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Monomeric rhodopsin is the minimal functional unit required for arrestin binding*

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Abstract

We have tested if arrestin binding requires the G protein-coupled receptor (GPCR) be a dimer or multimer. To do this, we encapsulated single rhodopsin molecules into nanoscale phospholipids particles (so called nanodiscs) and measured their ability to bind arrestin. Our data clearly show that both visual arrestin and β -arrestin 1 can bind to monomeric rhodopsin and stabilize the active metarhodopsin II form. Interestingly, we find the monomeric rhodopsin in nanodiscs has a higher affinity for wild-type arrestin binding than does oligomeric rhodopsin in liposomes or nanodiscs, as assessed by the stabilization of metarhodopsin II. Together, these results establish that rhodopsin self-association is not required to enable arrestin binding.

Keywords

rhodopsin; G protein-coupled receptor; arrestin; oligomerization; nanodiscs

Introduction

G protein-coupled receptors (GPCRs) are widely used by cells for transmitting extra-cellular signals to intra-cellular signaling cascades ¹; ². The ability of GPCRs to couple with and activate G proteins is blocked when the receptors are phosphorylated and bound by a protein called arrestin ³; ⁴; ⁵. Increasingly, the importance of GPCR-arrestin interactions is becoming apparent as studies discover their role in receptor internalization ⁶; ⁷, susceptibility to drug tolerance ⁸; ⁹, retinal disease states ¹⁰, and G protein-independent signaling ¹¹.

At present, it is still not clear exactly how arrestin interacts with the cytoplasmic face of a GPCR. In fact, even the stoichiometry of the arrestin-GPCR interaction is not established, and a number of different and conflicting models have been proposed ¹²; ¹³ (some possible arrangements are shown in Fig. 1A). One report has provided evidence that the overall stoichiometry of arrestin:rhodopsin binding is 1:1 ¹⁴, although that study could not determine

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if the actual stoichiometry of the complex was 1:1 versus 2:2, 4:4 or even higher ratios of arrestin:rhodopsin¹³.

Questions about the minimal stoichiometry required for arrestin-GPCR interaction are compelling, given the known ability of GPCRs to self-associate into dimers and/or even higher order species. For example, rhodopsin, a light-sensitive GPCR, has been found *in vivo* conditions to form dimers and higher order species in the rod outer segments (ROS)¹⁵. Moreover, opsin has been shown to self-associate when expressed in COS1 cells¹⁶, and our lab has found that purified rhodopsin self-associates when it is reconstituted into lipid vesicles (liposomes)¹⁷. Other rhodopsin-like (family A) GPCRs are also reported to form oligomers in lipid environment^{13, 18, 19, 20}. In the case of family C GPCRs, it is well known that dimerization or oligomerization of the receptors plays important functional roles²¹. Together, these observations raise the possibility that a multimeric form of rhodopsin (and other family A GPCRs) is a requirement for interaction with arrestin¹², but this possibility has never previously been directly tested.

Thus, in the present manuscript, we set out to directly test if a dimeric or oligomeric GPCR is required for arrestin binding. To do this, we reconstituted single phosphorylated rhodopsins into small lipid particles, called nanodiscs (Fig. 1B), and measured their ability to bind and interact with visual arrestin and β -arrestin 1. Nanodiscs consist of lipids and membrane scaffold proteins (MSP), a derivative of apolipoprotein A-1²². Recently, several laboratories have used nanodiscs in a similar approach to unequivocally demonstrate that a monomeric GPCR is the minimal unit required for activating a G protein, at least for the GPCRs rhodopsin^{23, 24, 25}, the β -adrenergic receptor²⁶ and the μ -opioid receptor²⁷. To enable comparison of our monomeric rhodopsin results with oligomeric rhodopsin, we also reconstituted rhodopsin molecules into liposomes (Fig. 1C), as well as nanodiscs containing multiple rhodopsin molecules.

Our data clearly show that monomeric, light-activated, phosphorylated rhodopsin in nanodiscs can interact with both visual arrestin and β -arrestin 1, resulting in the stabilization of the active rhodopsin form, called metarhodopsin II (meta-II). Interestingly, we find that monomeric rhodopsin in nanodiscs shows a higher affinity for wild-type visual arrestin than oligomeric rhodopsin in liposomes as assessed by meta-II stabilization. Our data also show that phospholipids with acidic head groups enhance the interaction of monomeric rhodopsin with visual arrestin, in agreement with our previous studies of rhodopsin-arrestin interaction in detergent²⁸. Together, our data provide the first direct evidence of interaction between a monomeric GPCR and arrestins, and they indicate that a monomeric rhodopsin is the minimal functional unit needed for arrestin binding.

