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VITAMIN A DEFICIENCY REDUCES THE CONCENTRATION OF VISUAL PIGMENT PROTEIN WITHIN BLOWFLY PHOTORECEPTOR MEMBRANES

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Summary

Visual pigment extracts prepared from rhabdomeric membranes of vitamin A deficient blowflies contain a 5—10 times lower concentration of rhodopsin than extracts from flies which were raised on a vitamin A rich diet. Spectrophotometry showed that digitonin-solubilized rhodopsin from blowfly photoreceptors R_{1-6} has an absorbance maximum at about 490 nm, but no unusually enhanced β-band in the ultraviolet. The extracts did not contain detectable concentrations of other visual pigments nor was there any evidence for the presence of photostable vitamin A derivatives.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated that the concentration of opsin in the rhabdomeric membrane is significantly reduced in vitamin A deficient flies compared to normal flies. The results indicate that the synthesis of opsin or its incorporation into the photoreceptor membrane is regulated by the chromophore concentration in the receptor cell. Furthermore, our findings open up the possibility that differences in the spectral absorption and excitability of photoreceptors from normal and vitamin A deficient flies result from the differing opsin content of the rhabdomeres.

Introduction

The ommatidium of the fly compound eye consists of six peripheral receptors (R_{1-6}) and two central receptors (R_{7+8}). In muscoid flies (e.g. *Drosophila*, *Calliphora*) the rhabdomeric membranes of the receptors R_{1-6} contain a rhodopsin with an absorbance maximum at about 490 nm which is converted by light into a relatively stable metarhodopsin (λ_{max} about 570 nm) [1—3]. Raising flies on a vitamin A deficient diet reduces the concentration of rhodopsin within rhabdomeres of R_{1-6} [4—6]. Like vertebrate rhodopsin, the

insect visual pigments, including the ultraviolet-sensitive pigment [7], appear to be composed of a colourless protein (i.e. opsin) and 11-*cis* retinaldehyde, a derivative of vitamin A. Thus, it is possible that the decrease of the rhodopsin concentration in vitamin A deficient flies merely reflects the reduced availability of the retinaldehyde chromophore. But it is also possible that, in addition, vitamin A deficiency decreases the concentration of opsin. Some evidence for a lower concentration of opsin in vitamin A deficient flies comes from freeze-fracture studies. The cytoplasmic half of freeze-fractured microvillar membranes from rhodopsin-rich rhabdomeres is densely covered with particles (3000—4200 per μm^2), whereas the density of such particles is reduced to 600—1030 per μm^2 in vitamin A deficient flies [6,8,9].

The unequivocal demonstration that vitamin A deficiency influences the opsin concentration within the microvillar membrane may help to interpret the various physiological effects of vitamin A deficiency. Therefore, we have analysed the opsin content of rhodopsin-rich (R^+) and rhodopsin-deprived (R^-) rhabdomeric membranes from blowfly photoreceptors.

Material and Methods

Preparation of rhabdomeric membranes and rhodopsin extracts. The flies used in the experiments were males of the blowfly *Calliphora erythrocyephala* Meig. (chalky mutant). R^+ flies were obtained by rearing blowfly larvae on bovine liver, whereas R^- flies were raised on bovine heart meat [4]. Retinas were dissected out of the compound eyes and illuminated with red light to photogenerate rhodopsin from the metarhodopsin formed during the previous exposure of flies to room light. The subsequent preparative steps were carried out under red light. Fly rhabdomeres which are broken by homogenization of retinas into pieces of different length cannot be separated from other cell debris by differential or density gradient centrifugation without a substantial loss of rhabdomeric membranes. Therefore, partly purified rhabdomeric membranes were prepared by pressing retinas against a steel net (mesh-width 50 μm) of 18 mm diameter and washing them repeatedly with destilled water and 0.1 M sodium phosphate buffer (pH 6.5) in a Millipore sampling manifold. Most of the rhabdomeres were attached to the net and remained intact. They were removed from the net by ultra sonication at 0°C in phosphate buffer, collected by centrifugation (80 000 $\times g$, for 30 min) and extracted with 3% (w/v) digitonin in 0.01 M phosphate buffer (pH 6.5). Each extract was centrifuged at 100 000 $\times g$ for 1 h before being used for spectrophotometry and polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out on 12.5% (w/v) polyacrylamide gels, containing sodium dodecyl sulfate (SDS) according to the method described by Laemmli [10]. Before application to the gels 5% (w/v) SDS and 2% (v/v) 2-mercaptoethanol were added to the digitonin extracts. For the identification of opsin on the gels the chromophore was linked to opsin by reduction with 0.5% (w/v) sodium cyanoborohydride (Serva, Heidelberg) [11]. Formation of *N*-retinylopsin was observed after photoconversion of rhodopsin into metarhodopsin. Addition of sodium cyanoborohydride to digitonin-solubilized rhodopsin, or to extracts containing SDS

