Organization of cGMP sensing structures on the rod photoreceptor outer segment plasma membrane

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Keywords: cyclic nucleotide gated channel, CNGA1, CNGB1, morphogenesis, photoreceptor, retina, rhodopsin

Abbreviations: CNGA1, cyclic nucleotide gated channel α-1; CNGB1, cyclic nucleotide gated channel β-1; Dend2, Dendra2; GARP, glutamic acid-rich protein; GC, guanylate cyclase; GCAP, guanylate cyclase activating protein; GPCR, G protein-coupled receptor; IS, inner segment; OS, outer segment; PDE6, phosphodiesterase 6; Rho, rhodopsin.

A diffusion barrier segregates the plasma membrane of the rod photoreceptor outer segment into 2 domains; one which is optimized for the conductance of ions in the phototransduction cascade and another for disk membrane synthesis. We propose the former to be named “phototransductive plasma membrane domain,” and the latter to be named “disk morphogenic plasma membrane domain.” Within the phototransductive plasma membrane, cGMP-gated channels are concentrated in striated membrane features, which are proximally located to the sites of active cGMP production within the disk membranes. For proper localization of cGMP-gated channel to the phototransductive plasma membrane, the glutamic acid-rich protein domain encoded in the β subunit plays a critical role. Quantitative study suggests that the disk morphogenic domain likely plays an important role in enriching rhodopsin prior to its sequestration into closed disk membranes. Thus, this and our previous studies provide new insight into the mechanism that spatially organizes the vertebrate phototransduction cascade.

Introduction

cGMP-gated channel plays a crucial role in vertebrate vision. Within the rod outer segment (OS), cGMP concentration is regulated by rhodopsin and downstream phototransduction enzymes which are highly enriched in the photosensitive disk membranes.1 Changes in the cGMP concentration are sensed by cGMP-gated channels located on the OS plasma membrane.2 The synthesis and hydrolysis of cGMP is fine-tuned by calcium ions which are conducted by the cGMP gated channel.3 Accordingly, the lifetime of active state rhodopsin is regulated by rhodopsin phosphorylation, which is subject to regulation by calcium binding protein recoverin.4,5 The lifetime of rhodopsin is then correlated with the activity of phosphodiesterase which hydrolyzes cGMP to 5’-GMP. cGMP is synthesized by guanylate cyclase (GC) which is subject to regulation by another calcium binding protein — guanylate cyclase activating protein (GCAP).6,7 Furthermore, cGMP-gated channel is regulated by calmodulin and calcium ions.8 Thus, cGMP and calcium ions play critical roles in the regulation of cGMP-gated channels. We recently found that cGMP-gated channel is enriched to the striated structures of the OS plasma membrane, and it is excluded from the bottom of the OS.9 Currently it is unclear if such unique architectures of rod OS and the distribution of cGMP-gated channel have an impact on the performance of the phototransduction signaling cascade.

The OS membranes are divided into 2 major subdomains, plasma membrane and disk membrane. The disks contain the majority of phototransduction components that regulate cGMP concentration in a light dependent manner.10 The plasma membrane contains cGMP-gated channels which mediate the last step of the phototransduction cascade.2 The previous immunofluorescence and electron microscopy studies suggest that rhodopsin is present both in the disk and the plasma membranes.11 The high disk concentration of rhodopsin allows effective photon capture, as reduction in the rhodopsin contents can lead to compromised sensitivity of rod photoreceptors.12 Rhodopsin content has been compared and estimated in purified preparations of disk and plasma membranes. According to Molday et al.,13 rhodopsin constitutes ~85% of total disk membranes, whereas, rhodopsin was estimated to constitute ~50% of total plasma membrane proteins. This study, however, appears to have led to an overestimation of rhodopsin content as the plasma membrane preparation contained substantial amounts of disk membrane material such as peripherin/rds.13 The disparity in the disk and plasma membrane concentration would be generated by the cellular trafficking and compartmentalization mechanisms.

