Flavonoids enhance rod opsin stability, folding and self-association by directly binding to ligand-free opsin and modulating its conformation

Joseph T. Ortega¹, Tanu Parmar¹ and Beata Jastrzebska¹#

¹Department of Pharmacology, Cleveland Center for Membrane and Structural Biology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106

Running Title: Effects of flavonoids on rhodopsin properties

#To whom correspondence may be addressed: Beata Jastrzebska, Ph.D. Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave, Cleveland, OH 44106-4965, USA; Phone: 216-368-4631; Fax: 216-368-1300; E-mail: bxj27@case.edu

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ABSTRACT: Rhodopsin (Rho) is a visual G protein-coupled receptor (GPCR) expressed in the rod photoreceptors of the eye, where it mediates transmission of a light signal into a cell and converts this signal into a nerve impulse. More than a 100 mutations in Rho are linked to various ocular impairments, including retinitis pigmentosa (RP). Accordingly, many efforts are directed toward developing ligands that target Rho and improve its folding and stability. Natural compounds may provide another viable approach to such drug discovery efforts. The dietary polyphenol compounds, ubiquitously present in fruits and vegetables, have beneficial effects in several eye diseases. However, the underlying mechanism of their activity is not fully understood. In this study, we used a combination of computational methods, biochemical and biophysical approaches, including bioluminescence resonance energy transfer (BRET), and mammalian cell expression systems to clarify the effects of four common bioactive flavonoids (quercetin, myricetin, and their mono-glycosylated forms, quercetin-3-rhamnoside and myricetin) on rod opsin stability, function and membrane organization. We observed that by directly interacting with ligand-free opsin, flavonoids modulate its conformation, thereby causing faster entry of the retinal chromophore into its binding pocket. Moreover, flavonoids significantly increased opsin stability, most likely by introducing structural rigidity and promoting receptor self-association within biological membranes. Of note, the binding of flavonoids to an RP-linked P23H opsin variant partially restored its normal cellular trafficking. Together, our results suggest that flavonoids could be utilized as lead compounds in the development of effective non-retinoid therapeutics for managing RP-related retinopathies.

G protein-coupled receptors (GPCRs) are the largest group of membrane receptors that transmit signals across the plasma membrane into the cell (1, 2). GPCRs are activated by multiple ligands such as small organic molecules, protons, ions, peptides, lipids, and light (3). Binding of the ligand triggers a conformational change in the receptor, allowing for coupling of the specific heterotrimeric G protein that further amplifies the
signal and activates downstream effectors leading to the biological responses (1,4).

GPCRs are expressed in most tissues and contribute to almost all physiological processes in the human body. They are also important targets of about 30-40% of all medications available on the market (5). Structural information available for many GPCRs enables the designing of more specific drugs targeting these particular receptors. However, often due to high homology of the orthosteric ligand binding pockets among GPCRs within the specific class, selectivity is limited and can lead to unwanted side effects. Thus, more work is needed to develop novel, more explicit therapeutic compounds. GPCR activity can also be modulated allosterically, either by endogenous modulators or exogenous natural products and synthetic molecules. Discovering new allosteric modulators of GPCRs is another promising avenue to explore for achieving higher drug-receptor specificity. In fact, several such modulators are already FDA-approved medications (6-8).

Rhodopsin (Rho) is a light-sensing GPCR. Its major function is the absorption of light photons and transduction of their absorption into a neural impulse (9,10). Over 100 mutations identified in Rho are linked with various ocular impairments, including congenital stationary night blindness (CSNB) and retinitis pigmentosa (RP) (11,12). There is a growing interest in finding novel ligand molecules that would improve the folding and stability of particular Rho mutants (13,14). However, small molecule synthetic drugs can often cause toxicity despite their specific modulatory effects. Thus, utilization of natural compounds could be an alternative approach to drug discovery efforts.

The medical use of plants or plant extracts to prevent or treat various diseases has a long history and is still commonly practiced in developing countries (15-18). The natural dietary polyphenol compounds, in particular flavonoids, ubiquitously present in fruits and vegetables, improve sight in several eye-related diseases including age-related macular degeneration (AMD), glaucoma, RP and diabetic retinopathy, most likely due to their anti-oxidant, anti-inflammatory or anti-apoptotic properties (19-23). Only limited studies indicate that flavonoid compounds can interact with GPCRs. For example, myricetin can act as an agonist of the glucagon-like peptide-1 (GLP-1) receptor, which plays a critical role in the regulation of glucose homeostasis (24). Also, iso-flavone and flavone compounds are able to inhibit the histamine H3 receptor that is expressed mainly in the central nervous system (25,26). In the case of Rho, as found in this study and demonstrated previously, flavonoids can interact with the opsin apoprotein resulting in the structural changes that enhance the access of native retinal to the ligand-binding pocket (27-29). Moreover, flavonoids interacting with GPCRs can modify their membrane organization through the formation of aggregates (30). However, the effect of flavonoids on the stability, function and oligomeric membrane organization of Rho is not entirely clear. Therefore, here we describe studies of the effects of four common bioactive flavonoids, quercetin 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxyxchromen-4-one and myricetin 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one, and their monoglycosylated forms (31,32), on rod opsin stability, its function, and membranous supramolecular assembly in vitro and in living cells.

**RESULTS**

**Computational analyses of the interaction between opsin and flavonoids**

The crystal structure of bovine rod opsin (PDB code 3CAP (33)) was used as a template to identify the possible interaction sites of quercetin, myricetin, quercetin-3-rhamnoside, and myricetrin with the ligand-free opsin, and the pocket analysis was performed with the CASTp software (Fig. 1a). Three potential binding pockets were identified, of which one was the orthosteric retinal-binding pocket and two were located on the surface of opsin. The extracellular binding pocket 1 was present between transmembrane helix (TM) 5, TM6 and extracellular loop (ECL) 2, and the extracellular binding pocket 2 was located between TM2, TM3, and ECL1 (Fig. 1a). Blind Docking (34) of quercetin (as a model flavonoid) to opsin structure co-validated the results obtained with the CASTp software and defined the coordinates of the binding sites (Table 1). Quercetin was accommodated best within the orthosteric binding site with the lowest binding free energy of -9.3
kcal/mol. The calculated binding energies of quercetin to the external binding sites were higher as compared to the orthosteric site: -7.9 kcal/mol and -6.5 kcal/mol for pockets 1 and 2, respectively. Based on the best binding energies and the conformation of the pocket, the orthosteric site and the pocket 1 were selected to develop the further analyses. Molecular docking of myricetin, quercetin-3-rhamnoside, and myricetin, into the opsin structure, revealed that all these flavonoid compounds, comparably to quercetin, accommodated into the orthosteric binding pocket with lower binding energy than to the external binding sites. Additionally, the glycosylated flavonoids displayed a change in the isoflavone group orientation within the orthosteric binding pocket as compared to their aglycone forms. The aglycone compounds, quercetin, and myricetin, but not the glycosylated quercetin-3-rhamnoside and myricetin, could also fit into the TM5-TM6-ECL2 external binding site (pocket 1) and their isoflavone group protruded outside the binding site (Fig. 1b and c). The enlarged size of the glycosylated flavonoid molecules occupied pocket 1, while 60% was exposed to the solvent, thus suggesting low stability of the interaction between protein and the compound over time. All flavonoid compounds formed mainly hydrogen bonds and π-π interactions with the protein side chains, within both the orthosteric site and the external binding pocket 1. However, fewer such interactions were detected in the external binding pocket as compared to the orthosteric binding site. The details of the interactions between flavonoids and opsin molecule are shown in Fig. 2 and listed in Table 1.

**Effects of flavonoids on the stability of opsin**

Ligand-free opsin is highly unstable *in vitro*, and *in vivo*, its excess accelerates retinal degeneration (35-37). The binding of the natural ligand or its analogs significantly increases the stability of opsin (38). The melting temperature of opsin in the rod outer segment (ROS) disk membranes was reported at 55.9 °C, while a 16-degree higher temperature of 71.9 °C is required to denature Rho (38). Based on our computational analyses, flavonoids could interact with the opsin apoprotein by either binding into the orthosteric site or the spatially distant, extracellular vestibule of the receptor, suggesting that flavonoids potentially could have stabilizing effects on opsin similar to those of retinal chromophore. Interestingly, treatment of ROS membranes containing opsin with aglycone flavonoids resulted in an increase of opsin’s melting temperature from 55.4±0.4 °C to 61.0±0.2 °C in the presence of quercetin and to 58.9±0.4 °C in the presence of myricetin (Fig. 3a and Table 2). An increasing, dose-dependent stabilizing effect of quercetin was detected at 0.1-10 µM concentrations range with a half-maximal effective dose the EC50=0.95±0.05 µM. The effect of quercetin was inversely proportional at higher (100-500 µM) concentrations. In the presence of myricetin, the stabilization effect was dose-dependent and observed within the 100-500 µM concentrations range, reaching the highest melting temperature at the highest concentration evaluated. The calculated EC50 of myricetin was 180±8.9 µM. Despite this stabilizing effect of both quercetin and myricetin, neither of them could reach the level of stabilization produced by the 9-*cis*-retinal isochromophore. Surprisingly, glycosylated flavonoids, quercetin-3-rhamnoside, and myricetin showed no beneficial effects on opsin stability (Fig. 3a and Table 2). Moreover, binding of quercetin and myricetin to opsin prior to its incubation with 9-*cis*-retinal and regeneration of isoRho revealed cooperative effects with an increase of melting temperature to nearly 80 °C at the highest doses for both flavonoids, while the glycosylated flavonoids showed no beneficial effect (Fig. 3b and Table 3). Interestingly, none of these tested flavonoids showed any stabilizing effects for retinal-bound isoRho (Fig. 3b, black).