Results

We prepared rhodopsin in nanodiscs by mixing purified phosphorylated rhodopsin and solubilized lipids (a mixture of POPC and POPG at a 3:2 ratio) with MSP (Fig. 1B). Separately, we also prepared rhodopsin in liposomes by mixing purified phosphorylated rhodopsin and the solubilized lipid mixture in the absence of MSP (Fig. 1C). We chose this lipid mixture because it was used for previous nanodisc studies using rhodopsin and other GPCRs, and because it mimics the zwitterionic environment of a cell membrane, and is suited for retaining functional activity for GPCRs^{25, 26, 29}. After removal of detergents using Bio-beads, the samples were injected onto a size exclusion column. In the presence of MSP, the majority of the sample eluted with a Stokes' diameter of ~12 nm (as determined by protein standards), a typical value for nanodisc containing monomeric rhodopsin (Fig. 2A)^{23, 30}. Our images of the nanodiscs using electron microscopy were consistent with this size (Fig. 2B). We collected this fraction and concentrated it using centrifugal filter devices

(see “Materials and Methods” section). In the absence of MSP, the samples formed lipid vesicles (liposomes) and eluted at the void volume (Fig. 2A).

Analysis by SDS-PAGE shows that the nanodisc samples contain phosphorylated rhodopsin and MSP, and the liposome samples contain only rhodopsin (Fig. 2C). The absorption spectra of the nanodisc samples indicate the phosphorylated rhodopsin was successfully reconstituted into nanodiscs, and retained spectral functionality. In the dark, the absorption maximum was at 500 nm, and upon irradiation, this value shifted to a mixture of ~380 nm and ~480 nm, values typical for the light-activated form of rhodopsin (meta-II) and its precursor (meta-I), respectively (Fig. 2D). The absorption spectra of nanodiscs showed very little light scattering (Fig. 2D) in contrast to the liposome spectra (Fig. 2E). These facts are again consistent with the nanodiscs having a much smaller size than the liposomes. Furthermore, we observed no loss in absorbance of nanodisc samples even upon centrifugation at 100,000 g for 30 minutes (data not shown), demonstrating our rhodopsin-nanodisc samples are fully soluble, even in the absence of detergent.

We confirmed that the rhodopsin was monomeric in the nanodiscs, by carrying out fluorescence energy transfer (FRET) experiments. We have previously shown FRET can be used to measure rhodopsin self-association in liposomes¹⁷. Briefly, these FRET experiments involved mixing equivalent molar amounts of Cy3-labeled and Cy5-labeled phosphorylated rhodopsin samples together, and then reconstituting them either into nanodiscs or liposomes. The results from these experiments clearly showed that the FRET efficiency between Cy3- and Cy5- labeled rhodopsins was significantly higher in liposomes (26%) than in nanodiscs (9%) (Fig. 3, A and B), indicating that rhodopsin was predominantly dimeric (or oligomeric) in liposomes, and predominantly monomeric in nanodiscs. The higher FRET efficiency in liposomes and lower FRET efficiency in nanodiscs are consistent with previous studies using Cy3- and Cy5- labeled rhodopsin¹⁷ and β -adrenergic receptor²⁶.

We also confirmed that our nanodisc preparations contained monomeric rhodopsin, by using an approach described in two previous reports of monomeric rhodopsin in nanodiscs^{23, 25}. In this approach, we re-purified the rhodopsin-containing nanodiscs using ConA-Sepharose, measured the absorption spectra (Fig. 3C) and then calculated the rhodopsin-MSP stoichiometry by comparing the extinction coefficients for rhodopsin (at 280 nm and 500 nm) with that of MSP (at 280 nm)^{23, 25}. This analysis showed a molar ratio of rhodopsin:MSP of ~1:2.3. Since each nanodisc is formed by two MSP molecules (see figure legend of Fig. 3C), this result confirms that there was one rhodopsin per nanodiscs, consistent with previous studies of monomeric rhodopsin in nanodiscs^{23, 25}. In summary, both our FRET data and absorption spectral analyses indicate that our nanodiscs samples contained monomeric rhodopsin.

We next measured the ability of monomeric rhodopsin in nanodiscs and multimeric rhodopsin in liposomes to bind arrestin, by measuring the amount of “extra meta-II” formation. “Extra meta-II” formation is a well-defined process that occurs when the binding of arrestin or G protein to photo-activated rhodopsin causes a shift of equilibrium between meta-II and its precursor meta-I, toward meta-II^{31, 32, 33}. “Extra meta-II” can be easily quantified from difference spectra, which are obtained by simply subtracting the spectra of the rhodopsin in the dark from the spectra obtained after photo-activation. Typically, these difference spectra will show negative absorbance at ~ 500 nm (from the loss of dark-state rhodopsin absorbance) and an increase in 380 nm absorbance (due to the formation of the active MII species). Since meta-II absorbs at 380 nm and meta-I at 480 nm, when arrestin binding to and stabilization of MII shifts the MI/II equilibrium towards formation of more MII, one observes an increase in absorbance at 380 nm, or “extra meta-II”.