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Submitted: 09/15/2014; Accepted: 09/30/2014
http://dx.doi.org/10.4161/19336950.2014.973776

Addendum to: I Nemet, G Tian, Y Imanishi. Submembrane assembly and renewal of rod photoreceptor cGMP-gated channel: Insight into the actin-dependent process of outer segment morphogenesis. J Neurosci 2014; 34:8164-74; PMID:24920621; http://dx.doi.org/10.1523/JNEUROSCI.1282-14.2014.
The cGMP-gated channel consists of one β (CNGB1) and 3 α (CNGA1) subunits and is reported to interact with several other constituents of the rod OS. For example, cGMP-gated channel interacts with peripherin/rds, 4.1G, and ankyrin G. While the role of channel-4.1G interactions is unclear, synchronized renewal of channel together with disk membranes is consistent with the interaction of channel with a disk rim component including peripherin/rds. Channel-ankyrin G interaction was proposed essential and sufficient for the trafficking of cGMP-gated channel to the rod OS plasma membrane. Consecutive isoleucine and leucine residues in the C-terminal tail region of CNGB1 are identified to be the site of the interaction between ankyrin G and cGMP-gated channel. Together with other cytoskeletal components, ankryns often regulate the distribution of transmembrane proteins, including channels, on the plasma membrane. In rod photoreceptors, however, it is unclear if such ankyrin G binding is required for the confinement of cGMP-gated channel to the sub-plasma membrane locations.

To clarify the role of OS architecture and protein compartmentalization in cGMP signaling, we characterized the sub-disk membrane localizations of phototransduction enzymes, including GCAP1, in GCs and calcium dependent regulator of phototransduction. Mentalization of G protein-coupled receptor (GPCR) cascade, studies provide insight into the spatial distribution and compartmentation of both disk and plasma membranes. To understand how the first step of the phototransduction cascade is spatially organized, we quantitatively studied the concentration of rhodopsin in disk and plasma membrane compartments. To elucidate the role of CNGB1 subunit in the sub-membrane assembly of cGMP-gated channels, we dissected the roles of amino acid sequences and regions that were reported to be critical for channel trafficking and distribution. Those regions include GARP domain, VXPX cilia targeting motif, and ankyrin G binding sequence. Those studies provide insight into the spatial distribution and compartmentation of G protein-coupled receptor (GPCR) cascade, which culminates in the regulation of cGMP-gated channels.

Results and Discussions

Photoreceptor outer segment plasma membrane is divided into 2 subdomains

We propose the unique area of OS plasma membrane enriched with cGMP-gated channel to be named “phototransductive plasma membrane domain (or in short, phototransductive domain),” because the phototransduction cascade culminates in this plasma membrane region (Fig. 1A). As reported previously, cGMP-gated channel is observed in the sub-plasma membrane domain which encompass the tip and lateral portion of the OS, but is occluded from the sub-plasma membrane domain proximal to axoneme and at the bottom of the OS. Consistent with the distribution of cGMP-gated channels, cGMP binding sites, labeled by a fluorescent analog of cGMP, are located in the tip and lateral portion of the OS and they were poorly observed at the bottom of the OS. cGMP-gated channel is not observed at the bottom of the OS because it is directly trafficked to the lateral portion of OS plasma membrane, and because the lateral and bottom portions of the plasma membrane are clearly segregated by a diffusion barrier. Approximately 30% of cGMP-gated channel is mobile in the lateral OS plasma membrane. This mobile fraction did not enter the bottom of the OS plasma membrane, indicating that a diffusion barrier is present at the base of the OS (Fig. 1A) which prevents the components of the phototransductive plasma membrane domain from entering the area of disk membrane morphogenesis.

