Format for ANSWERING REVIEWERS

January 30, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7552-review.docx).

Title: Binding of Rhodopsin and Rhodopsin Analogues to Transducin, Rhodopsin kinase and Arrestin-1

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Name of Journal: *World Journal of Biological Chemistry*

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Major changes were incorporated into the manuscript following the comments and the concerns of the reviewers, and the manuscript has been improved accordingly:

1. Reviewer 1:

1.1. In order to compare the effect of phosphorylation on binding to arrestin-1, data displaying that basal amounts of arrestin-1 interacted with rhodopsin, isorhodopsin and the 13-cis-retinal-rhodopsin complex, both in the dark and under illumination, are now shown and quantified in Fig. 7 (Fig. 6 in the original version).

1.2. A section was added to discuss the effect of phosphorylation on binding to arrestin-1 and its potential molecular mechanism.

1.3. Panels A, B and C are now labeled in Fig. 3.

1.4. Black and white bar graphs are labeled in Figs. 5, 6 and 7 (Figs. 4, 5 and 6 in the original version).

1.5. Dark and light labeling is now properly tagged in Fig. 6A (Fig. 5A in the original version).

2. Reviewer 2:

2.1. The referee mentions that we "failed to suggest the physiological importance of such study" and asks "what's the potential importance to study the 9-cis and 13-cis isomers?". To study ligand binding pockets in proteins, specific analogues with systematically altered chemical property in their structural moieties have usually been employed to establish structure-activity relationships with regard to their functional groups. Although rhodopsin uses the 11-cis form of retinal exclusively as the chromophore, under certain pathological conditions the 9-*cis* configuration of retinal is observed, which generates isorhodopsin. In contrast, the 13-*cis* configuration of retinal is never observed in vision and as such is not physiologically relevant in the visual process. Nevertheless, all-*trans*-retinal is an essential component of type I, or microbial, opsins such as bacteriorhodopsin, channelrhodopsin, and halorhodopsin, and in these receptor proteins, light causes the all-*trans*-retinal to become 13-*cis*-retinal, which then cycles back to all-*trans*-retinal in the dark state. In these microbial opsins, 13-*cis*-retinal is physiologically crucial. In the present manuscript, the 9-*cis*- and 13-*cis*-retinal analogues have been used to probe the rhodopsin chromophore binding pocket and to study ligand-protein relationships to better

understand the photochemical *cis-trans* isomerization of rhodopsin. We focused on the interactions of these rhodopsin analogues with transducin, rhodopsin kinase and arrestin-1, since very little is known about the binding and association of rhodopsin analogues with other proteins of the visual cascade. This issue is discussed now in the revised manuscript.

2.2. As requested, the purified components, such as transducin, rhodopsin and arrestin-1, are now examined and the results are provided (Figs. 2 and 4). Fig. 2A shows the protein composition of crude and washed ROS by SDS-PAGE analysis; and Figs. 2B and C illustrate the presence of rhodopsin in crude and washed ROS by Western blot and absorption spectra, respectively. In addition, transducin and arrestin were purified to homogeneity (Figs. 4A and C), and the functionality of rhodopsin kinase, which is contained in the enriched fraction of rhodopsin kinase, was also confirmed (Fig. 4B).

2.3. The discussion section was improved. Now it is focused in discussing the results, their meaning, importance and suggestions.

2.4. Figure legends were improved and now provide sufficient explanation including experimental conditions that let readers understand the figures independently of the text.

2.5. Each experiment was repeated three times independently and the number of repeats is now indicated in all figure legends.

2.6. I disagree with the reviewer when mentions that it will be more convincing to show the co-immunoprecipitation results of transducin, rhodopsin, etc. Co-immunoprecipitation is just one of multiple experimental procedures that can be used to evaluate protein-protein interactions. Here, we used specific functional assays that are generally employed when evaluating the interactions and associations between proteins involved in the photoexcitation cascade. The tests performed in the present work that include radioactive (GMpNp binding and phosphorylation) and sedimentation analyses, are more precise and unambiguous that a co-immunoprecipitation. Co-immunoprecipitation assays will require a high amount of purified proteins and will just confirm our findings.