Prolonged incubation of opsin at room temperature results in loss of its ability to bind the retinal chromophore. After 4 h at room temperature, only 50% of opsin could bind 9-*cis*-retinal and form isoRho when compared to opsin kept at 0 °C. However, in the presence of flavonoids, due to their stabilizing effects, the same 50% regeneration of isoRho could be reached after extended incubation times. The regeneration half-time of 8 h and 5 h was observed for 1 µM and 100 µM quercetin, respectively. In the case of myricetin, a low 1 µM concentration did not improve opsin’s...
half-life. However, at 100 μM concentration, the regeneration half-time extended to about 14 h (Fig. 3c). These results correlate with the changes of the opsin’s melting temperature detected in the presence of quercetin and myricetin.

Effects of flavonoids on the binding of retinal chromophore

To examine whether flavonoids change the rate of 9-cis-retinal binding to rod opsin, the pigment regeneration after treatment of opsin membranes with each flavonoid was monitored by UV-visible spectroscopy (Fig. 4). The maximum absorption at 487 nm that appeared after incubation of opsin with 9-cis-retinal is due to the formation of the Schiff base linkage. This was used to quantify the regenerated isoRho and calculate the rate of its regeneration. As noted, treatment with flavonoids increased the rate of regeneration in the presence of each tested flavonoid, in a concentration-dependent manner from about 4 to 2 min at the highest (500 μM) flavonoid concentrations (see Table 4 for details). This finding suggests that the binding of flavonoids results in a conformational change within the opsin structure permitting better accommodation of the retinal chromophore, and thus its faster binding as compared to non-treated opsin.

The binding of flavonoids to the orthosteric retinal-binding pocket was further confirmed by using the Trp fluorescence to monitor the changes within the chromophore-binding pocket in bovine opsin. In Rho, the intrinsic Trp fluorescence at 330 nm is quenched by the natural ligand 11-cis-retinal and increases upon its light-induced release from the retinal-binding pocket. This increased Trp fluorescence could be quenched by the retinal analogues, including 9-cis-retinal. Molecular docking revealed close proximity of flavonoid accommodated in the orthosteric binding pocket to Trp265 (Fig. 2). Interestingly, the quenching of opsin’s Trp fluorescence at 330 nm was observed in the presence of each tested flavonoid in a concentration-dependent manner. Almost full quenching of the Trp fluorescence was reached at the highest flavonoid concentration. Thus, this result suggests that flavonoids can either bind into the chromophore-binding pocket of opsin or their binding to the allosteric site changes the conformation of the orthosteric ligand-binding site. None of the flavonoids prohibited the binding of 9-cis-retinal. Addition of 9-cis-retinal to the flavonoid-bound opsin samples promoted further decrease of the Trp fluorescence and resulted in the full quenching of Trp fluorescence independent of the flavonoid concentration, suggesting that both flavonoid and 9-cis-retinal could accommodate into the opsin molecule (Fig. 5).

Effects of flavonoids on the spectral properties of isoRho

The UV-visible absorption spectra of flavonoid-bound, immunoaffinity-purified isoRho revealed the clear effect of aglycone compounds on its spectral properties, while glycosylated flavonoids had no significant effect. Titration with an increasing concentration of both quercetin and myricetin caused about 20% decrease of the absorption maximum and an increase in the ratio of absorption at 280 nm to 487 nm at the highest concentration. This finding suggested incomplete binding of 9-cis-retinal, presumably due to the presence of the flavonoids within the chromophore-binding pocket or a conformational change, introduced by an allosteric modulation, affecting the total amount of the regenerated isoRho (Fig. 6a). Additionally, an increase in absorbance at about 360 nm was observed, characteristic for flavonoids and related to increasing flavonoid concentrations. A similar increase in the absorbance at 360 nm was also observed in the immunoaffinity-purified, ligand-free opsin samples treated with these flavonoids (Fig. 6b). No peak within the visible light wavelengths was detected in these flavonoid-bound opsin samples, indicating that flavonoids do not act as visual chromophore analogues when bound to opsin.

The stable binding of quercetin and myricetin to opsin was confirmed by HPLC analysis. Both quercetin and myricetin were detected in flavonoid-bound immunoaffinity-purified opsin and isoRho samples, with the specific retention time at 19.6 min for quercetin and 8.5 min for myricetin (Fig. 6c). The small change in the retention time of quercetin extracted from the protein samples as compared to the standard (18.5 min) could be due to the oxidation...
of this molecule during samples preparation. Pure quercetin and myricetin were used as standards.

**Effects of flavonoids on function of isoRho**

To evaluate the effect of the aglycone flavonoids on the function of the visual receptor, G protein activation *in vitro* and *in situ*, as well as chromophore release (Meta II decay) assays were performed. The rates of light-induced Gactivation by flavonoid-bound purified isoRho were measured *in vitro* as a Trp fluorescence change due to the Gdissociation from the isoRho-G complex upon uptake of GTPγS. Only a slight increase (3-5%) in the activation rates was detected for both quercetin and myricetin-bound isoRho treated with low (1-10 μM) flavonoid concentrations when compared to isoRho. For isoRho samples treated with higher (100-500 μM) flavonoid concentrations, the Gactivation rates were either similar to those of isoRho for quercetin or significantly (30-60%) decreased at 250-500 μM myricetin, possibly due to receptor structural alterations caused by the flavonoid compounds (Fig. 7a and Table 5). Interestingly, in the absence of G, isoRho also couples to G1 in the heterologous expression systems to induce the Gs/*h* signaling cascade. Thus, we evaluated the effect of quercetin and myricetin on cAMP accumulation levels in response to light stimulation in HEK-293 cells stably expressing opsin. Cells were treated with quercetin or myricetin at two non-toxic (1 or 100 μM) concentrations (Fig. 7b) followed by their regeneration with 9-cis-retinal (Fig. 7c). The levels of cAMP were significantly reduced in cells treated either with 9-cis-retinal alone or 9-cis-retinal followed by the flavonoid treatment, which were exposed to light. However, no difference between these samples was detected. The cellular levels of cAMP were not changed by flavonoids alone either in the dark or after light stimulation. Thus together, these findings suggested no major effect of flavonoids on the Rho signaling activation within the physiologic relevant range of concentrations.

The presence of flavonoids had only mild effect on the rates of light-induced chromophore release (Meta II decay), for both quercetin and myricetin-bound isoRho treated with low (1-10 μM) flavonoid concentrations, and they were 3-10% slower as compared to the decay rates of isoRho (Fig. 7d, Table 6). However, in samples treated with higher (100-500 μM) flavonoid concentrations, chromophore release was 13-25% faster than in isoRho, most likely due to the structural changes introduced by the bound flavonoid compounds.

Binding of flavonoids had only a minor effect on chromophore release induced by the conformational changes in the chromophore-binding pocket. Flavonoid-bound purified isoRho released chromophore at rates comparable to those of purified isoRho when incubated at a high constant temperature of 55 °C (Fig. 8 and Table 7).

**Effects of flavonoids on the expression and distribution of opsin**

Although flavonoids increased opsin stability, the question arose whether they also improve the folding and membrane targeting of misfolded opsin mutants? To answer this question, the effects of quercetin and myricetin on the expression, glycosylation pattern and membrane localization of opsin was tested in NIH-3T3 cells stably expressing a P23H rod opsin mutant. The results were compared to WT rod opsin. In humans, the P23H Rho mutation associates with autosomal dominant retinitis pigmentosa (adRP) due to structural instability of opsin. In mammalian cells, P23H rod opsin features immature glycosylation and impaired membrane trafficking, which could be corrected by 9-cis-retinal isochromophore added to the cell culture during opsin biosynthesis (Fig. 9b and Fig. 10a and b) or a recently discovered non-retinoid chaperone YC-001 (13). To test if a comparable effect could be induced by flavonoids, cells expressing either WT or P23H rod opsin were treated with either 1 or 100 μM quercetin or myricetin (concentrations within non-toxic range (Fig. 9a and e)), and then membrane localization of opsin was determined in non-permeabilized cells by fluorescent immunostaining and high-content image analysis. Interestingly, treatment with flavonoids (100 μM quercetin and 1 μM myricetin) resulted in the noticeable movement of the P23H rod opsin mutant to the cell surface as compared with the non-treated cells (Fig. 9b, c, and d). Moreover, the relative ratio of the cell surface to the total protein expression also was significantly increased in these conditions (P<0.0001). As noted, 9-cis-retinal resulted also in an increased membrane fluorescence of WT opsin as compared...
with the non-treated cells (Fig. 9f). Moreover, both quercetin and myricetin at either 1 or 100 µM concentration resulted in similar, although slightly lesser effects as compared with 9-cis-retinal-treated cells (Fig. 9f). Quantification of the fluorescence at the plasma membrane in non-permeabilized cells and the total opsin expression in the permeabilized cells indicated increased membrane localization of WT opsin related to the increased total opsin levels upon treatment with 9-cis-retinal and both flavonoids (Fig. 9g and h). However, the relative ratio of the cell surface to the total protein expression for WT opsin was similar in all the cells regardless of the applied treatment (Fig. 9g and h). Together these findings indicated that flavonoids could enhance biosynthesis of both WT opsin and the P23H RP-linked opsin mutant and improve their membrane targeting in the absence of retinal chromophore.

The SDS-PAGE gel and immunoblotting analyses showed that flavonoids did not affect the glycosylation pattern of WT and P23H rod opsin mutant (Fig. 10). Opsin deglycosylation resulted in a change of opsin migration within the SDS-PAGE gel from about 72 to 55 kDa for P23H rod opsin and 50 to 26 kDa for WT rod opsin. Interestingly, higher concentrations of quercetin led to the formation of WT rod opsin oligomers or aggregates appearing at about 100 and 140 kDa, which were resistant to the treatment with PNGaseF deglycosylase. Thus, this finding suggested the potential role of quercetin in modulation of rod opsin dimeric/oligomeric organization.