Because of the diffusion barrier, the bottom of the OS constitutes another unique plasma membrane domain responsible for deriving disk components. We propose this bottom OS region to be named “disk morphogenic plasma membrane domain (or in short, disk morphogenic domain)” (Fig. 1A). It is important to note that there are 2 proposed models regarding the process of disk membrane morphogenesis (evagination/rim and endosome models). In both models, disk membranes are synthesized at the base of the OS, and rhodopsin and other disk membrane constituents are proposed to originate from the OS plasma membrane. Rhodopsin is targeted to the disk morphogenic domain for ultimate incorporation into the disk membranes. For effective incorporation and enrichment of rhodopsin into disks, rhodopsin shall be prevented from entering the phototransductive plasma membrane. Current consensus is that rhodopsin carrier vesicles initially fuse to the distal inner segment (IS) plasma membrane. It is estimated that rhodopsin is carried through ciliary plasma membrane by intraflagellar trafficking, and reaches the plasma membrane at the bottom of the OS.

This trafficking also involves Myosin VIIa motor protein, and deletion of Myosin VIIa leads to slowed intraflagellar trafficking and an increase in the steady state level of rhodopsin on the plasma membrane of the connecting cilia. Based on the evagination/rim formation model, evaginations are the precursor of disk membranes. Consistent with this model, disruption of F-actin leads to aberrant overgrowth of plasma membrane evagination at the base of the rod OS. In these evaginations, newly synthesized rhodopsin accumulated, but it did not enter significantly into the phototransductive plasma membrane region, indicating that rhodopsin in the disk membrane precursors is largely prevented from entering the phototransductive plasma membrane. Our study clearly demonstrates that the overgrown evaginations are not covered by the phototransductive plasma membrane. Therefore along with previous electron microscopy studies, our observations suggest that those are structures open to the extracellular environment.

The diffusion barrier may also allow the disparity of rhodopsin concentrations observed between the disk and phototransductive plasma membrane. To visualize the disk and plasma membrane localized rhodopsin-Dendra2 fusion protein (Rho-Dend2-1D4), the OS was ruptured by low osmotic pressure. This rupturing leads to disk membranes opening like a “fan” around a whirled plasma membrane structure and allowed us to spatially discriminate disk (Fig. 1B asterisk) and plasma membrane (Fig. 1B arrow) domains.

By using fluorescence derived from Rho-Dend2-1D4, we estimated the concentration ratio of rhodopsin localized in disk and plasma membranes. It was noted that the majority of plasma
membrane in this preparation did not contain detectable amounts of Rho-Dend2-1D4. If we overestimate the plasma membrane contents of rhodopsin, by intentionally selecting only the ruptured OS which contained rhodopsin in the plasma membrane, the rhodopsin concentration in the plasma membrane was 7.62 ± 5.15% (n = 20) of the concentration in the disk membranes. This observation is inconsistent with the previously estimated high concentration of rhodopsin in the plasma membrane, the concentration as high as 50% of what's seen in the disks.13

The above estimations were made using Rho-Dend2-1D4 heterologously expressed in Xenopus rods. To estimate the concentration ratio of endogenous rhodopsin, rod OSs were hypotonically ruptured, chemically fixed, and then stained with 11D5 antibody which was raised against the C-terminal tail region of rhodopsin28 (Fig. 1C). As the samples had to be processed through chemical fixation and permeabilized by detergent, the disk structures were compromised, but this indirect immunofluorescence method allowed the comparison of disk (Fig. 1C, asterisk) and plasma membrane (Fig. 1C, arrow) localized rhodopsin. Level of endogenous rhodopsin plasma membrane was 26 ± 10% (n = 14) of the concentration in the disk membrane. Thus, regardless of the methods introduced, the plasma membrane content of rhodopsin is lower than the previous estimation (50% of disk contents). The difference between the previous and our estimations may be possibly derived from a species dependent difference, as this study was conducted for Xenopus laevis and the previous study was for bovine. However, it is also likely that previous study overestimated the rhodopsin plasma membrane contents due to contamination of the plasma membrane preparation by the disk membrane contents as demonstrated by the quantification of a disk specific component peripherin/rds. Within the plasma membrane preparation, peripherin/rds content was between 15–25% of the amount found in the disk membranes. Thus if 25% of disk membrane contaminant was subtracted from the estimated levels in plasma membrane, rhodopsin plasma membrane concentration would be as low as 25%, the value closer to 7.62 ± 5.14% and 26 ± 10% in our study. Overall, the discrepancy in the rhodopsin concentration and high disk content of rhodopsin is consistent with an idea that the diffusion barrier prevents disk membrane components from entering the phototransductive plasma membrane.
Compartmentalization of cGMP and Ca$^{2+}$ signaling into restricted volumes of the photoreceptor outer segment

