Biochemical and Biophysical Research Communications 461 (2015) 665–670

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effective delivery of recombinant proteins to rod photoreceptors via lipid nanovesicles

Sabrina Asteriti [a, 1], Giuditta Dal Cortivo [b, 1], Valeria Pontelli [c], Lorenzo Cangiano [a], Mario Buffelli [c, d, *, 2], Daniele Dell'Orco [b, d, *, 2]

[a] Dept. of Translational Research, University of Pisa, Pisa, Italy
[b] Dept. of Life Sciences and Reproduction, University of Verona, Strada Le Grazie 8, Verona, Italy
[c] Dept. of Neurological and Movement Sciences, University of Verona, Strada Le Grazie 8, Verona, Italy
[d] Center for Biomedical Computing, University of Verona, Strada le Grazie 8, 37134 Verona, Italy

ARTICLE INFO

Article history:
Received 9 April 2015
Available online 25 April 2015

Keywords:
Liposomes
Retina
Recoverin
Phototransduction
Protein therapy

ABSTRACT

The potential of liposomes to deliver functional proteins in retinal photoreceptors and modulate their physiological response was investigated by two experimental approaches. First, we treated isolated mouse retinas with liposomes encapsulating either recoverin, an important endogenous protein operating in visual phototransduction, or antibodies against recoverin. We then intravitrally injected *in vivo* liposomes encapsulating either rhodamin B or recoverin and we investigated the distribution in retina sections by confocal microscopy. The content of liposomes was found to be released in higher amount in the photoreceptor layer than in the other regions of the retina and the functional effects of the release were in line with the current model of phototransduction. Our study sets the basis for quantitative investigations aimed at assessing the potential of intraocular protein delivery via biocompatible nanovesicles, with promising implications for the treatment of retinal diseases affecting the photoreceptor layer.

1. Introduction

Blindness remains one of the most dreaded diseases due to its dramatic impact on the quality of life of patients. Many early-onset forms of retinal disease are caused by inherited mutations in key proteins of the phototransduction cascade, which lead to deregulation of calcium levels and, sooner or later, to photoreceptor degeneration. In recent years a large number of studies have focused on the delivery of small therapeutic compounds, or even entire genes, to the retina (see for instance [1]). An alternative to gene therapy that would allow a better control of the outcome in terms of desired functional protein levels in photoreceptors, would be the direct delivery of intact and functional proteins. A 'protein therapy' approach could exploit the many advantages of the recombinant protein technology, allowing to compensate for the lack of native proteins and even outcompete the dysfunctional ones. One major drawback of protein therapy is that delivery can be rather challenging. Delivery of proteins via the blood stream, for instance, would lead to protein degradation, and successful attempts have been limited to compartmentalized areas of selected tissues like the brain or the heart. Proteins, however, could be encapsulated in lipid nanovesicles (liposomes) and delivered directly to the eye. Lipid-based nano-devices, including liposomes, have been shown to reduce the toxicity and increase the residence time of several active molecules in the eye, and can effectively protect their content from degradation [2–4]. Successful reports have shown the potential of liposomes for improving the treatment of a variety of eye diseases [5,6].

Here we explored the possibility of using 130 nm liposomes as carriers of high amounts of recombinant proteins in the posterior segment of the eye, in order to exert a specific physiological function. We used a lipid composition that ensures full biocompatibility while guaranteeing stability of the suspension. Liposomes were

* Corresponding authors. Center for Biomedical Computing, University of Verona, Strada le Grazie 8, 37134 Verona, Italy.
E-mail addresses: mario.buffelli@univr.it (M. Buffelli), daniele.dellorco@univr.it (D. Dell'Orco).
[1] Co-first author.
[2] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.04.088
0006-291X/

encapsulated with recombinant proteins affecting the phototransduction cascade. By *in vitro* acute treatment of mouse retinas and following electrophysiological recordings, we demonstrated that the release of the nanovesicle content within rod photoreceptors alters phototransduction in a way that is quantitatively in line with the current most comprehensive model of phototransduction kinetics. Moreover, we found that one intravitreal injection *in vivo* is sufficient to accumulate high amount of recombinant proteins in the photoreceptor layer.

2. Materials and methods

2.1. Preparation of recombinant proteins

Recombinant myristoylated recoverin (Rec) was expressed and purified as described in a previous work [7]. Details on the specific protocols are reported in the Supplementary Materials.

2.2. Preparation and characterization of liposomes with different lipid content

Lipid films for the preparation of lipid vesicles following hydration with physiological buffers were obtained by mixing suitable amounts of L-α-phosphatidylcholine from egg (PC, Sigma Aldrich) and cholesterol (Ch, Sigma Aldrich) dissolved in chloroform in order to obtain 8:1 w/w (PC:Ch 8:1) and PC:Ch 60:40 mixtures. Samples were aliquoted to get 2 mg lipid films per aliquot and dried down by vacuum in a speed-vac concentrator. Dried lipid films were frozen and stored at −80 °C until use. Liposomes incapsulating rhodamine B (RhB-Lip), Rec (Rec-Lip) and antibodies against recoverin (anti-Rec-Lip) were prepared and characterized by dynamic light scattering (DLS) as detailed in the Supplementary Materials.