**Effect of flavonoids on the membrane oligomeric organization**

Rod opsin has the ability to self-associate, forming dimers and/or higher-ordered oligomers within the membranes (39,40). In order to understand if flavonoids could modulate the oligomeric state of rod opsin in live cell membranes, the bioluminescence energy transfer (BRET) assay was performed in HEK-293 cells stably expressing both opsin•Rluc (donor) and opsin•Venus (acceptor). Likewise for HEK-293S GtT1- and NIH3T3 both, quercetin and myricetin were not toxic to this stable cell line within 0.1-100 µM concentrations range. A significant (P≤0.0001) time-dependent increase of the BRET signal was observed in the cells incubated with either quercetin or myricetin (100 µM), while only small increase was detected in the non-treated control cells after 16 h, likely due to increased cell number (Fig. 11a, left panel). However, no change in the BRET signal was found at earlier time points (2, 4 and 8 h) in the non-treated cells (Fig. 11a, left panel, black bars). Thus, these results suggested the stimulating effect of flavonoids on opsin dimerization/oligomerization. The treatment with quercetin for 2 h resulted in ~30% elevation of the BRET signal, which increased to ~45% after 16 h incubation as compared to the non-treated control cells (Fig. 11a, left panel, blue bars). The effect of myricetin was slightly less prominent with ~18% and ~30% increase of the BRET signal after 2 and 16 h incubation, respectively (Fig. 11a, left panel, red bars). These flavonoid-stimulated dimers/oligomers were sensitive to the detergent and could be disrupted upon addition of DDM, resulting in a decrease of the BRET signal (Fig. 11a, middle and right panels). Interestingly, neither quercetin nor myricetin had an effect on dimerization/oligomerization of isoRho in the cells regenerated with 9-cis-retinal prior to the treatment with flavonoids, thus supporting the *in vitro* results, indicating that flavonoids could affect properties of ligand-free opsin, but not chromophore-bound isoRho (Fig. 11b). Furthermore, to confirm these results the formation of flavonoid-stimulated rod opsin dimer/oligomers was also determined in the transiently transfected HEK-293 cells with a 1:4 donor to acceptor ratio. As determined previously (41,42) and in this study (Fig. 11c), the BRET signal in cells co-expressing opsin•Rluc and opsin•Venus in a 1:4 ratio was maintained within a linear range. Interestingly, quercetin and myricetin enhanced BRET at all examined donor to acceptor ratios (1:2, 1:4, 1:6, and 1:8) with the highest effect at the 1:2 and 1:4 ratios. The effect of flavonoids was less prominent at the opsin expression levels, resulting in the saturation of the BRET signal (Fig. 11c). Likewise, in the cells stably expressing opsin•Rluc and opsin•Venus (acceptor), the BRET signal upon incubation with either quercetin or myricetin (100 µM) was detected. Small increase of BRET was detected in the non-treated control cells after 16 h, likely due to increased cell number (Fig. 11d, left panel, black bars).
DISCUSSION

Steady biosynthesis and folding of Rho are critical for the formation of ROS in the retinal tissue of the eye, its correct morphology, and its function. Imbalanced biogenesis or misfolding of this visual receptor associates with retinal degeneration and visual impairments in several pathologies including RP (43). Pharmacological chaperones could preclude these pathological effects by enhancing Rho stability, improving folding and restoring its function. Thus, development of novel analogs of the native retinal chromophore, 11-cis-retinal, gained appreciable interest within the past decade (44-48). Multiple investigations showed the therapeutic potential of 9-cis-retinal and 11-cis-6-membered-ring-retinal analogs against retinopathies based on in vitro and in vivo studies in animal models of retinal diseases (49,50). Supplementation with a precursor of 11-cis-retinal chromophore, vitamin A, also displayed beneficial effects in patients with RP (51).

However, prolonged retinoid therapy could disturb the retinoid homeostasis and cause toxicity (52). To overcome this limitation, the development of non-retinoid pharmacological chaperones is indispensable. A discovery of a novel non-retinoid modulator of rod opsin, YC-001, exhibiting promise in treating retinal disorders linked with disrupted Rho homeostasis was recently reported (13). However, further studies developing a specific retina-targeting delivery system of YC-001, enabling steady release of this compound in the eye, is still required due to its fast clearance rates. An alternative promising approach for preventing or delaying retinal degeneration could be the use of natural products as a source of the bioactive compounds such as flavonoids. Indeed, these polyphenolic plant compounds could improve sight in several eye-related diseases (19-23). However, the mechanism of action of these compounds is still not fully understood. As suggested previously, flavonoids could potentially interact with rod opsin and act as allosteric modulators of its function (27-29,53). Yet, a comprehensive study deciphering the underlying mechanism enhancing vision by this class of compounds is lacking. Thus, to clarify the role of flavonoids in modulating rod opsin properties, we studied the effect of four flavonoids: quercetin, myricetin, and their glycosylated forms, quercetin-3-rhamnoside, and myricetrin, on opsin stability, function, and its oligomeric membrane organization by using a combination of computational, biochemical and biophysical approaches.

Previous molecular docking studies predicted prospective binding sites on the surface of rod opsin (27,53) in addition to the retinal-binding pocket. Our bioinformatic analysis of the interaction site(s) between flavonoids and ligand-free opsin revealed two potential binding regions with the lowest binding energy: located within the helical bundle at an orthosteric chromophore-binding pocket, and an external binding pocket, positioned between TM5, TM6, and ECL2. Interestingly, the external binding pockets could be found only in the ligand-free opsin but not in the retinal-bound Rho structure, most likely due to the structural rearrangements involving movements of TM5 and TM6 occurring in response to the light stimulus. Followed by these computational analyses, first we investigated the effect of flavonoids on the stability of rod opsin. Ligand-
free rod opsin is highly unstable. However, its stability greatly enhances upon binding of either the native 11-cis-retinal or 9-cis-retinal isochromophore (38). Interestingly, aglycone flavonoids, quercetin, and myricetin, but not their glycosylated forms, both displayed enhancement of the opsin stability effects, presumably due to an increased rigidity of the protein structure by the bound flavonoid. An increased chemical stability in the presence of quercetin was also reported for the G90V RP-linked Rho mutant associated with reduced protein stability (27). Interestingly, this stabilizing effect was achieved at different concentrations range of quercetin and myricetin. Quercetin positively modulated opsin stability at low µM concentrations, while at higher concentrations it displayed an invert effect. On the other hand, myricetin showed dose-dependent opsin-stabilizing effect with its maximum at the highest concentration evaluated. This difference in the dose-response can suggest different binding modes of quercetin and myricetin to the opsin structure. While dual effect of quercetin is likely related to its binding to both the orthosteric binding site and the allosteric binding site, the effect of myricetin is rather associated with its binding to the orthosteric binding pocket. Furthermore, flavonoids and 9-cis-retinal displayed a cooperative effect, resulting in an even higher melting temperature, as compared to isoRho. This finding signified additional tightening of the opsin structure by bound flavonoids. In contrast, the presence of flavonoids did not affect the stability of isoRho, indicating that specific flavonoid-binding site(s) emerge only upon the conformational rearrangements associated with chromophore release from the retinal-binding pocket. In fact, potential allosteric binding site emerge only in the opsin conformation. Moreover, only in opsin the chromophore-binding pocket is vacant and could be occupied by the flavonoid compound.

The bound flavonoid did not prevent regeneration of isoRho with 9-cis-retinal but rather enhanced the rates of retinal binding to opsin in agreement with the previously reported effects of quercetin (27) and the anthocyanin, cyanidin-3-glucoside (28), on the regeneration of the visual pigment. Based on the 1H-NMR study reported for cyanidin-3-glucoside, this enhanced pigment regeneration was mediated by the direct interaction of flavonoid with opsin, resulting in the modulation of the structural conformation and dynamics of opsin by restricting the mobility of some residues (53). Interestingly, both quercetin and myricetin resulted in a similar, dose-dependent increase in the rate of pigment regeneration. Thus, the allosteric modulation of opsin properties by myricetin cannot be excluded.

Despite the fact that flavonoids can directly interact with opsin, modulating its structure, increasing its stability and the regeneration of the visual pigment, their effects on receptor function are rather modest. At physiological concentrations, flavonoids tested in this study had no significant impact on the rates of G, activation, the activation of the signaling cascade in cultured cells or the light-stimulated chromophore release. Although, myricetin at higher (250 and 500 µM) concentrations resulted in the inhibition of the G activation rates these high doses of myricetin are unlikely to be considered for a therapeutic application due to its high cellular toxicity at concentrations above 100 µM. Furthermore, the effect of flavonoids on the function of the specific RP-linked Rho mutants yet needs to be elucidated.

Both an increase of rod opsin stability and enhancement of retinal binding mediated by flavonoids potentially contribute to vision improving effects in the RP-linked diseases (22,54). A large group of RP-causing Rho mutations is accompanied by misfolding and arrest in the secretory pathway of the inner segments of rod photoreceptor cells, preventing proper transport of Rho to the ROS, and thus leading to the photoreceptor cells death. The P23H RP Rho mutant is associated with decreased protein stability and misfolding. However, these defects could be mitigated by pharmacological treatments not only with 9-cis-retinal isochromophore but also with non-retinoid modulator YC-001 (13). Both compounds increase the stability of P23H rod opsin mutant, its folding and membrane targeting. Flavonoids, due to their opsin-stabilizing effects, could potentially provide analogous benefits to this RP-linked Rho mutant. Indeed, incubation of cells stably expressing P23H rod opsin with flavonoid compounds improved its mobility to the plasma membrane as compared to non-treated control cells.
However, while treatment with 9-cis-retinal or YC-001 improved the glycosylation pattern of P23H opsin, this effect was not detected for flavonoids. Nevertheless, as noted in this study, flavonoids enhanced the expression level of opsin, both WT and the P23H opsin mutant. Thus, the mechanism of increased membrane mobility mediated by flavonoids potentially relates to the increased expression and higher cellular levels of this protein.