Within the phototransductive plasma membrane, cGMP-gated channel is concentrated in striated features proximal to the calyceal processes (see Nemet et al.9). This localization pattern is consistent with the distribution of cGMP binding sites, which were studied by a fluorescent cGMP analog and were concentrated on striated features.20 Striated features were also labeled previously by anti-spectrin antibody, which is known to cross react to cGMP-gated channel.29 While this past study did not discriminate spectrin and cGMP-gated channel, the result is consistent with cGMP-gated channel localizing to the striated plasma membrane features. The striated features are juxtaposed to the disk membrane incisures, and shall be of functional significance. cGMP concentration profile is determined by the rate of its hydrolysis and synthesis. For example, phosphodiesterase 6 (PDE6), an enzyme that catalyzes hydrolysis of cGMP to 5'-GMP, is localized to the disk rim and incisure regions in frog photoreceptors.30 While PDE6 is localized uniformly around the circumference of the disk, the involuted structure of the incisures accommodates more enzyme per area of proximal phototransductive plasma membrane. Thus the disk incisures may allow quick hydrolysis of cGMP in the limited cytoplasmic volumes that are proximately located to the striated features.

GCs are the enzymes responsible for cGMP synthesis from GTP. There are 2 types of GCs, GC1 and GC2, expressed in cone and rod photoreceptors. Deletions of GC1 gene lead to primarily cone dystrophy, whereas deletions of both GC1 and GC2 lead to cone and rod dystrophy.31,32 Given the important role of GCs in rods, we tested the localization of endogenous GC1 and GC2 using a polyclonal antibody raised against a peptide sequence which is well conserved both in GC1 and GC2. GCs also localize primarily to the rim and incisure regions of rod disk membranes (Fig. 2A). This observation is consistent with the previously reported localization of murine GC1 fused to EGFP, heterologously expressed in Xenopus.
laevi rod photoreceptors,33 GCs are regulated by Ca2+ and Ca2+-
binding protein GCAP. GCAP1 also localized primarily to the
rim and incisures (Fig. 2B) where it constitutively binds to GC1
and GC2 and regulates their activity. Calcium ions, which regu-
late the activity of GCs, are conducted by cGMP-channel. Thus
cGMP-gated channels and GCs regulate each other by a Ca2+-
cGMP mediated feedback loop, which can be regulated more
quickly in the cytoplasmic area surrounded by incisure and stri-
ated features. Striated localization of cGMP-gated channels in
the phototransductive plasma membrane would allow the spatial
confinement of the feedback mechanism so that channels can
quickly respond to the changes in the cGMP concentration, and
can provide rapid feedback to GC for timely reestablishment of
cGMP level after completion of phototransduction (Fig. 2C).

Dissection of CNGB1 regions which dictate the trafficking
and localization of cGMP-gated channels