Our results show that addition of WT visual arrestin to nanodiscs clearly increases the amount of meta-II in the difference spectra (note the increase in ~380 nm species in Fig. 4A). Importantly, the increase in meta-II was dependent on arrestin concentration and the difference spectra in the presence of various concentration of arrestin showed an isosbestic point at ~420 nm (Fig. 4A), both spectral properties typical for “extra meta-II” formation induced by arrestin binding^{32, 34}.

Interestingly, we observed much less increase in meta-II formation when we added WT visual arrestin to our multimeric rhodopsin samples in liposomes (Fig. 4B), in which ~85 % of the rhodopsin was oriented with its cytoplasmic faces on the outside of the liposomes (Supplemental Fig. S1). This intriguing result could indicate that visual arrestin prefers binding to monomeric rhodopsin rather than to multimeric rhodopsin. However, the assay we used to obtain this result formally cannot rule out the possibility of arrestin binding to multimeric rhodopsin, merely that if it does so, it does not produce extra meta-II. Either way, this is a curious results - previous studies of rhodopsin in ROS membranes have demonstrated significant “extra meta-II” formation occurs upon arrestin binding^{32, 33, 34}. Thus, our results may suggest that the “extra meta-II” signal observed in ROS membranes is due to arrestin binding to monomeric rhodopsin formed transiently in the ROS membranes, and very few of these transient monomers exist in our reconstituted liposomes.

In addition, we measured “extra meta-II” formation induced by a constitutively active R175E mutant of visual arrestin, which can bind to both unphosphorylated rhodopsin and phosphorylated rhodopsin^{7, 35}. The R175E mutant induced significant “extra meta-II” formation both in nanodiscs and in liposomes, to an extent much greater than WT arrestin (Fig. 4, C and D). This result is not surprising, since another constitutively active form of arrestin, named p⁴⁴ (Arr^{1-370A}), is reported to induce larger “extra meta-II” formation than WT arrestin³⁴. We also find that arrestin mutant R175E can induce “extra-meta II” formation of unphosphorylated rhodopsin in nanodiscs (supplemental Fig. S2). Taken together, our data show that WT visual arrestin and the constitutively active arrestin mutant R175E possess typical abilities for forming a complex with rhodopsin in nanodiscs.

The concentration dependence of arrestin binding is shown in Fig. 4, E and F. The plot shows the amount of “extra meta-II” formation (difference absorbance between 390 nm and 426 nm) in nanodiscs and liposomes is dependent on arrestin concentration. In both nanodisc and liposomes, the R175E arrestin mutant showed higher affinity (lower EC₅₀ value) than WT arrestin. Interestingly, the R175E mutant appears to have a similar affinity for rhodopsin in either nanodiscs or liposomes (EC₅₀ = ~0.6 μM), although WT arrestin appears to show higher affinity for rhodopsin in nanodiscs (EC₅₀ = ~1.4 μM) than for rhodopsin in liposomes (EC₅₀ = ~7.9 μM). Based on these results, it is tempting to speculate that WT visual arrestin prefers to bind to monomeric rhodopsin rather than to oligomeric rhodopsin, but the R175E mutation destroys this discrimination.

To enable better comparison between our various results, we estimated the amount of meta-II induced by arrestin binding to nanodiscs according to the methods described in Weitz and Nathans³⁶. In the absence of arrestin (black line in Fig. 4A), meta-II formation in nanodiscs containing predominantly monomeric rhodopsin was calculated to be 18% {[meta-II]/([meta-I] + [meta-II]) = 0.18}. In the presence of 4 μM arrestin WT (purple line in Fig. 4A) and R175E mutant (purple line in Fig. 4C), meta-II formation were 50 % and 76 %, respectively. Thus, the amount of meta-II increased by 32% upon binding of WT arrestin, and 58% upon binding of the mutant R175E.

We used these values to assess whether the “extra meta-II” formation we see (Fig. 4, A and C) might be due to arrestin binding to a small amount of dimeric (or oligomeric) rhodopsin

present as a “contamination” in our monomeric rhodopsin nanodisc samples. We tested this possibility by preparing nanodiscs containing predominantly dimeric (or oligomeric) rhodopsins, which we obtained by changing the rhodopsin/MSP/lipid ratio (rhodopsin:MSP:lipid=1:1:41.25), as described in previous studies^{23, 24}. These nanodisc samples exhibited very high FRET efficiency (33 %) between the Cy3- and Cy5- labeled rhodopsins, indicating each of these nanodiscs predominantly contained two or more rhodopsin molecules (Fig. 5, A and B). The 33 % FRET efficiency for these samples is much higher than the 9 % efficiency for the monomeric rhodopsin/nanodiscs (Fig. 3A). It is even higher than the 26 % efficiency for the labeled rhodopsin reconstituted into liposome (Fig. 3A). These dimeric/oligomeric rhodopsin-nanodisc samples showed a shift in the meta-I/meta-II equilibrium towards more meta-I (Fig. 5C), consistent with the previous report²³. Addition of WT arrestin to these oligomeric rhodopsin nanodisc samples induced very little “extra meta-II” formation, and addition of arrestin mutant R175E produced a small amount of “extra meta-II” (Fig. 5C).