In the native tissue, such enhanced expression of Rho with increased ability of retinal binding could be helpful in maintaining the proper balance of functional protein. Indeed, enhanced expression of Rho was observed in a mouse model of endotoxin-induced uveitis upon treatment with anthocyanin-rich bilberry extract and resulted in suppression of the shortening of ROS in photoreceptor cells, and thus preservation of retinal degeneration and vision loss (54).

Enhanced rod opsin stability facilitated by flavonoids could be related to opsin’s increased self-association and formation of the condensed oligomers within the cell membrane. Accumulated research on the Rho supramolecular membrane organization documented that Rho forms dimers organized in higher-ordered oligomers tightly packed in the native ROS membranes of photoreceptor cells (39,40,55,56). In the heterologous expression system, opsin also exists in equilibrium between monomers and dimers that could be shifted towards increased dimer population by manipulating the opsin expression level (57). Rho within the isolated ROS membranes or in a form of the extracted oligomers is much more stable than monomeric Rho (40,42,55). Additionally, exogenous phospholipids increase the stability of detergent-solubilized Rho (58). In the presence of quercetin, oligomers of opsin were detected in the SDS-PAGE gel electrophoresis, suggesting that flavonoids, especially quercetin, stimulated either aggregation or oligomerization of opsin. In fact, small organic molecules, as well as natural products, could promote protein aggregation, often resulting in false inhibition or activation of these proteins, including GPCRs receptors (30). Thus, to discriminate between non-specific flavonoid-stimulated aggregation and oligomerization of opsin, a BRET assay, commonly used in studying GPCR oligomerization, was performed in live cells either stably or transiently expressing opsin. Indeed, quercetin and to a lesser extent, myricetin, promoted oligomerization of opsin in a time-dependent manner. Interestingly, no effects on oligomer formation were observed in the cells regenerated with 9-cis-retinal prior to the treatment with flavonoids, thus supporting other results indicating that flavonoids bind to and modulate the properties of the ligand-free opsin only. Moreover, the flavonoid-stimulated increased BRET signal occurred rather due to the formation of opsin oligomers than aggregates as they were disrupted with the DDM detergent, in opposition to the RP-linked opsin aggregates that are not sensitive to such treatment (59). Thus, this finding suggests that increased opsin stability could be related to the formation of tightly packed oligomers within the biological membranes.

Altogether, this study suggests that flavonoid compounds enhance rod opsin stability through direct binding to the ligand-free opsin molecule and modulates its conformation by rigidifying its structure and promotes its self-association within the phospholipid bilayer. Different patterns of responses to the treatment with quercetin and myricetin suggest differential binding of these flavonoids to opsin. While the effects of quercetin are likely associated with its dual effect through the binding to both the orthosteric binding pocket and allosteric site, the effect of myricetin is rather related to its accommodation in the orthosteric binding pocket. The difference in the interaction pattern of quercetin and myricetin with Trp265 within the binding pocket also emerged from the molecular docking. While quercetin interacted with Trp265 via Van der Waals interactions, myricetin formed much stronger π-π stacking interactions. However, more advanced structural studies, including crystallography, molecular dynamic simulations, and/or mass-spectrometry-based techniques are required to learn about flavonoid-opsin interactions in more details. Moreover, this work supports the therapeutic potential of flavonoids as lead compounds to discover novel non-retinoid medications for treating or delaying retinal degeneration associated with disturbed stability and imbalanced homeostasis of the rod photoreceptors. However, more studies are necessary to fully understand the pharmacological
potential and the underlying mechanism of flavonoids in the prevention of retinopathies associated with Rho misfolding.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents**

Alexa Fluor 594-conjugated goat anti-mouse secondary antibody was purchased from Life Technologies (Grand Island, NY). BODIPY FL L-Cystine (BFC) (DMSO) was obtained from Invitrogen (B20340). n-Dodecyl-β-D-maltoside (DDM) was purchased from Affymetrix Inc. (Maumee, OH). The cAMP Direct Biotrak EIA kit was obtained from GE Healthcare Life Sciences (Piscataway, NJ). Coelenterazine h, purchased from Cayman Chemical (Ann Arbor, MI), was dissolved in DMSO to make a 2.5 mM stock solution and stored at -80 °C. DAPI Fluoromount-G was purchased from SouthernBiotech (Miami, FL). Dimethyl sulfoxide (DMSO), GTPγS, 9-cis-retinal and phosphodiesterase inhibitor (Ro-20-1724) were obtained from Sigma (St. Louis, MO). EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Basel, Switzerland). Flavonoids: myricetin, quercetin, and their monoglycosylated forms myricetin and quercetin-3-rhamnoside were purchased from Sigma. Forskolin bought from Sigma was dissolved in DMSO to obtain a 24 mM stock solution and stored at -20 °C. MTT 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was purchased from Sigma.

**Molecular Docking**

The coordinates of the ligand-free bovine opsin at 2.9 Å resolution were obtained from the protein data bank, PDB code 3CAP (33). One monomeric unit of the protein was selected and the co-crystallized molecules and all crystallographic water molecules were removed from the coordinate set; hydrogen atoms were added and partial charges were assigned to all atoms. The protein was then submitted to the restrained molecular mechanics refinement with NAMD 2.12 software (60), using the CHARMM22 force field (61). After further energy minimization, the protein was suitable for bioinformatic analysis performed with CASTp 3.0 software using the [http://sts.bioe.uic.edu/castp/](http://sts.bioe.uic.edu/castp/) server. The potential ligand-binding pockets located in the extracellular part of the protein and the orthosteric ligand-binding site were selected and then verified by “a priori” docking approach with quercetin using the Achilles Blind Docking available at the [http://bio-hpc.ucam.edu/achilles/](http://bio-hpc.ucam.edu/achilles/) server. The 3D structures of the flavonoid compounds used in a docking study were obtained from the PubChem database. The molecular docking of these compounds into the opsin structure was performed with VINA/VegaZZ 3.1.0.21 software (62) with 30 iterations conducted for each compound. The results were prioritized according to the predicted binding free energy in kcal/mol. The results obtained from the docking simulation were visualized with the Biovia Discovery Studio Visualizer 17.2.0 software.

**Preparation of Opsin Membranes**

Rod outer segment (ROS) membranes were isolated from frozen bovine retinas under dim red light as described previously (63). The buffer composed of 10 mM sodium phosphate, pH 7.0 and 50 mM hydroxylamine was used to resuspend ROS membranes to Rho concentrations of ~3 mg/ml. The membranes then were exposed to white light with a 150 Watt bulb for 30 min at 0 °C. These membranes were then pelleted by centrifugation at 16,000 x g for 10 min. The supernatant was discarded and the membrane pellet was washed twice with 10 mM sodium phosphate, pH 7.0 and 2% BSA followed by 4 washes with 10 mM sodium phosphate, pH 7.0 and 2 washes with 20 mM BTP, pH 7.5 and 100 mM NaCl. To pellet the membranes after each wash, centrifugation at 16,000 x g for 10 min was applied.

**Thermal Shift Assay**

The BFC probe was diluted to a stock concentration of 10 mM in DMSO. A final working concentration of 2 mM BFC in 20 mM BTP, pH 7.5 and 100 mM NaCl was used in all experiments. The opsin membranes at a concentration of 0.01 mg/ml were loaded into a 96-well plate (Applied Biosystem). Then, specific flavonoid at 1, 10, 100, 250, and 500 μM concentration was added and the plate was incubated for 1 h at 4 °C. Alternatively, 30 min prior to the addition of flavonoid, the regeneration of isoRho with 9-cis-retinal was performed. Next, 5 μl of the BFC probe was added to each well, which contained 20 μl of opsin membrane with or
without the flavonoid compound. The plate was sealed with a ClearSeal film (HR4-521) from Hampton Research and incubated for 10 min on ice prior to measurement of their fluorescence. All measurements were performed with a StepOnePlus Real-Time PCR System (Applied Biosystems), and melting curve experiments were recorded using StepOne software version 2.3. The fluorescence in the SYBR, FAM, and ROX channels was recorded for each sample. The run was set to cool the plate to 4 °C within 10 s, kept at 4 °C for 1 min, and then increased 1 °C per min in a step-and-hold manner up to 99.9 °C. The multicomponent data were exported to a Microsoft Excel sheet and analyzed with Prism 6.0. The melting temperatures of bovine Rho and opsin within the membranes were 71.9 and 55.9 °C, respectively (38).

### Pigment Reconstitution and Purification by 1D4 Immunoaffinity Chromatography

Bovine opsin membranes were resuspended in 20 mM BTP containing 120 mM NaCl, pH 7.5 and incubated with a specific flavonoid at 1, 10, 100, 250, and 500 µM concentration for 1 h at 4 °C. Then, opsin was regenerated with 9-cis-retinal, which was added to the membrane suspension from a DMSO stock solution to a final concentration of 10 µM followed by an incubation in the dark for 1 h at 4 °C on a nutator. To solubilize the membranes, n-dodecyl-β-D-maltopyranoside (DDM) was added to 20 mM final concentration and incubated for 1 h at 4 °C on a nutator. The lysate was centrifuged at 16,000 x g for 1 h at 4 °C and isoRho was purified from the supernatant by 1D4 immunoaffinity chromatography (using anti-Rho C-terminal 1D4 antibody (a generous gift from Dr. K. Palczewski, University of California-Irvine, CA) immobilized on cyanogen bromide (CNBr)-activated agarose) (64). Three-hundred µl of 6 mg 1D4/ml agarose beads were added to the supernatant and incubated for 1 h at 4 °C on a nutator. The resin was then transferred to a column and washed with 10 ml of 20 mM BTP, 120 mM NaCl, and 2 mM DDM, pH 7.5. IsoRho was eluted with the same buffer, supplemented with 0.6 mg/ml of the 1D4 peptide (TETSQVAPA).

