previously examined plasma membrane asymmetry of mouse POS by quantifying surface exposure of the membrane phospholipid phosphatidylserine (PS) [2]. In these studies we first used recombinant annexin V to specifically label exposed PS in live, freshly dissected retina. Quantification of annexin V association with retinas either by

fluorescence microscopy of samples fixed after labeling or by immunoblotting analysis of recombinant annexin V of samples lysed after labeling revealed that PS exposure is significantly elevated at light onset, the time of POS shedding, as compared to 7 h later [2]. Furthermore, we monitored the subcellular localization of externalized PS on POS by imaging freshly dissected mouse retina samples live in the presence of a novel annexin-based biosensor with switchable states of fluorescence, termed pSIVA (“polarity-sensitive indicator of viability and apoptosis”) [3]. pSIVA imaging revealed for the first time PS externalization restricted to POS tips with discrete boundaries. Tips were slightly but significantly longer immediately after light onset than at all other times of day tested [2]. Most strikingly, changes in frequency of these tips correlated with circadian changes in POS renewal (Fig. 12.1). Altogether, our findings suggest that enhanced PS exposure precedes rod POS shedding and phagocytosis to promote these processes.

We have been intrigued by the precise retention of PS exposure to distal POS tips that suggest that rod outer segments possess mechanisms exposing PS solely at tips and preventing lateral diffusion of externalized PS toward the proximal end of POS. In this study, we set out to determine POS mechanisms that restrict PS mobility. Specifically, we tested if short-term treatment with pharmacological agents that selectively disrupt either the F-actin or the microtubule cytoskeleton alters PS restriction to POS tips in freshly dissected mouse retina.

12.2 Materials and Methods

12.2.1 Animals

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and reviewed and approved by the Fordham University Institutional Animal Care and Use Committee. Wild-type 129T2/SvEms mice originally obtained from Jackson Laboratories were reared and housed under cyclic 12 h light/12 h dark conditions and fed ad libitum. Three- to four-month-old male and female mice were used for experiments.

12.2.2 Tissue Preparation, Drug Treatment, pSIVA Labeling, and Fluorescence Imaging

Mice were sacrificed by CO₂ asphyxiation at specific times before or after light onset. Retinas were immediately dissected and either stained with pSIVA and imaged immediately as described below or treated with cytoskeleton disrupting agents before staining and imaging. To destabilize F-actin, one retina of a mouse was placed into Hank’s buffered saline solution (HBSS) containing 2.5 μ M cytochalasin D, and the other retina was placed into HBSS alone for incubation at room temperature for 15 min. To destabilize microtubules, one retina of each mouse was placed into HBSS containing 10 μ M nocodazole, and the other retina was placed into HBSS alone for incubation at 37°C for 15 min. Both agents were diluted from 100-fold stock solutions in dimethyl sulfoxide (DMSO, Sigma) directly before use. Retinas were rinsed in HBSS at room temperature for 5 min before being placed onto glass slides photoreceptor side up in HBSS supplemented with pSIVA at 1:100. Retinas were covered with a cover glass and imaged immediately on a Leica TSP5 laser scanning confocal microscopy system. Image acquisition parameters were

kept constant for all experiments. Maximal projections of image stacks were recompiled in Adobe Photoshop.

12.3 Results

12.3.1 Effects of Cytochalasin D on Localization of Externalized PS at POS Tips

We sought to examine the potential role of cytoskeletal components, namely F-actin microfilaments and microtubules, in restricting external plasma membrane PS localization to rod POS tips. To this end, we first examined the effects of cytochalasin D, a pharmacological agent that is well characterized to disrupt specifically the F-actin cytoskeleton. F-actin is present in POS, and it has been shown earlier to play an important role in disk morphogenesis at the proximal end of outer segments [4]. Disruption of F-actin in dissected mouse retinas with attached RPE disturbs the light-driven translocation of arrestin and transducin between inner and outer segment [5]. Whether or not F-actin also plays a role at distal POS tips of rods has not yet been studied. However, in human erythroleukemia cells in culture, the F-actin cytoskeleton contributes to PS exposure such that treatment with cytochalasin D diminishes PS externalization [6]. Here, we incubated freshly dissected whole retinas with cytochalasin D for 15 min followed by labeling with pSIVA and live imaging. We found in most samples that cytochalasin D-treated POS displayed very little disruption in localization of PS when compared to control POS (Fig. 12.2, compare panels b and a). In some samples, we observed a fraction of rods in which pSIVA labeled not the tips of POS but discrete bands along POS (Fig. 12.2, panel c). In these samples, we also found a significant number of POS that appeared to be disorganized. We conclude that cytochalasin D at the concentration applied does not specifically affect PS localization to POS tips but causes some cytotoxicity, the extent of which is variable among samples.