However, we must be cautious about inferring too much from these results, because we do not know for sure the orientation of the dimeric/multimeric rhodopsin in the nanodiscs. However, these results do make one thing clear. The arrestin induced “extra meta-II” signal we see in our monomeric rhodopsin nanodiscs (Fig. 4, A and C) is clearly larger than it is for the multimeric rhodopsin samples (Figs. 4, B and D and 5C). Thus, even if one postulates there could be some multimeric rhodopsin “contaminating” the monomeric rhodopsin nanodiscs preparations, our data show the “extra meta-II” signal we see for the monomeric rhodopsin samples cannot be due to arrestin binding only to a hypothetical multimeric rhodopsin “contaminant”, because multimeric rhodopsin produces less (or no) “extra meta-II” in the presence of arrestin, compared to the monomeric rhodopsin nanodiscs samples. Thus, based on our experiments, we can safely conclude that the “extra meta-II” formation we observed for monomeric rhodopsin samples (Fig. 4, A and C) has to be due to arrestin binding to at least some monomeric rhodopsin, thus demonstrating that monomeric rhodopsin can bind arrestin.

We also tested the effect of lipid composition on the interaction between arrestin and rhodopsin in nanodiscs. Previously we have found that phospholipids with acidic head groups enhance the interaction of rhodopsin in detergent micelles, resulting in a stabilized meta-II^{28, 37}. Of the lipids used in our present study, POPC has a basic head group, and POPG has an acidic head group. To test the effect of lipid head-group charge, we measured the ability of arrestin to bind to rhodopsin in nanodiscs containing only POPC. As shown in Fig. 6, rhodopsin in nanodiscs containing only POPC showed much less “extra meta-II” formation induced by WT arrestin and the R175E mutant compared to nanodiscs containing POPC and POPG. This result suggests that POPG can enhance arrestin-rhodopsin interaction and stabilize meta-II. These results are consistent with our above mentioned study showing a role for negatively charged lipid head groups on arrestin binding in detergent micelles²⁸.

Finally, to test if the ability to bind to a monomeric receptor is unique to visual arrestin, we tested the ability of β -arrestin 1 (Arr 2) to bind rhodopsin in nanodiscs. Although it is well established that β -arrestin can bind to phosphorylated rhodopsin^{38, 39}, it has not previously been established whether or not β -arrestin can bind to a monomeric GPCR. Our results in Fig. 7 show that addition of β -arrestin also induces significant “extra meta-II” formation for phosphorylated rhodopsin (Fig. 7, A and C), but not for unphosphorylated in nanodiscs (Fig. 7, B and D). Thus, these results show that as with visual arrestin, β -arrestin can bind to a monomeric rhodopsin.

Discussion

In this study, we found that arrestin shows robust binding to a monomeric rhodopsin in nanodiscs (Figs. 4 and 7). This result directly demonstrates that an oligomeric GPCR is not required for arrestin binding. Below, we discuss our results in relation to the stoichiometry of arrestin-rhodopsin complex and possible functional roles for GPCR oligomerization.

It is known that rhodopsin and other rhodopsin-like (family A) GPCRs can form dimers (or oligomers) in the lipid membrane^{13; 15; 16; 17; 18}. Visual arrestin can also form dimers and tetramers in a concentration dependent manner^{40; 41; 42; 43}. Thus, various stoichiometries of rhodopsin-arrestin complex can be imagined (see Fig. 1A). However, our data provide the first clear indication that monomeric rhodopsin is the minimal unit required for arrestin binding (Figs. 4 and 7). Our results, combined with recent spin labeling studies of arrestin showing only monomeric arrestin binds to rhodopsin⁴³, strongly suggested that the stoichiometry of arrestin:rhodopsin complex is 1:1.