Functional cGMP-gated channel is a heterotetramer consist-
ing of 1 β and 3 α subunits,14,15 and this heterotetramerization is
essential for the trafficking to the OS,34 which is a part of mod-
ified primary cilium. CNGB1 stays in the cytosol when expressed
alone and heterologously in cultured cells or oocytes.35,36 Co-
expression of β with α subunits allows for heterotetramer forma-
tion and plasma membrane localization. While CNGA1 can be
 targeted to the plasma membrane in cultured cells,35,36 it failed
to localize to the OS in dog and mouse models lacking functional
CNGB1.34,37,38 Therefore, CNGA1 itself does not contain suffi-
cient information for the OS targeting in rod photoreceptor cells
in vivo. Location of the OS targeting signal in CNGB1 is under
active debate, and accordingly 2 hypotheses were previously
tested. One hypothesis assumes that cGMP-gated channel does
not contain any trafficking signals, and that interaction of
CNGB1 subunit with ankyrin G is essential and sufficient for
OS targeting of cGMP-gated channels because ankyrin G is tar-
geted to the OS and brings the CNG channel along with it.18
Another hypothesis, which was tested for rat CNGB1 channel, is
that CNGB1 channel contains the VXPX motif39 (also compati-
able with RVXP motif40) at the C-terminal tail region, and traf-
cicked to the cilia similar to rhodopsin which also contains the
VXPX signal. This idea was tested using ciliated cells in culture.35
We obtained evidence that these 2 hypotheses are not well sup-
ported for rod cGMP-gated channel. First of all, bovine
CNGB1, for which ankyrin G binding motif was disrupted
(CNGB1ΔGARP), can reach the olfactory sensory cilia.35,41 There-
fore the cilium targeting mechanism of cGMP-gated channel
appear to vary among different sensory cells.

Summary

In this manuscript, we extended the discussion about the 2
major aspects of membrane biology involving cGMP-gated
channel. First, we focused on the compartmentalization of
cGMP-gated channel into the specific plasma membrane
compartment. cGMP-gated channel is localized to unique
phototransductive plasma membrane domain, which is segre-
gated from the disk morphogenic plasma membrane domain
by a diffusion barrier. The same diffusion barrier allows rhod-
opsin to concentrate in the disk membranes. Within the
phototransductive plasma membrane, cGMP-gated channels
are further enriched in striated plasma membrane features.
Such segregation and enrichment would allow effective opera-
tion of the phototransduction cascade. The molecular identity
of the diffusion barrier, and identity of the proteins tethernin-
cGMP-gated channel to the striated features remain to be
elucidated. Regarding the tethering of cGMP-gated channel,
interesting developments were made for elucidating the inter-
active partners of cGMP-channels in the rod OSs.17

Second, we focused on the trafficking mechanism of cGMP-
gated channel. The role of ankyrin G in the cGMP-gated channel
biology shall be revisited. Previous study suggested that disrup-
tion of the ankyrin G binding motif leads to defective OS traf-
ficking of human CNGB1 and retinitis pigmentosa.18 The same
mutation in bovine CNGB1 did not lead to the OS targeting
defect. Thus, there is a possibility that a species dependent differ-
ence affected the property of cGMP-gated channel mutants, for
example the protein stability. Unstable nature of human cGMP-
gated channel might have led to its IS mislocalization,18 and
cause retinitis pigmentosa.42 By analogy, rhodopsin is known to
demonstrate dramatic species dependency in structural stability,
where bovine rhodopsin mutant is more stable than the corre-
sponding human rhodopsin mutant.43 Instability of human
### Figure 3

GARP region of CNGB1 is required for CNG channel localization to the OS PM. **(A)** Partial protein sequences of rod CNGB1. Ankyrin G binding residues (IL, yellow) at C-terminus are highly conserved among species while VXXP motif is not (green); however, (R)VXPX motif at N-terminus is highly conserved among species (gray). **(B)** CNGB1 with mutated ankyrin G binding site (bCNGB1IL1360-1361AA-Dend2) properly localized to the rod OS similar to the WT (bCNGB1-Dend2) **(C)**. **(D)** R7VXP sequence is also non-essential for the channel trafficking to the OS, as replacement of R7VLP sequence to A7ALA (bCNGB1-R7VLP7-10AALA-Dend2) did not affect channel localization to the OS. **(E)** Trafficking signal is located in the CNGB1 GARP domain, as removal of GARP domain (bCNGB1ΔGARP-Dend2) led to its accumulation in the IS and localization to the apical membrane of the IS (arrow) as well as in the calycal processes (arrowheads and co-localization with F-actin (phalloidin) right panel). In **(B–E)**, left panels are live and unfixed retinas, right panels are fixed retinas stained with Dend2 antibody and **(E)** phalloidin (F-actin). Scale bar 5 μm.