Alternatively, the ability to stabilize rod opsin over a longer period of time was tested. Opsin membranes (2.5 µM) were incubated with or without 1 µM or 100 µM flavonoid for 1 h on ice, followed by their solubilization in 20 mM DDM. Solubilized opsin samples were incubated at room temperature for 0, 2, 4, 6, and 24 h before incubation with 10 µM 9-cis-retinal in the dark for isoRho regeneration. Then UV-visible spectra of these samples were measured.

### UV-visible Spectroscopy of Opsin and IsoRho

The concentration of Rho or opsin within membranes was measured after membrane solubilization with 20 mM DDM and pelleting insoluble material by centrifugation at 16,000 x g for 15 min at 4 °C. A UV-visible spectrophotometer (Cary 50, Varian, Palo Alto, CA) and the absorption coefficients ε_{485nm}=40,600 M⁻¹cm⁻¹ and ε_{280nm}=81,200 M⁻¹cm⁻¹ (65) were used respectively for Rho and opsin.

The concentration of isoRho was measured and quantified in freshly purified samples by using a UV-visible spectrophotometer and the absorption coefficient ε_{485nm}=43,600 M⁻¹cm⁻¹ (66).

### HPLC Flavonoid Detection

To detect the flavonoids bound to opsin, opsin membranes were incubated with quercetin or myricetin at a concentration of 100 µM for 1 h at 4 °C. These samples were divided and the half was regenerated with 9-cis-retinal. The excess flavonoid was removed by 1D4 immunoaffinity purification of opsin and isoRho. Protein-bound flavonoids were extracted from the purified samples in 1 ml of methanol: acetone (1:1, v/v) by vigorous shaking followed by centrifugation at 3,220 x g for 15 min at 4 °C to separate the precipitated material. The supernatants were filtered through a 0.22 µM filter to glass vials and dried in a Savant speedvac concentrator (Thermofisher, Waltham, MA). Then, these samples were dissolved in 300 µl of methanol, and 100 µl of each was injected into an Agilent 1260 Infinity II HPLC system. Flavonoids were separated on a reverse-phase analytical HPLC column (Gemini RPC18 5 µm, 4.6x250 mm) equilibrated with 50% (v/v) methanol in water (0.1% H₃PO₄) with a gradient elution from 50% to 80% methanol for 15 min and then an isocratic elution at a flow rate of 0.4 ml/min for 10 min. The absorbance signals at 258 and 360 nm were
collected and the flavonoids were identified by a comparison with an elution profile of authentic standards.

**Fluorescence Spectroscopy**

The binding of flavonoid into the opsin’s chromophore-binding pocket was determined by measuring the quenching of the intrinsic Trp fluorescence after the addition of increasing concentrations of the flavonoid compounds. Then 9-cis-retinal was added and the measurements were repeated. The emission spectra were recorded with a PerkinElmer L55 Luminescence Spectrophotometer at 20 °C between 300 and 450 nm after excitation at 295 nm. The excitation and emission slit bands were set at 5 and 10 nm, respectively. Changes in the intrinsic Trp fluorescence at 330 nm were plotted as a function of the flavonoid concentration. All experimental data were corrected for the samples’ background and self-absorption at excitation and emission wavelengths (inner filter effect correction).

**Gt Activation**

Gt was extracted from ROS membranes isolated from 200 dark-adapted bovine retinas followed by its purification as described in (67,68). Activation properties of the isoRho and flavonoid-bound isoRho were tested in a Trp fluorescence Gt activation assay. Briefly, a mixture of Gt and isoRho samples at a 10:1 ratio (250 nM Gt and 25 nM isoRho) diluted in a buffer consisting of 10 mM BTP, 120 mM NaCl, and 1 mM DDM, pH 7.0 was illuminated for 30 s with a Fiber-Light illuminator through a band-pass wavelength filter (480-520 nm) followed by a 5 min incubation with continuous low-speed stirring. Gt activation was recorded as the intrinsic fluorescence increase from Gt₀ upon addition of 5 µM GTPγS. Measurements were performed with a Perkin Elmer LS 55 Luminescence Spectrophotometer. Excitation and emission wavelengths were set at 300 nm and 345 nm, respectively (69-71). In the control experiments, no signals from isoRho without Gt were detected.

**Chromophore Release**

Changes in the intrinsic Trp fluorescence were measured with 50 nM purified isoRho or flavonoid-bound isoRho samples diluted in a buffer consisting of 10 mM BTP, 100 mM NaCl, and 1 mM DDM, pH 6.0 after their illumination with a Fiber-Light illuminator through a 420-520 nm band-pass filter for 15 s. Light exposure was conducted at a distance of 15 cm. Changes in the intrinsic Trp fluorescence were recorded for 60 min, and they correlate with the decrease in the protonated Schiff base concentration (72). These measurements were performed with a Perkin Elmer L55 Fluorescence Spectrophotometer at 20 °C. Spectrofluorometer slit settings were 8 nm at 295 nm for excitation and 10 nm at 330 nm for emission collection.

**Thermal Stability**

Purified isoRho or flavonoid-bound isoRho samples diluted in a final volume 0.4 ml of 20 mM BTP, 120 mM NaCl and 1 mM DDM, pH 7.5 were incubated at 55 °C in the dark and their spectra were recorded every 2 min for 1 h. Absorbance at the maximum wavelength at the initial time point was assumed to be 100%. The percentages of remaining pigments were normalized to their initial concentrations and then plotted as a function of time. From these plots, the half-lives (t₁/₂) of chromophore release were calculated.

**Cell Culture**

NIH-3T3 cells stably expressing WT mouse opsin, HEK-293S GnTI- cells stably expressing WT mouse opsin, and HEK-293 cells stably expressing both WT mouse opsin•Rluc and opsin•Venus were cultured in DMEM with 10% FBS (Hyclone, Logan, UT), and 1 unit/ml penicillin with 1 µg/ml streptomycin (Life Technologies) at 37 °C under 5% CO₂ according to the instructions from the ATCC Animal Cell Culture Guide.

**Cytotoxicity Assay**

The above-described cells were seeded in 96-wells plate at a density of 3x10⁴ cells/well. The next day, different concentrations of the flavonoid compounds were added to the cells and 24 h later the cell viability was evaluated by using the MTT cell proliferation assay (Sigma) (73). The percentage of dead cells under tested conditions was calculated.

**cAMP Detection**
HEK-293S GnTI cells stably expressing WT mouse opsin were plated in two 96-well plates at a density of 90,000 cells per well in 85 µL of DMEM medium containing 10% FBS and antibiotics. The cells were treated with flavonoids at different concentration for 16 h. Next, 9-cis-retinal was added for 2 h to regenerate isoRho in cells either treated or not with the flavonoid compounds. After that, forskolin (5 µM) and phosphodiesterase inhibitor (0.1 µM) were added to maximize the concentration of total cAMP within the cells. One plate then was kept in the dark, while the second plate was exposed to bright (150 Watt) light for 15 min from a 10 cm distance. Levels of accumulated cAMP were detected with the cAMP Direct Biotrak EIA kit (GE Healthcare Life Sciences) following the manufacturer's protocol and the absorbance readout at 630 nm by the Tecan M1100 plate reader (Tecan Life Sciences) at the final step of the assay, according to the protocol provided.

Membrane Localization of Rod Opsins in HEK-293 Cells

NIH-3T3 cells expressing WT opsin or P23H mutant opsin together with green fluorescent protein GFP (a generous gift from Dr. K. Palczewski, University of California-Irvine, CA) were plated in a 96-well plate at a density of 2.0x10^4 cells/well and cultured overnight. Next day, the cells were treated with the flavonoids at different concentration for 16 h. For the regeneration of isoRho, the cells were incubated with 9-cis-retinal for 2 h. The cell growth medium was removed from each well and the cells were washed with PBS 3 times. The cells were fixed with 3% formaldehyde freshly prepared in PBS for 20 min at room temperature and then they were washed with PBS 2 times. Next, the cells were incubated in 10% normal goat serum in PBS for 1 h at 37 °C. To detect opsin, cells were incubated with the B6-30 anti-Rho antibody that recognizes the N-terminal epitope (a generous gift from Dr. K. Palczewski), University of California-Irvine, CA, for 3 h at room temperature. Then, cells were washed 3 times with PBS for 10 min at room temperature. To detect total opsin, cells were permeabilized with Triton x-100 before immunostaining. Opsin immunostaining was visualized by incubating the cells with an antimouse antibody conjugated with Alexa Fluor 594 (Thermofisher) at a 1:100 dilution for 1 h at room temperature. After that, the cells were washed 3 times with PBS for 10 min at room temperature. The cell nuclei were stained with DAPI, following the manufacturer’s protocol. The plate was sealed with a transparent film and the cells were imaged with the Operetta High Content Imager (PerkinElmer) using a 20x long objective. Eight fields were taken of each well for cell images with four channels including bright field, GFP, Alexa Fluor 594, and DAPI. Images were analyzed with the Columbus storage and analysis system (PerkinElmer). DAPI fluorescence images were used to define nuclei and count cells. Bright field images and GFP fluorescence were employed to define cells and select populations of intact cell images. The plasma membrane was defined within ± 5% of the cell border.