This conclusion is completely consistent with our experimental work using purified rhodopsin in detergent (M. E. Sommer and DLF, unpublished data). It is also consistent with the results of a recent study which investigated the stoichiometry of rhodopsin and arrestin based on *in vivo* studies and pull-down assay using ROS membranes¹⁴. In the latter study, the authors concluded an overall stoichiometry of 1:1, but unfortunately their experimental methods could not determine if monomeric rhodopsin binds one arrestin molecule or if their data was due to higher order stoichiometries (such as 2:2 or 4:4 arrestin:rhodopsin, etc.)¹³. In light of our results presented here, it is clear that one rhodopsin molecule can bind one arrestin molecule, consistent with the fact that the maximal amount of arrestin in rod outer segment is close to the amount of rhodopsin¹⁴. However, it is important to note several caveats about our results. They do not rule out a role for arrestin binding to dimeric/multimeric rhodopsin, under some conditions, and they do not prove that wild-type arrestin is incapable of binding to dimeric or multimeric rhodopsin, only that if it does bind, it produces and stabilizes less “extra meta-II”.

We also find that monomeric rhodopsin in nanodiscs can interact with a β -arrestin, β -arrestin 1 (Fig. 7, A and C). This finding suggests a general possibility that monomeric forms of family A GPCRs are sufficient to interact with all of the arrestins, and that multimeric receptors are not required for arrestin binding as a general rule. However, as noted above, our results do not rule out other possible roles for GPCR oligomerization in arrestin function (discussed below).

Previously, studies of rhodopsin, the β -adrenergic receptor and μ -opioid receptor in nanodiscs have all shown that monomeric GPCRs can activate G proteins efficiently^{23; 24; 25; 26; 27}. Those results, along with the results we report here, strongly suggest that dimerization (or oligomerization) is not necessary for interaction of a GPCR with a G protein, or with arrestin, at least for family A GPCRs. Thus, the question remains- what are the functional roles of dimerization/multimerization for family A GPCRs? Our data in Fig. 4 may provide a tantalizing hint. The data show that wild-type arrestin has a higher affinity for monomeric rhodopsin in nanodiscs than it does for oligomeric rhodopsin in liposomes (Fig. 4, E and F). Thus, it is tempting to speculate that the arrestin-rhodopsin interaction may in fact be regulated by a dynamic transition of the receptor between monomeric and oligomeric states. This type of regulation would be consistent with a recent study of the dopamine receptor, which found that agonist-induced G protein activation of the receptor is negatively regulated within its dimer⁴⁴. Another possibility is that the interaction of GPCRs with other GPCR affiliated proteins may require dimerization of GPCRs⁴⁵. Clearly, further

biochemical and biophysical studies are required to elucidate these myriad possible functional roles for GPCR dimerization.

Materials and Methods

Materials

POPC and POPG were obtained from Avanti Polar Lipids, octyl glucoside and sodium cholate were obtained from Anatrace. Cy3-maleimide and Cy5-maleimide were purchased from GE Healthcare. Frozen bovine retinas were purchased from Lawson and Lawson, Inc. All other chemicals and reagents were purchased from Sigma.

Preparation of ROS and purification of rhodopsin

ROS and highly phosphorylated ROS were prepared from bovine retinas as described previously⁴⁶. Rhodopsin was purified from ROS as described previously¹⁷.

Labeling of rhodopsin with Cy3- and Cy5- maleimide

Solubilized rhodopsin in buffer A (20 mM HEPES, 140 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1 mM MnCl₂, pH 7.0) containing 4% octyl glucoside was mixed with ConA-Sepharose (GE Healthcare) for 3~4 hours at 4 °C. The mixture was washed using 20 mL of buffer A containing 1.46 % octyl glucoside and then mixed with a 7-fold excess of the Cy3- or Cy5- maleimide label. The slurry was then nutated for 16 hours at 4 °C, and the mixture then washed extensively using buffer A containing 1.46 % octyl glucoside. Finally, rhodopsin was eluted with buffer A containing 1.46 % octyl glucoside and 0.3 M methyl- α -D-mannopyranoside. The labeling efficiency was calculated from the absorbance spectra and the known extinction coefficients of rhodopsin and the labels as previously described¹⁷.

Construction, expression and purification of MSP

We designed a synthetic gene for the MSP based on the published work of Sligar's group³⁰. Essentially, their MSP, which they call MSPE3D1, is an optimized derivative of human apolipoprotein A-1 (apo A-1). MSPE3D1 contains additional helices introduced into the middle of apo A-1, in order to increase the length of the MSP "belt" around the phospholipids bilayer, and it also includes a His-tag and TEV protease cleavage site on the N-terminus to facilitate purification³⁰. In addition to those features, in our gene, we mutated three tryptophan residues at positions 41, 77 and 143 in MSP1E3D1 with phenylalanine residues in order to decrease intrinsic Trp fluorescence. The molecular coefficient of MSPE3D1 is reported as 29,910²³, and that of our tryptophan substituted mutant MSPE3D1-F1 is a 13,410. We also optimized the codons to increase expression levels and simplify cloning strategies. This optimized "Trp-less" MSP gene, which we call MSPE3D1-F1, was expressed and purified according to ref.³⁰. Briefly, the MSP1E3D1-F1 was expressed in *Escherichia coli* BL21 and purified using Ni-NTA-sepharose (Qiagen). The 6x His tag on the N-terminus was cleaved using TEV protease and the cleaved MSP was obtained as the flowthrough from the nickel column.