2.3. Electrophysiology

Dark adapted mice were anesthesized by i.p. injection of urethane 20% W/V in 0.9% saline. Both retinas were rapidly extracted through corneal incisions into cold bicarbonate-buffered Ames' medium, the vitreous removed with forceps and each laid vitreal side down in a Petri dish containing 2 ml of Ames' supplemented with 10% specific liposome solution. The experimenter was blind as to the contents of the two dishes, which were prepared and labeled anonymously by another person. Both dishes were placed uncovered in the same chamber, which was continuously aerated with a 95% O_2/5% CO_2 mixture, and incubated at 37 °C for 2 h. Thereafter, the retinas were rinsed with Ames', laid down next to each other on filter paper and sliced as previously described [8]. Slices were transferred to the recording chamber and superfused with bicarbonate-buffered Ames' medium at ~24 °C. Recordings were targeted to slice areas displaying intact rod outer segments using a blind 'perforated loose seal' technique that does not alter rod response kinetics [9]. Recordings from the two retinas were interleaved, so as to compensate for any time-dependent effects on the properties of rods. Full field flashes of 1–10 ms duration and 520 nm wavelength were delivered as previously described [9]. Data are reported as mean and SEM or 95% confidence interval ($CI_{95\%}$). Statistical significance was assessed with the Mann-Whitney-Wilcoxon (MWW) test, the extra-sum-of-squares F-test (F-test) and the Kruskal–Wallis (KW) test.

2.4. Animals, surgery procedures for intravitreal injection, perfusion procedures, and immunoflourescence

The adult C57BL/6J mice (>P30) were anesthetized with 2,2,2-tribromoethanol (Sigma–Aldrich, St Louis, MO, USA); ip dose of 0.4 g/kg body weight and the eyes of control and experimental animals were injected intravitreally with either empty liposomes (2 mg/ml) or RhB-Lip (2 mg/ml) or Rec-Lip (1 mg/ml): each injection consisted of 1 μl of liposome stock solution (see above). Injections were performed, as described in Ref. [10], by using a heat-pulled glass capillary connected to a picospritzer (Parker Instrumentation, NH, USA).

At the end of the experiment, mice were sacrificed by terminal anesthesia and transcardially perfused with 4% paraformaldehyde in PBS, pH 7.4. The eyes were removed, postfixed and retina cryosections (10–20 μm thickness) were permeabilized, stained with antibodies and by DAPI (see Supplementary Materials for details). Acquisitions were performed by confocal microscope Leica SP5 and processed as described in the Supplementary Materials.

2.5. Mathematical modeling

A mathematical model developed previously [11] and describing the kinetics of the phototransduction cascade in mouse rods was used to simulate the effects of an excess or the lack of Rec on the photoresponses as a consequence of the delivery of Rec-Lip or anti-Rec-Lip. The conditions for the Rec knock-out case were simulated by setting the initial concentration of Rec to zero, while the excess of Rec was simulated by assuming a 4-fold higher initial concentration of Rec (i.e., 4×10^7 molecules instead of 1×10^7 molecules) with subsequent recalculation of the equilibria involving different states of Rec and rhodopsin kinase (RK) in the dark, similarly to what was previously done in Ref. [12]. *In silico* experiments for both wild type and liposome-modified rods were run as elucidated previously [13], allowing to assess numerical values for T_{sat}, the length of time that a saturating photoresponse remains at greater than 90% of its maximum current suppression, and τ_D, the dominant time constant of recovery from a saturating response, measured as the slope of T_{sat} over logarithmically increasing stimulus intensities (also called Pepperberg plot; see Ref. [14]).

3. Results

To investigate the effectiveness of protein delivery to photoreceptors via liposome encapsulation we used Rec, an endogenous protein regulating the duration of the phototransduction cascade in photoreceptor cells, or its antibody (anti-Rec). This choice allowed us to obtain a quantitative measure of protein incorporation in rods by performing single cell recordings of their flash responses, as well as to detect the protein distribution in the retina with immunofluorescence. We integrated the morphological analysis by also examining the delivery of liposomes loaded with the non-protein fluorescent marker rhodamine B (RhB).

3.1. Liposome suspensions characterization

Although liposomes were prepared following an identical procedure, the quality of the final suspension was found to strongly depend on the encapsulated content. While PC:Ch 60:40 liposomes loaded with RhB were significantly monodisperse with an average diameter of 124 nm as assessed by DLS (Fig. S1B), the same type of liposomes loaded with Rec led to two main populations in the suspension, corresponding to average diameters of 161 nm and 608 nm (Fig. S1A). This could be due to the fact that Rec in its Ca^{2+}-