| Species       | Partial Protein Sequence                                      |
|---------------|----------------------------------------------------------------|
| Human         | 1216 ... AEPEEHSRVICMSPGPEGEQIIISVQPMEEREKAE                   |
| Bovine        | 1339 ... ARPEEHPSVRIHVTLGAPSEQILLYKEVEPQKEEKEKEEKEEKEE       |
| Rat           | 1339 ... APGLPSVRIHPSQDSEQIPEVEPEEKKEKEEKEKEEKEE             |
| Rabbit        | 1284 ... ARPEEHPSVRITSPGESPSEQIIISVQGPEEKDEE                |
| Guinea pig    | 1307 ... MGTEEPQVIRYPPEQEQTSVEQTMQEEKEEAAE               |
| Rh. monkey    | 1212 ... AQPEEHSRVIRMSPGPEGEQIIISVQPEEKDEE                  |
| Human         | 1 MLGWVQRLPQQPPGTPKTKQME................................EEVEPEP |
| Bovine        | 1 MLGWVQRLPQQPPGTPKTKQME................................EEVEPEP |
| Rat           | 1 MLGWVQRLPQQPPGTPKTEEGAPQPETSEQKBEANPQPEPEVQPEP            |
| Rabbit        | 1 MLGWVQKVLQPPGTPRTKVEEE................................EEEEAEP  |
| Guinea pig    | 1 MLGWVQKVLQPPGTPKSIQME................................EEEEAEP  |
| Rh. monkey    | 1 MLGWVQRLPQQPPGTPKTKQME................................EEVEPEP  |

**Figure 3.** GARP region of CNGB1 is required for CNG channel localization to the OS PM. **(A)** Partial protein sequences of rod CNGB1. Ankyrin G binding residues (IL, yellow) at C-terminus are highly conserved among species while VXXP motif is not (green); however, (R)VXPX motif at N-terminus is highly conserved among species (gray). **(B)** CNGB1 with mutated ankyrin G binding site (bCNGB1IL1360-1361AA-Dend2) properly localized to the rod OS similar to the WT (bCNGB1-Dend2) **(C)**. **(D)** R7VXP sequence is also non-essential for the channel trafficking to the OS, as replacement of R7VLP sequence to A7ALA (bCNGB1-R7VLP7-10AALA-Dend2) did not affect channel localization to the OS. **(E)** Trafficking signal is located in the CNGB1 GARP domain, as removal of GARP domain (bCNGB1ΔGARP-Dend2) led to its accumulation in the IS and localization to the apical membrane of the IS (arrow) as well as in the calycal processes (arrowheads and co-localization with F-actin (phalloidin) right panel). In **(B–E)**, left panels are live and unfixed retinas, right panels are fixed retinas stained with Dend2 antibody and **(E)** phalloidin (F-actin). Scale bar 5 μm.
CNGB1 may possibly be caused by compromised ankyrin G-CNGB1 interaction prior to entering the secretory pathway. Our finding that GARP domain is essential for cGMP-gated channel trafficking is largely consistent with recent developments elucidating the trafficking signal of CNGB1.\(^4^4\) Complementary to our study in which the entire GARP region was deleted, it appears that a partial deletion of the GARP region does not appear to affect the localization of CNGB1.\(^4^5\) Thus, further studies of cGMP-gated channel will lead to a discovery of a novel cilia trafficking signal.