denatured rhodopsin, did not produce detectable amounts of *N*-retinyl-opsin. After electrophoresis gels were scanned at 334 nm to locate the opsin band. The gels were then fixed and stained with coomassie blue. Molecular weights were determined by running marker proteins together with portions from rhodopsin-rich extracts on the same gel [12].

Results

Repeated extraction with digitonin solubilized more than 90% of the rhodopsin present in the partly purified rhabdomeric membranes. Extraction of membranes from 100 R^+ flies gave approximately $3 \cdot 10^{-10}$ mol of rhodopsin, the corresponding extracts from R^- flies contained a 5—10 times lower amount of rhodopsin. Generally, the extracts were contaminated to the same extent by a compound absorbing maximally at about 410 nm. Therefore, we recorded the absorbance difference between extracts from R^+ and R^- flies. Such a difference spectrum is shown in Fig. 1a and can be seen to fit closely a rhodopsin nomogram with λ_{max} at 490 nm [13]. Spectral differences between these extracts, other than those which can be attributed to the difference in the concentration of a rhodopsin (λ_{max} 490 nm), were never observed.

In order to be able to determine the position of opsin on SDS polyacrylamide gels the chromophore was bound covalently to opsin by reduction with sodium cyanoborohydride. The difference spectra shown in Fig. 1b indicate that metarhodopsin (λ_{max} 570 nm) is rapidly converted into *N*-retinylopsin. This is confirmed by the detection of a high concentration of a retinyl-protein after SDS gel electrophoresis of R^+ extracts, whereas the concentration of this protein in R^- extracts is substantially reduced (Fig. 2, lower tracings). The binding of the chromophore to opsin presumably results, as in vertebrates [14], from the reduction of a Schiff's base linkage between retinaldehyde and the ϵ-amino group of a lysine residue.