2.7. As explained in 2.5, all experiments including those that are shown in Figs. 6 and 7 (Figs. 5 and 6 in the original version) were repeated three times. Error bars and statistical significance are now incorporated in the figures.

2.8. In all cases, rhodopsin (or the corresponding rhodopsin analogue) was used as the internal control. The amount of reconstituted rhodopsin (or rhodopsin analogue) was employed to normalize all reported values. However, although the ratio of interest protein against internal control was used, we decided to report our results as arbitrary units in Figs. 6B and 7C given that these results were determined by densitometry.

3. Reviewer 3:

3.1. As explained in 2.2, the proteins used as substrates for in vitro assay were verified either functionally or by immunoblotting using specific antibodies.

3.2. As described in 2.4, figure legends were improved and now include more information, such as experimental conditions, labeling, etc. In addition, P-values were also incorporated in bar graphs (See 2.7).

3.3. Positive and negative controls are included in all figures.

4. Reviewer 4:

4.1. As requested by the referee, the reproducibility of the experiments was validated by expressing the quantitative data as mean ± SD of several experiments. Error bars are now defined and included in all figures.

4.2. As explained in 1.4, white and dark bars of the histograms are now clearly labeled in Figs. 5, 6 and 7 (Figs. 4, 5 and 6 in the original version).

4.3. The data presented in Fig. 6 (Fig. 5 in the original version) was generated from two

different SDS-PAGE experiments, which were carried out in triplicates. The different lanes were separated in order to arrange and organize the figure in a comprehensible and logical manner.

5. Reviewer 5:

5.1. We agree with the reviewer when mentions that the interactions of rhodopsin and its analogues (especially 13-*cis*-rhodopsin) with transducin, rhodopsin kinase and arrestin-1 should be further studied by other assays like binding anisotropy, in order to analyze binding constants and binding kinetics. Unfortunately, there is not an anisotropy instrument in the whole country of Venezuela.

5.2. The 13-*cis*-retinal isomer is neither the main not one of the natural rhodopsin photointermediates in the body.

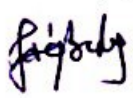
5.3. The referee asked: Why 13-*cis*-rhodopsin behaves like an active rhodopsin?. We don't know the answer to this question, but feel that finding that 13-*cis*-retinal-rhodopsin is pseudo-active in the dark is the most important result of this work. It is plausible that the structure of 13-*cis*-retinal-rhodopsin adopts a tridimensional conformational fold that mimics the active photoproduct of rhodopsin and, consequently, is capable of interacting with transducin, rhodopsin kinase and arrestin-1 even without illumination.

5.4. The reviewer comments that "it is mentioned in the discussion part that opsin can activate transducin 10 fold higher in the presence of retinal that resembles 13-*cis* isomeric form. It may be important to show that the same occurs with the reconstituted 13-*cis* retinal". Although it was only incorporated in the last figure (Fig. 7), experiments using only opsin were also carried out during our whole investigation. Opsin behaved as inactive rhodopsin (or rhodopsin under dark conditions) when measuring binding to transducin, rhodopsin kinase and arrestin-1. In the other hand, 13-*cis*-rhodopsin behaved like active rhodopsin even in the dark.

5.5. The referee requested the incorporation of the absorption spectra of meta I in Fig. 3 as a control. Although it has been reported that upon illumination rhodopsin thermally relaxes through a series of distinct photointermediates, each with characteristic UV/visible absorption maxima, most of these intermediates can only be trapped by using ultra freezing temperatures. This is the case for meta I, which can be freeze-trapped at - 40 ℃ and at temperatures below the phase transition temperature of the surrounding lipids. Consequently, it is not viable for us to generate the meta I photointermediate under our normal laboratory setting.

Thanks in advance for reconsidering this manuscript for publication in the *World Journal of Biological Chemistry*.

Sincerely yours,



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