Preparation and purification of nanodiscs

Phosphorylated or unphosphorylated rhodopsin (solubilized in 1.46 % octyl glucoside), MSP and lipid (solubilized in 0.5 M sodium cholate) were mixed with ~2/3 volume of Bio-beads SM-2 (Bio-Rad) overnight at 4 °C. The molar ratio of Rh:MSP:lipid was set to 0.1:1:75. In order to make nanodiscs containing predominantly dimeric (or oligomeric) rhodopsins (see Fig. 5), the molar ratio was changed to 1:1:41.25 according to ref.²⁴. The Bio-beads were removed by centrifugation (1,000 g, 1 min). Liposome samples were prepared without MSP in the same methods described above. After reconstitution of the

rhodopsin into nanodiscs or liposomes, the samples were injected onto a Superdex 200 column (GE Healthcare) (column volume of 23.55 mL) run at 0.5 mL/min. For the nanodisc samples, fraction was collected that corresponds to a diameter of ~12 nm (see Fig. 2A). The diameter of nanodisc was determined by Gel Filtration Calibration Kit HMW (GE Healthcare) (see Fig. 2A). The collected sample was concentrated by Amicon Ultra 0.5 mL Centrifugal Filters (10,000 MWCO, Millipore).

Electron Microscopy of nanodiscs

Nanodiscs (~1 mg/mL) were lifted onto ultrathin carbon film/holey carbon 400 Mesh copper grids (Ted Pella 01824) for 3 min, wicked, rinsed in water for 1 minute, stained for 45 sec in filtered 1.33 % uranyl acetate, wicked, stained again and air dried. Samples were imaged at 100 kV on a Philips CM120 TEM transmission electron microscope. Images were collected as 1024 × 1024 pixel 14-bit gray-scale Gatan Digital Micrograph 3 (DM3) files on a Gatan 794CCD multiscan camera and converted into 8-bit gray-scale TIF images using the program Digital micrograph 3.4.

Expression and purification of arrestins

Both the bovine visual arrestin cDNA containing a single glycine inserted at position 2 (a generous gift from Dr. Vsevolod Gurevich) and the bovine β -arrestin 1 cDNA were cloned in the pET15b vector (Invitrogen). Both these proteins were expressed in *E. coli* BL21(DE3) and purified using a two step ion-exchange chromatography as described previously²⁸.

The constitutively active visual arrestin mutant R175E was cloned at the C-terminal of a modified 77 amino acid prodomain region of subtilisin BPN' (proR8FKAM), in pG58 expression vector (a generous gift from Dr. Kevin Ridge)^{47, 48}. The R175E arrestin fused with the prodomain was expressed in BL21(DE3)-RP cells (Stratagene). The cells were grown in 1 L of LB media in the presence of 100 μ g/mL ampicillin at room temperature to A₅₅₀ of 0.3, and then induced with 30 μ M IPTG for 12–14 h at room temperature. The cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl, 5 mM β -mercaptoethanol, 0.1 mM PMSF, and a protease inhibitor tablet (Roche) and then disrupted by sonication. The supernatant, obtained after centrifuging the cell lysate at 100,000 g for 45 min, was loaded onto a 5 mL Profinity eXact column (Bio-Rad). The column was washed with 20 column volumes of 100 mM sodium phosphate, pH 7.2 and 10 column volumes of 100 mM sodium phosphate, 300 mM sodium acetate, pH 7.2. The cleavage of arrestin from the prosubtilisin tag was initiated by passing one column volume of 100 mM sodium phosphate, pH 7.2 containing 100 mM sodium fluoride (elution buffer). The fluoride-mediated cleavage reaction was allowed to occur for 1 hr at room temperature. Tag-free arrestin was eluted off the column by passing 5 column volumes of the elution buffer and was further purified by cation exchange chromatography using a 1 mL HiTrap Heparin column.

FRET measurements to test for presence of oligomeric rhodopsin

Fluorescence excitation and emission spectra were recorded using a PTI steady-state fluorescence spectrophotometer. Emission spectra were measured by exciting the donor (Cy3) at 520 nm (0.5-nm bandpass) while scanning the fluorescence intensity of the acceptor (Cy5) (10-nm bandpass) at 10 °C. Excitation spectra were measured by collecting emission from the acceptor (Cy5) at 670 nm (10-nm bandpass) while scanning the excitation spectrum of the donor (Cy3) (0.5-nm bandpass). All measurements were performed in the dark state, immediately after light activation (with >500 nm light) at 10 °C. Total sample volume was 250 μ L in a 4mm*4mm cuvette. Further details about measuring and calculating FRET are provided in our previous paper¹⁷.