The Bioluminescence Resonance Energy Transfer (BRET) Assay

Stable HEK-293 cells expressing both opsin•Rluc (donor) and opsin•Venus (acceptor) were plated at a density of 8x10^4 cells/ml into a 96-well plate. The next day, these cells were treated with flavonoids at a 100 µM final concentration for 2, 4, 8 or 16 h. During this treatment, cells were cultured at 37 °C with 5% CO₂ and 90% humidity. Next, 9-cis-retinal at a final concentration of 10 µM was added, and the plates were wrapped with aluminum foil and incubated for 2 h at 37 °C with 5% CO₂ and 90% humidity. Then, the culture medium was aspirated and replaced with 200 µl PBS/well. Cells were resuspended and transferred from the 96-well cell culture plate to a white-walled opaque 96-well plate (Corning Life Sciences). DDM in PBS at a final concentration of 5 mM or 0.1 mM was added to the wells (59). Coelenterazine h was diluted to 25 µM in PBS from a 2.5 mM stock solution. Each well of the 96-well plate was injected with 25 µl of diluted coelenterazine h followed by dual luminescence readings at 480 and 530 nm. The measurements were performed with a Tecan M1100 plate reader (Tecan Life Sciences). The BRET signal was calculated as the ratio of emission at 530 nm and 480 nm. The BRET signal was normalized to the signal obtained for non-treated cells. The average of three independent experiments was used to obtain the final plot.
Alternatively, HEK-293 cells were plated into two 12-well plates at ~25 x 10^4 cells/ml. The plates were cultured at 37 °C with 5% CO_2 and 90% humidity. The next day, cells were transiently transfected either with both opsins•Rluc (donor) and opsins•Venus (acceptor) at a 1:4 earlier employed ratio of donor to acceptor constructs (41) or opsins•Rluc construct only using polyethylenimine (74,75). Constructs of mouse opsins fused to Venus (opsins•Venus) and Renilla luciferase (opsins•Rluc) in the pcDNA3.1Zeo vector were a generous gift from Dr. N.A. Lambert (Georgia Regents University, GA).

The cells were treated with the flavonoid compounds as described above for the stable cells. On the following day, 48 h post transfection, the culture medium was aspirated and replaced with 500 µl PBS/well. Cells were resuspended and 200 µl of this suspension was transferred to a white-walled opaque 96-well plate (Corning, NY). DDM in PBS at a final concentration of 5 mM or 0.1 mM was added to the assigned wells (59). The measurement of the BRET signal was performed as described above for the stable cell line.

**Statistical Analyses**

The cytotoxicity assays, image-based analyses with the Operetta High Content Imager, quantification of cAMP levels, and the BRET analyses included three biological replicates, and were performed at least twice. Positive and negative controls were included in each assay. Effects of the tested flavonoid compounds were analyzed in a dose-dependent manner. The thermal stability, opsins binding, regeneration of isorhodopsin, thermal stability at 55 °C and G_{i} activation assays, each experiment was repeated three times. The parameters derived from these experiments were averaged and standard deviations (S.D.) were calculated. The effect of each compound was either plotted in a dose-dependent or time-dependent manner as compared with controls. Statistical analyses were performed with GraphPad Prism 7 software. For two-group comparisons, the one-way ANOVA test with Dunnett’s multiple comparisons post-test was employed. For the means of 2 variables of more than 2 groups, the two-way ANOVA with Tukey’s or Sidak’s multiple comparisons post-test was used. Differences were considered statistically significant at a P value of <0.05 (*P<0.05, **P<0.001, ***P <0.0001).
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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

B.J. and J.T.O. conceived and designed the experiments. B.J., J.T.O and T.P. conducted the experiments. B.J. and J.T.O. wrote the manuscript. B.J. coordinated and oversaw the research project. All authors discussed the results and commented on the manuscript.

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Abbreviations

adRP, autosomal dominant retinitis pigmentosa; AMD, age-related macular degeneration; BFC, BODIPY FL L-Cystine; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; BTP, Bis-tris propane; cAMP, cyclic adenosine monophosphate; CSNB, congenital stationary night blindness; DDM, n-dodecyl β-D-maltoside; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; ECL, extracellular loop; GFP, green fluorescence protein; GPCRs, G protein-coupled receptors; GTPγS, guanosine triphosphate gamma S; isoRho, isorhodopsin; MTT 3-(4,5-Dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide; PVDF, polyvinyl difluoride; ROS, rod outer segments; Rho, rhodopsin; RP, retinitis pigmentosa, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TM, transmembrane helix; UV-visible, ultraviolet-visible; WT, wild type
**Table 1.** The details of the interactions between flavonoids and opsin protein side chains within the binding pockets

| Compound                  | Orthosteric                                                                 | Extracelular pocket 1                                      |
|---------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------|
| Quercetin                 | H-Bond: Ala117, Thr118, Glu122, and ILE189                                   | H-Bond: Thr193, Glu196, Asn199, and Glu201                |
|                           | Electrostatic interactions: Glu181, Tyr268, Ala292, and Lys296               | Electrostatic interactions: Pro194 and Phe276              |
| Quercetin-3-Rhamnoside    | H-Bond: Glu122, Glu181, Tyr191, and Lys296                                   | NA                                                        |
|                           | Electrostatic interactions: Ala117, Tyr191, Tyr192, and Lys296               |                                                           |
| Myricetin                 | H-Bond: Thr118, Glu122, and Tyr192                                           | H-Bond: Tyr191, Glu196, Asn199, and Gln279                |
|                           | Electrostatic interactions: Glu181,Trp265, Tyr268, Ala292, and Lys296        | Electrostatic interactions: Pro194, Glu196, Glu201, and Phe276 |
| Myrticetrin               | H-Bond: Ile189 and Tyr192                                                    | NA                                                        |
|                           | Electrostatic interactions: Ala117, Tyr268, Pro291, Ala292, and Lys296       |                                                           |
**Table 2. Melting Temperature of opsin in the presence of flavonoids**

| Concentration (µM) | Quercetin | Quercetin-3-Rhamnoside | Myricetin | Myricetrin |
|-------------------|-----------|------------------------|-----------|------------|
| Opsin (NT)        | 55.4±0.4  | 55.4±0.4               | 55.4±0.4  | 55.4±0.4   |
| isoRho (NT)       | 72.1±1.2  | 72.1±1.2               | 72.1±1.2  | 72.1±1.2   |
| 0.01              | 54.9±0.4  | 55.1±1.1               | 54.4±0.1  | 55.0±0.1   |
| 0.1               | 55.6±0.2  | 55.0±1.2               | 54.4±0.5  | 55.0±0.9   |
| 0.5               | 57.2±0.3***| 54.4±1.1               | 54.6±0.6  | 55±0.8     |
| 1                 | 61.0±0.2***| 54.1±1.1               | 54.9±0.2  | 55.8±1.2   |
| 10                | 59.8±0.3***| 53.8±1.1               | 54.8±1.1  | 55.1±0.3   |
| 100               | 59.0±0.4***| 54.5±1.2               | 56.0±1.2  | 54.8±1.1   |
| 250               | 58.4±0.5***| 54.8±0.5               | 57.1±1.5**| 54.6±1.1   |
| 500               | 55.7±0.4  | 54.7±0.4               | 58.9±0.4***| 53.7±1.1   |

Melting Temperature was determined for non-treated (NT) controls: opsin, isoRho, and opsin incubated with the flavonoid compound at 0.01-500 µM concentrations before the measurement. Each flavonoid-treated opsin sample was compared to the non-treated opsin sample. Statistical significance (P) was calculated with the one-way ANOVA test and Dunnett’s post-test for multiple comparisons (GraphPad Prism 7 software). Statistically significant effect of flavonoid was denoted with asterisks. ***P≤0.0001; **P≤0.001.
### Table 3. Melting temperature of isoRho in the presence of flavonoids

| Concentration (μM) | Melting temperature (°C) |
|-------------------|--------------------------|
|                   | Quercetin | Quercetin-3-Rhamnoside | Myricetin | Myricetrin |
| isoRho 0.1        | 72.1±1.2  | 72.1±1.2               | 72.1±1.2  | 72.1±1.2  |
| isoRho 1          | 72.3±1.3  | 72.4±1.2               | 72.8±1.2  | 71.3±1.2  |
| isoRho 10         | 75.4±0.4**| 72.4±1.1               | 72.4±1.1  | 71.1±1.1  |
| isoRho 100        | 75.3±0.3**| 72.7±1.1               | 72.2±1.3  | 70.6±1.1  |
| isoRho 250        | 78.2±0.3***| 72.8±1.2               | 75.9±2.3  | 70.9±0.3  |
| isoRho 500        | 79.5±0.4***| 73.4±1.2               | 79.5±1.4***| 71.1±1.4  |

Melting Temperature was determined for isoRho and opsin incubated with the flavonoid compound at 1-500 μM concentration followed by its regeneration with 9-cis-retinal before the measurement. Each flavonoid-treated sample was compared to the non-treated isoRho sample. Statistical significance (P) was calculated with the one-way ANOVA test and Dunnett’s post-test for multiple comparisons (GraphPad Prism 7 software). Statistically significant effect of flavonoid was denoted with asterisks. ***P≤0.0001; **P≤0.001.
Table 4. Regeneration rates of isoRho in the presence of flavonoids

| Concentration (μM) | Quercetin | Quercetin-3-Rhamnoside | Myricetin | Myricetin |
|-------------------|-----------|------------------------|-----------|-----------|
| NT                | 4.2±0.2   | 4.0±0.1                | 4.2±0.1   | 4.1±0.1   |
| 1                 | 4.5±0.2   | 4.0±0.1                | 3.8±0.3*  | 3.1±0.1** |
| 10                | 3.5±0.1** | 4.0±0.1                | 3.4±0.1** | 3.6±0.1*  |
| 100               | 3.1±0.1***| 3.8±0.1                | 3.4±0.1***| 3.0±0.1** |
| 250               | 2.5±0.1***| 2.6±0.1***             | 2.6±0.1***| 3.0±0.1** |
| 500               | 2.0±0.1***| 2.8±0.3***             | 2.8±0.1***| 2.4±0.1***|