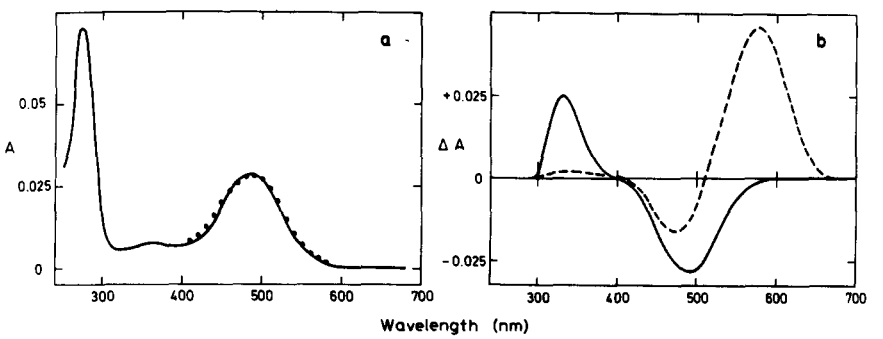

Fig. 1. Absorbance characteristics of digitonin extracts from blowfly photoreceptor membranes. (a) Absorbance difference recorded after placing an extract from 200 rhodopsin-rich retinas into the sample cuvette, and the corresponding extract from vitamin A deficient flies into the reference cuvette. The closed symbols represent a nomogram for a visual pigment with maximum absorbance at 490 nm [13]. (b) Absorbance changes recorded after sodium cyanoborohydride was added to both extracts (final conc. 0.5%) and the rhodopsin-rich extract had been illuminated with blue light (472 nm); (- - - - -), absorbance change after 2 min of illumination at 8°C, resulting primarily from the formation of metarhodopsin (λ_{max} 570 nm); (———), absorbances change after 6 min of illumination at 25°C, indicating that *N*-retinylopsin (λ_{max} 333 nm) was formed.

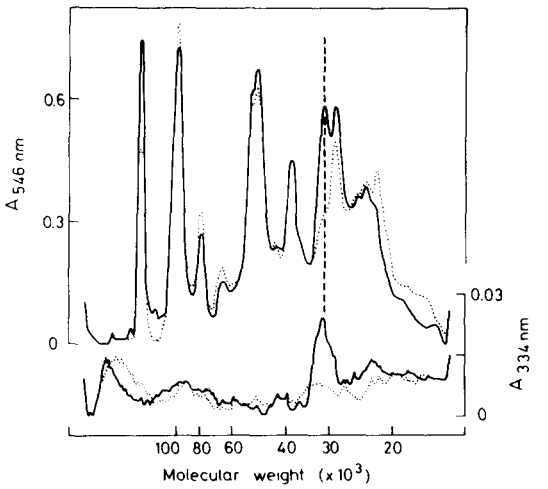

Fig. 2. Identification of blowfly opsin from receptors R_{1-6} after SDS gel electrophoresis. Digitonin extract from rhodopsin-rich membranes (———); digitonin extract from membranes of vitamin A deficient flies (· · · · ·). Retinaldehyde chromophores were bound to opsin by reduction with cyanoborohydride. The position of *N*-retinylopsin in coomassie-stained gels (upper tracings) is indicated by the absorbance peak observed after scanning the unstained gels at 334 nm (lower tracings).

SDS gel electrophoresis indicates that the chromophore was bound exclusively to a protein with an apparent molecular weight of 32 500 ± 1600 (Fig. 2). The molecular weight obtained in this way for the *N*-retinylopsin of blowfly receptors R_{1-6} was confirmed by using different acrylamide concentrations and different buffer systems. The value is close to that estimated for an insect ultraviolet-visual pigment [10], but lower than the value of 37 000 reported for *Drosophila* opsin [15].

The membrane material from rhodopsin-rich flies gives a distinct protein band in the position of *N*-retinylopsin (Fig. 2). This band is also present if rhodopsin has not been converted into *N*-retinylopsin prior to electrophoresis. As demonstrated most clearly in Fig. 3, the concentration of opsin is significantly reduced in the extract which was prepared from vitamin A deficient

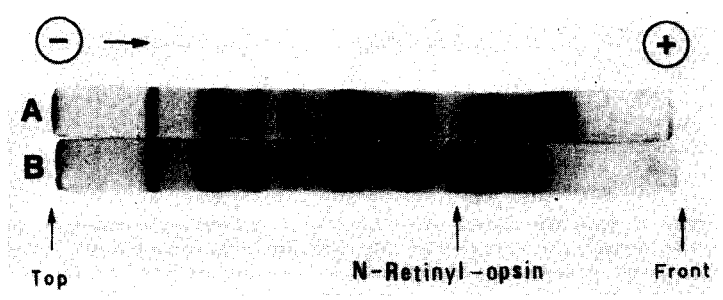

Fig. 3. Protein pattern obtained by SDS gel electrophoresis of digitonin extracts from blowfly photoreceptor membranes. 100 µl extract from rhabdomeres of vitamin A deficient blowflies applied to gel (A) contained about $0.26 \cdot 10^{-10}$ mol rhodopsin. 100 µl extract from rhodopsin-rich rhabdomeres applied to gel (B) contained about $1.5 \cdot 10^{-10}$ mol rhodopsin. The position of opsin was obtained from its absorbance at 334 nm after being converted into *N*-retinylopsin.