Measurement of absorption spectra

Absorption spectra were recorded using a UV-1601 spectrophotometer (Shimadzu). All measurements were performed in the dark state, immediately after 15 sec light activation (with >500nm light) at 10 °C using a T-Q/FOI-1 150w fiber optic illuminator (Techni-Quip).

Orientation of rhodopsin in liposomes as determined by proteolysis

The orientation of rhodopsin in liposomes was assessed using the endoprotease Asp-N, as described previously^{17, 49}. Asp-N specifically cleaves between Gly-329 and Asp-330 in the C-terminus of rhodopsin⁵⁰. Briefly, the liposome samples were incubated with Asp-N at a molar ration 1:20 (Asp-N/rhodopsin) in the dark at room temperature for 4 hours. The reaction was terminated by the addition of SDS-PAGE loading buffer and the samples then subjected to SDS-PAGE and the amount of cleavage determined by densitometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ConA	concanavalin A
FRET	fluorescence energy transfer
GPCR	G protein-coupled receptor
meta-I	metarhodopsin I
meta-II	metarhodopsin II
MSP	membrane scaffold protein
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
POPG	1-palmitoyl-2-oleoyl phosphatidylglycerol
ROS	rod outer segments

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Fig. 1. Cartoon scheme outlining the strategy behind this study

(A) Models showing some possible stoichiometries of the arrestin-rhodopsin complex. Other stoichiometries can also be imagined. (B) In the presence of MSP (purple circles), nanodiscs containing monomeric rhodopsin and lipids are formed. (C) In the absence of MSP, liposomes containing dimeric (or oligomeric) rhodopsin and lipids are formed.

Fig. 2. Characterization of phosphorylated rhodopsin reconstituted into nanodiscs by size exclusion chromatography, absorption spectroscopy and electron microscopy

(A) Purified phosphorylated rhodopsin reconstituted into nanodiscs was resolved on a Superdex 200 size exclusion column (*solid line*, “nanodisc”). In separate experiments, liposome samples were also resolved (*broken line*, “liposome”). For preparing monomeric rhodopsin in nanodiscs, the fraction eluting from 1050 sec to 1290 sec (shaded area) was collected and concentrated. The calibrated Stokes’ diameter scale and void volume (“void”) for this column are shown at the top. (B) Electron micrograph of nanodiscs. Expanded images are shown at the bottom of this panel. Scale bars are also shown. (C) SDS-PAGE analysis of a peak collected in panel A shows both phosphorylated rhodopsin (“Rh-P”) and MSP proteins (*center lane*, “nanodisc”). The nanodisc sample contains Rh-P and MSP at a 1:10 molar ratio (see “Materials and Methods”). A liposome sample (without MSP) was also loaded as a control, and shows only phosphorylated rhodopsin (*left lane*, “liposome”). Molecular sizes of standard proteins are also indicated (*right lane*). Proteins were visualized using Coomassie staining. (D) Absorption spectra of phosphorylated rhodopsin in nanodiscs collected from the size exclusion chromatography indicate the rhodopsin in nanodiscs retains its light-sensitive properties. Solid (“dark”) and broken (“+light”) lines indicate spectra before and after irradiation, respectively. (E) Absorption spectra of phosphorylated rhodopsin in liposomes. Solid (“dark”) and broken (“+light”) lines indicate spectra before and after irradiation, respectively. The *insets* in panels D and E show the difference spectra of the samples (after irradiation minus before irradiation). Spectra in panels D and E were recorded at 10 °C and pH 7.4. Lipid composition in nanodisc and liposome is POPC/POPG at a ratio 3:2.

Fig. 3. FRET and absorption spectral analyses indicate rhodopsin is monomeric in these nanodiscs

(A) FRET studies showing strong and weak Rh-Rh energy transfer in liposomes and nanodiscs, respectively. Fluorescence excitation spectra of nanodiscs (red line) and liposomes (green line) containing Cy3- and Cy5- labeled rhodopsin. Calculated FRET efficiencies¹⁷ are 26% (in liposomes) and 9% (in nanodiscs). The spectra are normalized to a maximal fluorescence intensity of 1.0. (B) Fluorescence emission spectra of nanodiscs (red line) and liposomes (green line) containing Cy3- and Cy5- labeled rhodopsin. The arrow heads in panels A and B indicate the strong FRET signal observed in liposomes. Spectra were recorded at 10 °C and pH 7.4. Lipid composition in nanodisc is POPC/POPG at a ratio 3:2. The spectra are normalized to a maximal fluorescence intensity of 1.0. (C) Absorption spectrum of rhodopsin in nanodisc, purified from excess MSP using ConA-Sephadex. The extinction coefficient of rhodopsin at 500 nm is 40,000 and purified rhodopsin should have an $A_{280}:A_{500}$ ratio at 1.7²⁵. The molecular coefficient of our tryptophan substituted mutant MSPE3D1-F1 is 13,410. In the present example, the $A_{280}:A_{500}$ ratio of purified nanodisc was 2.47, indicating a molar ratio of rhodopsin:MSP of about 1:2.3, which is consistent with one rhodopsin per the two MSP proteins required to assemble a nanodisc.