Regeneration of isoRho with 10 μM 9-cis-retinal after opsin incubation with the flavonoid compound at 1-500 μM concentration. The isoRho regeneration half-time (t₁/₂) of each flavonoid-treated sample was compared to the non-treated (NT) control sample. Statistical significance (P) was calculated with the one-way ANOVA test and Dunnett's post-test for multiple comparisons (GraphPad Prism 7 software). Statistically significant effect of flavonoid was denoted with asterisks. ***P≤0.0001; **P≤0.001; *P≤0.05.
Table 5. Effects of flavonoids in $G_i$ activation rates

| Concentration ($μM$) | Quercetin ($k \times 10^{-3} s^{-1}$) | Myricetin ($k \times 10^{-3} s^{-1}$) |
|-----------------------|--------------------------------------|--------------------------------------|
| NT                    | 2.7±0.4                              | 2.8±0.4                              |
| 1                     | 3.0±0.2                              | 3.1±0.2                              |
| 10                    | 2.9±0.3                              | 2.9±0.3                              |
| 100                   | 2.8±0.3                              | 2.8±0.3                              |
| 250                   | 2.7±0.1                              | 1.8±0.1**                            |
| 500                   | 2.6±0.2                              | 1.2±0.2***                           |

The $G_i$ activation rates ($k$) were determined for purified isoRho samples, either non-treated (NT) or treated with the flavonoid compound at 1-500 $μM$ concentration prior to the regeneration with 10 $μM$ 9-cis-retinal. Statistical significance ($P$) was calculated with the one-way ANOVA test and Dunnett's post-test for multiple comparisons (GraphPad Prism 7 software). Statistically significant effect of flavonoid was denoted with asterisks. ***$P$≤0.0001; **$P$≤0.001.
Table 6. Effects of flavonoids on Meta II decay rates

| Concentration (μM) | Quercetin (min) | Myricetin (min) |
|-------------------|-----------------|-----------------|
| NT                | 12.9±1.4        | 14.8±2.2        |
| 1                 | 13.2±1.9        | 15.4±1.7        |
| 10                | 13.4±1.9        | 16.6±0.2        |
| 100               | 11.2±0.1        | 13.2±2.5        |
| 250               | 9.6±0.8*        | 11.9±2.3        |
| 500               | 9.7±0.6*        | 12.7±0.7        |

The Meta II decay rates ($\tau$) were determined for purified isoRho samples, either non-treated (NT) or treated with the flavonoid compound at 1-500 μM concentration prior to the regeneration with 10 μM 9-cis-retinal. Statistical significance ($P$) was calculated with the one-way ANOVA test and Dunnett's post-test for multiple comparisons (GraphPad Prism 7 software). Statistically significant effect of flavonoid was denoted with asterisk. *$P$$\leq$0.05
FIGURES AND FIGURE LEGENDS

Figure 1. Bioinformatic analysis of the interaction between bovine rod opsin and the flavonoids. (a) Assessment of the potential flavonoid binding sites within the bovine rod opsin structure (PDB: 3CAP) performed with the CASTp 3.0 software is shown on the left. These binding sites were co-validated using a blind docking approach. Three potential binding sites were found: 1) the orthosteric site or retinal-binding site (shown in red), 2) the external binding site between TM5, TM6 and ECL2 pocket 1 (shown in purple) and 3) the external binding site between TM2, TM3 and ECL1, pocket 2 (shown in yellow). The binding energies for each binding site calculated were as follows: 1) -9.3 kcal/mol, 2) -7.9 kcal/mol and 3) -6.5 kcal/mol. Chemical structures of quercetin, quercetin-3-rhamnoside, myricetin, and myricetrin are shown on the right. (b) Molecular docking of quercetin into the orthosteric binding pocket (left) and pocket 2 (middle), and docking of quercetin-3-rhamnoside into the orthosteric binding pocket (right) of rod opsin. (c) Molecular docking of myricetin into the orthosteric binding pocket (left) and pocket 2
(middle), and docking of myricetin into the orthosteric binding pocket (right) of rod opsin. Quercetin and myricetin could accommodate within the retinal-binding pocket and the external binding pocket located between TM5, TM6, and ECL2, while their glycosylated forms, quercetin-3-rhamnoside, and myricetin could accommodate only into the retinal-binding pocket.

**Figure 2.** Two-dimensional representation of the interactions between flavonoids and specific residues in the protein side chains. In the orthosteric binding-pocket, the flavonoid compounds form hydrogen bond interactions with the following residues: Ala117, Thr118, Glu122, Glu181, ILE189, Tyr191, Tyr192, and Lys296, while in the external pocket 1 with Thr193, Glu196, Tyr191, Asn199, Glu201, and Gln279. The main electrostatic interactions within the orthosteric binding pocket involve Ala117, Glu181, Tyr191, Tyr192, Trp265, Tyr268, Ala292, Pro291, and, Lys296, while in the external pocket 1 Pro194 Glu196, Glu201, and Phe276. The 2D-representations of the low energy structures obtain from the docking simulation were visualized with the *Biovia Discovery Studio Visualizer 17.2.0* software.
**Figure 3.** Effect of flavonoids on thermal stability of opsin and isoRho. (a) The opsin membranes were incubated with increasing concentrations (0.01, 0.1, 1, 10, 100, 250 and 500 µM) of quercetin, quercetin 3-rhamnoside, myricetin, or myricetrin for 1 h at room temperature. After that, the temperature of melting was determined by using a fluorescence BFC probe. Opsin membranes not incubated with flavonoids and opsin membranes incubated with 9-cis-retinal were used as controls. The values of fluorescence were plotted as a function of temperature and the melting temperature was calculated using Prism GraphPad 7.04 software. The derived melting temperature for each experimental condition is shown as a function of concentration. The values of the melting temperatures derived for non-treated controls (opsin and isoRho) were 55.4±0.4 and 72.1±1.2 °C, respectively. This experiment was repeated 3 times. Error bars represent standard deviation (S.D.). All values of the melting temperatures derived from these experiments and statistical significance of the effect of flavonoids are shown in Table 2. (b) The opsin membranes were incubated with different concentrations (1, 10, 100, 250 and 500 µM) of quercetin, quercetin-3-rhamnoside, myricetin, or myricetrin for 1 h at room temperature, and then regenerated with 10 µM 9-cis-retinal (color), or isoRho was regenerated first, and then membranes were incubated with the flavonoid compounds (black). The melting temperature was determined in these samples by using a BFC fluorescence probe. These experiments were performed in triplicates. Error bars represent S.D. All values of the melting temperatures derived from these experiments and statistical significance of the effect of flavonoids are shown in Table 3. (c) Regeneration of isoRho from aged opsin. Opsin-containing membranes were incubated with quercetin or myricetin for 1 h on ice, solubilized with DDM, and then kept at room temperature for 2, 4, 6 or 24 h before regeneration with 10 µM 9-cis-retinal for 1 h. The percentage of regenerated isoRho was calculated. The regeneration of isoRho at time 0 h was set as 100%. NT, non-treated; 9cR, treated with 9-cis-retinal; Q-1, treated with 1 µM quercetin; Q-100, treated with 100 µM quercetin; M-1, treated with 1 µM myricetin; M-100, treated with 100 µM myricetin. These experiments were performed in triplicates. Error bars represent S.D. The statistical significance of the effect of flavonoids on the stability of aged opsin as compared with non-treated control was as follows: at 2 h for Q-1 and Q-100 $P ≤ 0.001$ and $P = \text{NS}$, for M-1 and M-100 $P ≤ 0.05$ and $P = \text{NS}$ (not significant); at 4 h for Q-1 and Q-100 $P ≤ 0.001$ and $P = \text{NS}$, for M-1 and M-100 $P = \text{NS}$ and $P ≤ 0.001$; at 6 h for Q-1 and Q-100 $P ≤ 0.0001$ and $P ≤ 0.005$, for M-1 and M-100 $P ≤ 0.05$ and $P ≤ 0.001$; at 24 h for Q-1 and Q-100 $P = \text{NS}$ and $P = \text{NS}$, for M-1 and M-100 $P = \text{NS}$ and $P ≤ 0.0001$. 