flies. With the exception of the opsin band and a protein band with a molecular weight of about 20 000, the protein pattern of the extracts from R^+ and R^- flies are identical and can be taken as an indication of the reproducibility of the method used to prepare the extracts. The rhodopsin content of R^- photoreceptors can be increased most efficiently by the injection of 11-*cis* retinaldehyde [16]. The electrophoretic pattern of photoreceptor membrane proteins prepared from R^- flies injected with 11-*cis* retinaldehyde reveals that in parallel with the rhodopsin content the concentration of protein in the opsin band increases up to the level observed in R^+ flies. Whether the injection of 11-*cis* retinaldehyde also affects the 20 000 dalton band has not yet been measured. Thus, our results demonstrate that vitamin A deficiency not only reduces the number of chromophores required for the formation of rhodopsin, but also decreases the opsin content of fly photoreceptor membranes.

Discussion

The finding of a reduced opsin concentration in R^- flies supports the hypothesis that a substantial fraction of the particles seen in freeze-fractured microvillar membranes [6,8,9] is equivalent to rhodopsin. Together with the observation that a formation of rhodopsin-containing membranes can be induced particularly by injecting 11-*cis* retinaldehyde into the eyes of R^- flies [16], our data suggest that the synthesis of opsin or its incorporation into the photoreceptor membranes is regulated by the concentration of chromophore within the receptor cell.

With the exception of changes in the particle density, electron microscopy has failed to reveal any structural differences between rhabdomeres now known to differ in their opsin content [6,9]. The absence of degenerative processes raises the question as to how the microvilli, depleted of most of their opsin, maintain their structural integrity. Opsin in the membrane could be replaced by lipids or proteins: it is, therefore, of interest to note that extracts from R^- flies contained varying concentrations of a protein (apparent molecular weight about 20 000) which was not extracted from R^+ membranes.

Vitamin A deficiency in flies has been shown to decrease the ultraviolet sensitivity maximum of R_{1-6} [17], which is usually indicated by a second peak in the receptor potential action spectrum at about 350 nm, and to eliminate the prolonged depolarizing afterpotential [18,19], which is recorded from rhodopsin-rich receptors after a certain percentage of rhodopsin has been converted into metarhodopsin. As illustrated by Fig. 1a, extracts from R^+ and R^- flies differ only in the concentration of rhodopsin (λ_{max} 490 nm) present. Thus, the characteristic double-peaked spectral sensitivity curve of rhodopsin-rich receptors R_{1-6} is not due to the presence of two distinct visual pigments. The high sensitivity of R_{1-6} to ultraviolet light could be caused by a photostable pigment that absorbs light quanta and transfers the energy to rhodopsin, as suggested by Kirschfeld et al. [20]. Accordingly, R^+ flies should contain a higher concentration of such a pigment than R^- flies, since not only the ultraviolet sensitivity, but also the absorbance of the rhabdomeres appears to be vitamin A dependent [16]. Our finding that digitonin extracts from rhodopsin-rich rhabdomeres do not have a significantly higher content of a photostable

vitamin A derivative does not rule out the existence of a sensitizing pigment, as the pigment may be water-soluble or not extractable by digitonin. However, another possibility remains: that rhodopsin itself exhibits unusual absorbance properties when densely packed into the rhabdomeric membrane. Pigment-pigment interactions in rhodopsin-rich membranes might create absorption properties similar to that which exist in the insect ultraviolet-visual pigments with maximum absorbance at 350 nm [1]. These interactions may also stabilize the photoproduct which, by an unknown mechanism, causes the prolonged depolarization of rhodopsin-rich fly photoreceptors [19].

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