12.3.2 Effects of Nocodazole on Localization of Externalized PS at POS Tips

We next turned our attention to the potential relevance of microtubules in restricting externalized PS to POS tips. Microtubules are abundant in rod POS, and distal POS tips in toads have been reported to contain distinct microtubule-like elements [7]. We incubated freshly dissected mouse retinas in nocodazole, which interferes with microtubule polymerization, and labeled them with pSIVA. We found that retinas treated with nocodazole displayed no obvious differences in PS localization at POS tips when compared to control retinas (Fig. 12.3). This experiment suggests that retention of externalized PS to POS tips is independent of microtubules that are sensitive to nocodazole.

12.4 Discussion

In this study, we tested how short-term incubation of freshly isolated mouse retina with pharmacological agents disturbing F-actin or microtubules affected the distribution of externalized PS on distal rod POS tips.

To manipulate F-actin, we applied cytochalasin D, which prevents F-actin polymerization and promotes depolymerization of existing F-actin filaments. We found that cytochalasin D as applied had negligible effect on samples that did not show signs of cytotoxicity. These data suggest that cytochalasin D does not specifically perturb PS retention at POS tips.

To manipulate microtubules, we applied nocodazole, which prevents microtubule polymerization. We found that nocodazole as used had no effect on the localization of externalized PS at POS tips. These data suggest that nocodazole-sensitive microtubules are not required for restriction of externalized PS to POS tips. It is important to note, however, that POS contain a distinct population of microtubules, some of which are not affected by nocodazole [8]. Further studies are needed to determine their potential role in positioning of externalized PS on rod POS.

Acknowledgments

We are grateful for the Young Investigator Travel Award from the National Eye Institute that supported the participation at the RD2012 Symposium of Linda Ruggiero. We thank all members of the Finnemann lab for helpful discussions. This study was supported by NIH grant EY13295.

Abbreviations

HBSS	Hank's buffered saline solution
PS	Phosphatidylserine
pSIVA	Polarity-sensitive indicator of viability and apoptosis
POS	Photoreceptor outer segments
RPE	Retinal pigment epithelium

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Fig. 12.1.

PS-biosensor imaging of live mouse rods reveals precise retention of externalized PS to distal POS tips at all times of day and increased frequency of POS exposing PS after light onset. Fields show representative confocal maximal projections of pSIVA labeling of rod POS of live retinal tissue freshly dissected from wild-type mice at different times with respect to light onset as indicated. Scale bar, 20 μm . (© Ruggiero et al. 2012, originally published in Proc Natl Acad Sci USA [2])

Fig. 12.2.

Cytochalasin D F-actin destabilizing agent has no effect on precise retention of externalized PS to distal POS as detected by PS-biosensor imaging of live mouse rods in samples that do not display signs of cytotoxicity. Fields show representative confocal maximal projections of pSIVA labeling of rod POS of live retinal tissue freshly prepared from wild-type mice at light onset. **a** Control retina incubated in buffer alone. **b** Retina incubated in buffer with cytochalasin D showing normal pSIVA labeling and no cytotoxicity. **c** Retina incubated in buffer with cytochalasin D showing displaced PS exposure (*arrows*) and cytotoxicity (*arrowheads*). Note that the image shown in **b** is representative of the majority of samples. Scale bar, 20 μm

Fig. 12.3.

Nocodazole microtubule destabilizing agent has no effect on precise retention of externalized PS to distal POS as detected by PS-biosensor imaging of live mouse rods. Fields show representative confocal maximal projections of pSIVA labeling of rod POS of live retinal tissue freshly prepared from wild-type mice at light onset. **a** Control retina incubated in buffer alone. **b** Retina incubated in buffer with nocodazole. Scale bar, 20 μ m