Fig. 4. Arrestin can bind to monomeric rhodopsin as indicated by “extra meta-II” formation induced by the presence of WT arrestin and the constitutively active arrestin mutant R175E (A–D) Difference absorption spectra, obtained by subtracting the spectra before irradiation from the spectra after irradiation in the presence or absence of visual arrestin. (A) Phosphorylated rhodopsin (625 nM) in nanodiscs with visual arrestin WT; (B) phosphorylated rhodopsin (625 nM) in liposomes with visual arrestin WT; (C) phosphorylated rhodopsin (625 nM) in nanodiscs with visual arrestin R175E mutant; (D) phosphorylated rhodopsin (625 nM) in liposomes with visual arrestin R175E mutant. Arrestin concentration is indicated. Surprisingly, more “extra meta-II” formation was observed for arrestin binding to monomeric rhodopsin in nanodiscs than for oligomeric rhodopsin in liposomes. (E, F) Plots of the difference absorbance between 390 nm and 426 nm (from the difference spectra) as a function of arrestin concentration. Red circles and blue triangles indicate the values in the presence of visual arrestin WT and R175E mutant, respectively. (E) nanodisc samples; (F) liposome samples. Rhodopsin concentration was 625 nM. Spectra were recorded at 10 °C and pH 7.4. Lipid composition in nanodisc is POPC/POPG at a ratio 3:2. The error bars reflect S.E. for two separate experiments.

Fig. 5. Fluorescence and absorption spectroscopic properties of nanodisc containing predominantly multiple rhodopsin molecules per nanodisc

(A) Fluorescence excitation spectrum. Calculated FRET efficiency is 33%. For comparison, the spectra are normalized to a maximal fluorescence intensity of 1.0. (B) Fluorescence emission spectrum. The arrow heads in panels A and B indicate the strong FRET signal. The spectra are normalized to a maximal fluorescence intensity of 1.0. (C) Difference absorption spectra (obtained by subtracting the spectra before irradiation from the spectra after irradiation) in the presence of visual arrestin WT (red line, “+WT”), R175E mutant (blue line, “+R175E”) or absence of visual arrestin (black line, “-Arr”) are shown. Rhodopsin and arrestin concentrations were 625 nM and 4 μ M, respectively. Spectra were recorded at 10 $^{\circ}$ C and pH 7.4. Lipid composition in nanodisc is POPC/POPG at a ratio 3:2.

Fig. 6. A lipid with an acidic head group (POPG) enhances arrestin binding to monomeric rhodopsin in nanodiscs

(A, B) Difference absorption spectra after minus before irradiation in the presence of visual arrestin WT (red line, “+WT”), R175E mutant (blue line, “+R175E”) or absence of visual arrestin (black line, “-Arr”) are shown. The relative amount of POPC/POPG is indicated. Rhodopsin and arrestin concentrations were 625 nM and 2 μ M, respectively. Spectra were recorded at 10 $^{\circ}$ C and pH 7.4. (C, D) Difference absorbance between 390 nm and 426 nm in the difference spectra under the same conditions as in panels A and B. The relative amount of POPC/POPG is indicated. Black, red and blue bars indicate the values in the absence of arrestin, and in the presence of arrestin WT and R175E mutant, respectively. The error bars reflect S.E. for two separate experiments.

Fig. 7. β -arrestin can also bind to monomeric rhodopsin in nanodiscs, as indicated by “extra meta-II” formation in the absorption spectra

(A) Difference absorption spectra of phosphorylated rhodopsin (625 nM) in nanodiscs with (solid line, “+ β -Arr”) or without (broken line, “- β -Arr”) β -arrestin (2 μ M). (B) Difference absorption spectra of unphosphorylated rhodopsin (625 nM) in nanodiscs with (solid line, “+ β -Arr”) or without (broken line, “- β -Arr”) β -arrestin (2 μ M). Spectra were recorded at 10 $^{\circ}$ C and pH 7.4. Lipid composition in nanodisc is POPC/POPG at a ratio 3:2. (C, D) Difference absorbance between 390 nm and 426 nm in the difference spectra of phosphorylated (C, *Rh-P*) and unphosphorylated (D, *Rh*) rhodopsin under the same conditions as in panels A and B. Light and dark gray bars indicate that values in the absence of β -arrestin and in the presence of β -arrestin, respectively. The error bars reflect S.E. for two separate experiments.