by guest on April 26, 2019
Figure 4. Regeneration of isoRho in the presence of flavonoids. (a) The regeneration of isoRho was determined upon prior incubation of the opsin membranes with quercetin, quercetin 3-rhamnoside, myricetin, and myricetrin at different concentrations (1, 10, 100, 250 and 500 µM). Changes in the absorption maximum were plotted as a function of time. Saturation was reached at 20 min, and the absorption maximum at this time point was assumed to be 100%. Insets, the half-times \( t_{1/2} \) of regeneration plotted as a function of the flavonoid concentration. Error bars represent S.D. All values of \( t_{1/2} \) derived from these experiments and statistical significance of the effect of flavonoids are shown in Table 4.
Figure 5. Effect of flavonoids on the chromophore-binding pocket in rod opsin. The binding of flavonoids into the retinal-binding pocket of opsin was determined by quenching of the intrinsic Trp fluorescence upon addition of increasing concentrations (1, 10, 100, 250 and 500 µM) of quercetin, quercetin-3-rhamnoside, myricetin, or myricetrin, to the opsin membranes (blue). After incubation with the flavonoid compound (at each concentration) 10 µM 9-cis-retinal was added and the Trp fluorescence was recorded (red). These measurements were performed in triplicates. Error bars represent S.D.
Figure 6. Spectral properties of flavonoid-bound isoRho and detection of flavonoids. (a) UV-visible spectra of isoRho regenerated with 10 μM 9-cis-retinal after incubation of the opsin membranes with different concentrations of flavonoids and purified by 1D4 affinity chromatography are shown. (b) Spectral properties of flavonoid-bound opsin. UV-visible spectra of opsin samples after incubation of the opsin membranes with different concentrations of quercetin or myricetin, followed by their purification by 1D4 affinity chromatography are shown. (c) Detection of quercetin and myricetin in purified isoRho samples. Extracted flavonoids were separated by using reverse phase HPLC chromatography and identified by comparison to the HPLC profile of quercetin and myricetin standards. These experiments were performed in triplicates.
Figure 7. Effect of bound flavonoids on isoRho functional properties. (a) G_{i} activation by flavonoid-bound isoRho. Opsin membranes incubated with quercetin or myricetin at different concentrations followed by regeneration with 10 µM 9-cis-retinal were solubilized with DDM, and then applied to immunopurification. G_{i} activation by photoactivated flavonoid-bound isoRho was recorded as an increase of the intrinsic Trp fluorescence and plotted as a function of time. The pseudo first-order kinetic rates (k) of G_{i} activation were derived from the function \( A(t) = A_{\text{max}}(1 - \exp^{-kt}) \), where \( A_{\text{max}} \) is the maximal G_{i} fluorescence change, and \( A(t) \) is the relative fluorescence change at time \( t \). Each measurement was repeated three times. Error bars represent S.D. All values of \( k \) derived from these experiments and statistical significance of the effect of flavonoids are shown in Table 5. (b) The cytotoxicity of quercetin (blue) and myricetin (red) on HEK-293S GnTI- cells stably expressing WT opsin was determined by using the MTT assay. The cells were treated with flavonoid compounds at concentrations within a range of 0-1000 µM for 24 h. The results are expressed as a percentage of cytotoxic effect in comparison to non-treated control cells. (c) The effect of quercetin and myricetin on light stimulated accumulation of cAMP in cells expressing opsin. HEK-293S GnTI- cells stably expressing WT opsin were incubated with the flavonoid compounds overnight and then 9-cis-retinal 2 h before the measurement. Forskolin was added to the cells to saturate their cAMP levels followed by
light illumination. Control cells, non-treated with flavonoids or 9-cis-retinal underwent the same procedure. cAMP levels were detected as described in the Experimental Procedures. Each condition was performed in triplicate and the experiment was repeated twice. Error bars represent S.D. Statistically significant effect of flavonoid was denoted with asterisks (**P≤0.001). NT, non-treated; 9cR, treated with 9-cis-retinal; Q-1, treated with 1 µM quercetin; Q-100, treated with 100 µM quercetin; M-1, treated with 1 µM myricetin; M-100, treated with 100 µM myricetin. (d) The Meta II decay of flavonoid-bound isoRho. Opsin within membranes was incubated with quercetin, quercetin-3-rhamnoside, myricetin, or myricetrin, followed by its regeneration with 10 µM 9-cis-retinal and then immunoaffinity purified. The chromophore release (Meta II decay) of flavonoid-bound isoRho after light illumination for 15 s is shown as a change in the intrinsic Trp fluorescence at 330 nm plotted as a function of time. These experiments were performed in triplicates. Insets, the rates (τ) of regeneration plotted as a function of the flavonoid concentration. Error bars represent S.D. All values of τ derived from these experiments and statistical significance of the effect of flavonoids are shown in Table 6.

Figure 8. Thermal stability of isoRho regenerated after incubation of the opsin membranes with flavonoids. Thermal stability of isoRho regenerated followed by an incubation of the opsin membranes with quercetin or myricetin at different concentrations (1, 10, 100, 250 and 500 µM) was determined in the immunoaffinity-purified samples. Samples were incubated at 55 °C in the dark and their absorbance spectra were recorded every 5 min for 60 min. The changes in the absorbance maximum were calculated as a percentage of residual pigment assuming the absorbance at the initial point as 100%. The changes were plotted as a function of time, and the half-lives (t1/2) of chromophore release were calculated using these plots. These measurements were performed in triplicates. Error bars represent S.D.
Figure 9. Effect of flavonoids on opsin membrane localization. (a) The cytotoxicity of quercetin (blue) and myricetin (red) on NIH-3T3 cells stably expressing P23H rod opsin was determined by using the MTT assay. The cells were treated with flavonoid compounds at concentrations within a range of 0-1000 µM for 24 h. The results are expressed as a percentage of cytotoxic effect in comparison to non-treated control cells. (b) Fluorescence images of the NIH-3T3 cells expressing P23H rod opsin mutant treated either with quercetin or myricetin (at a final concentration of 1 or 100 µM) or 5 µM 9-cis-retinal for 16 h. The images were taken with a high-content imaging
operetta microscope at 20x magnification. Scale bar, 50 µm. To detect opsin at the plasma membrane cells were immunostained with B6-30 antibody recognizing opsin’s N-terminus and Alexa594-labeled secondary antibody (orange). The nuclei of the cells were stained with DAPI (blue). (c) Quantification of the fluorescence intensity in the plasma membrane and the total fluorescence intensity in cells expressing P23H rod opsin mutant. (d) Ratio of the fluorescence intensity at the plasma membrane and the total fluorescence in cells expressing P23H rod opsin mutant. Statistically significant change in the fluorescence intensity is shown with asterisks, (***P≤0.0001). (e) The cytotoxicity of quercetin (blue) and myricetin (red) on NIH-3T3 cells expressing WT rod opsin was determined by using the MTT assay. The cells were treated with flavonoid compounds at concentrations within a range of 0-1000 µM for 24 h. The results are expressed as a percentage of cytotoxic effect in comparison to non-treated control cells. (f) Fluorescence images of the NIH-3T3 cells expressing WT rod opsin treated either with quercetin or myricetin (at a final concentration of 1 or 100 µM) or 5 µM 9-cis-retinal for 16 h. The images were taken with a high-content imaging operetta microscope at 20x magnification. Scale bar, 50 µm. To detect opsin at the plasma membrane cells were immunostained with B6-30 antibody recognizing opsin’s N-terminus and Alexa594-labeled secondary antibody (orange). The nuclei of the cells were stained with DAPI (blue). (g) Quantification of the fluorescence intensity in the plasma membrane and the total fluorescence intensity in cells expressing WT rod opsin. (h) Ratio of the fluorescence intensity in the plasma membrane and the total fluorescence in cells expressing WT opsin. NT, non-treated; 9cR, treated with 9-cis-retinal; Q-1, treated with 1 µM quercetin; Q-100, treated with 100 µM quercetin; M-1, treated with 1 µM myricetin; M-100, treated with 100 µM myricetin. Error bars represent S.D.
Figure 10. Effect of flavonoids on the glycosylation pattern of P23H rod opsin mutant and WT rod opsin. NIH-3T3 cells stably expressing the P23H rod opsin mutant were treated either with quercetin (a) or myricetin (b) at 1 or 100 µM final concentration, or 5 µM 9-cis-retinal for 16 h. NIH-3T3 cells stably expressing WT rod opsin were treated either with quercetin (c) or myricetin (d) at 1 or 100 µM final concentration, or 5 µM 9-cis-retinal for 16 h. The cells were lysed and 50 µg of total protein were separated using SDS-PAGE gel, followed by transfer to PVDF membrane. Opsin was detected with the 1D4 anti-Rho C-terminal antibody (top panels). Anti-GAPDH antibody was used as a loading control (bottom panels). All samples were deglycosylated with PNGaseF prior to the loading onto the gel. The experiment was repeated 3 times. These are representative immunoblots. NT, non-treated; 9cR, treated with 9-cis-retinal; Q-1, treated with 1 µM quercetin; Q-100, treated with 100 µM quercetin; M-1, treated with 1 µM myricetin; M-100, treated with 100 µM myricetin.
Figure 11. Effect of flavonoids on rod opsin dimerization/oligomerization within the cell membrane. (a) The effect of flavonoids on rod opsin membrane dimerization was tested with the BRET assay. The BRET signal was recorded in HEK-293 cells stably expressing both mouse opsin•Rluc and opsin•Venus, which were incubated with quercetin or myricetin at a concentration of 100 µM for 2, 4, 8 or 16 h. To disrupt opsin dimerization, DDM at 5 mM or 0.5 mM was added to the cell suspension prior to the BRET measurements. The results are expressed as net BRET for each condition. (b) Alternatively, isoRho was regenerated with 5 µM 9-cis-retinal for 2 h before the treatment with flavonoid compounds for 2 or 16 h. DDM at 5 mM concentration was added to the cell suspension to disrupt opsin dimerization prior to the BRET measurements. (c) The effect of flavonoids on the BRET signal in transiently transfected HEK-293 cells with different donor to acceptor ratios. The BRET signal was recorded in HEK-293 cells transiently transfected with opsin•Rluc (donor) and opsin•Venus (acceptor) constructs at different donor to acceptor ratios (1:2, 1:4, 1:6, 1:8). Twenty-four hours post-transfection, cells were incubated or not with flavonoids at a concentration of 100 µM for 16 h and then the BRET signal was measured in the cell suspension in each condition. (d) Time-dependent effects of flavonoids on the BRET signal in HEK-293 cells transiently transfected with opsin•Rluc and opsin•Venus constructs at a 1:4 donor to acceptor ratio. Twenty-four hours after transfection these cells were incubated with quercetin or myricetin at a concentration of 100 µM for 2, 4, 8, or 16 h, and then
the BRET signal was recorded in the cell suspension. To disrupt opsin dimerization, DDM at 5 mM was added to the cell suspension prior to the BRET measurements. (e) Specificity of the flavonoid-related effect on opsin-opsin interaction. The BRET signal was recorded in HEK-293 cells transiently transfected with opsin•Rluc (donor) only or in cells co-transfected with opsin•Rluc (donor) and Kras•Venus (acceptor) constructs used as a negative control and compared with the BRET signal recorded in cells co-transfected with opsin•Rluc (donor) and opsin•Venus (acceptor). The BRET signal detected in cells co-expressing opsin•Rluc and Kras•Venus was due to co-localization in the cell membrane and was much smaller than BRET detected in cells co-expressing opsin•Rluc and opsin•Venus. No effect of flavonoids on the BRET signal in cells co-expressing opsin•Rluc and Kras•Venus was detected. The results are shown as the BRET1 signal or NET BRET (ΔBRET). BRET was calculated as the emission ratio at 530 and 480 nm. Net BRET was calculated as the emission ratio at 530 and 480 nm (BRET1 signal) subtracted by the emission of donor only at 480 nm. ΔBRET = (530/480 ratio – 480 nm). Each experiment was performed in triplicate. Error bars represent S.D. Statistically significant change in BRET was indicated with asterisks. *P≤0.05, **P≤0.001 ***P≤0.0001.
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Joseph T. Ortega, Tanu Parmar and Beata Jastrzebska

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