**Methods**

**Constructs**

Full-length bovine cyclic nucleotide-gated channel β-1 (bCNGB1) cDNA was a generous gift from Dr. Andrew F.X. Goldberg (Eye Research Institute, Oakland University, Rochester, MI) and Dr. Robert Molday (department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada). The coding region of bCNGB1 was cloned into a TOPO vector which contained the Xenopus rhodopsin promoter upstream and Dendra 2 (Clontech Laboratories) downstream of the cloning site (bCNGB1-Dend2). Mutations and deletions of bCNGB1 were generated by standard cloning methods. Human rhodopsin was fused to Dend2 and 1D4 epitope (Rho-Dend2–1D4) as described previously.\(^4^6\)

**Generation of transgenic *Xenopus laevis***

All animal experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and based on the protocol approved by the institutional animal care and use committee at Case Western Reserve University. Transgenic *Xenopus laevis* expressing bCNGB1-Dend2, and Rho-Dend2-1D4 were generated as previously described\(^9\),\(^4^6\) by using the intra-cytoplasmic sperm injection method. In all experiments, tadpoles were 12–16 d old (stage range 46–47\(^4^8\)). Unless otherwise specified, a minimum of 4 animals were used in each experiment. Albino *Xenopus laevis* was used for all the procedures.

**Immunofluorescence microscopy**

For retinal flat mounts, the neural retina was excised from each eye in modified Wolf medium (55% MEM; Invitrogen; 31% Earle’s sodium-free BBS, 10% FBS, 30 mM NaHCO\(_3\), 700 mg/l D-glucose). Rod OS was ruptured by exposing retinas for 5 min to a hypotonic buffer (2 mM HEPES, 2 mM EGTA, pH 7.4). Rupturing enabled spatial separation of disk membranes from the OS plasma membrane for microscopy observation. Neural retinas were fixed in methanol/DMSO (80:20; v/v) for 15 min at −20°C or in 4% w/v formaldehyde in PBS for 1 h at room temperature and processed for immunofluorescence. Hypotonically-treated retinas were fixed in 4% w/v formaldehyde containing 0.01% w/v glutaraldehyde in PBS for 1 h at room temperature. Fixed retinas were blocked in 1.5% normal goat serum diluted in PBS with 0.1% (v/v) Triton X-100 (PBST) for 1 h, incubated with primary antibody overnight, and then secondary antibody for 1 h at room temperature. Antibodies against GCAP1 (UW83, raised against bovine GCAP1\(^4^9\),\(^5^0\)), GC1/2 (UW85, raised against GTRMRHMPVPRIRIG peptide) were generous gifts from Dr. Krzysztof Palczewski at Case Western Reserve University. Monoclonal anti-rhodopsin 11D5 was a kind gift from Dr. Dusanka Deretic at University of New Mexico Health Sciences Center.\(^2^8\) Rabbit polyclonal antibody against Dendra2 (Dendra2-138) was raised against the entire peptide sequence of Dend2.\(^4^6\) Donkey anti-rabbit Alexa Fluor 488 (ImmunoResearch Laboratories, Cat# 711-545-152) was used as secondary antibody. For visualizing F-actin, Alexa Fluor 546 conjugated phalloidin (Invitrogen), at a concentration of 22 nM, was incubated with retinas overnight at room temperature in PBS with 0.1% (v/v) Triton X-100.

**Confocal microscopy**

Living, fixed, or hypotonically-treated retinas were imaged using the HCX PL APO CS 40.0 x 1.25 oil UV objective of a Leica TCS SP2 laser scanning confocal microscope as previously described.\(^9\)

**Images analysis**

Intensity profiles for Rho-Dend2-1D4 and endogenous rhodopsin were determined in a single optical section by using ImageJ software. Some x-y panel images of the OS cross-sections were processed by 2D blind deconvolution using AutoDeblur and AutoVisualize 9.3 (MediaCybernetics).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Mr. Richard Lee for excellent technical assistance in the preparation of this paper and for reading the manuscript. We also thank Mr. Christopher M Strauch for reading the manuscript prior to submission and for his helpful suggestions. We would like to thank Dr. Andrew F.X. Goldberg from Oakland University and Dr. Robert Molday from University of British Columbia for full-length bovine CNGB1 cDNA, Dr. Krzysztof Palczewski from Case Western Reserve University for providing GCAP1, GC1/2 polyclonal antibodies and Dr. Dusanka Deretic from University of New Mexico Health Sciences Center for providing 11D5 antibody.

**Funding**

This work was supported by the US. National Institutes of Health grants EY020826 and EY011373.

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