LIGHT-ACTIVATED PHOSPHORYLATION OF CEPHALOPOD RHODOPSIN

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1. Introduction

Light absorption in the transducer region of vertebrate rod visual cells induces a Mg^{2+}-dependent, kinase-catalyzed phosphorylation of the visual pigment protein [1–3]. This light-activated rhodopsin phosphorylation is a reversible process [4–7] which is induced by an early event in the photolytic sequence of rhodopsin [8]. Unlike phosphorylation of proteins from other neuronal membranes, e.g., synaptic membranes [9], rhodopsin phosphorylation appears not to be regulated by cyclic nucleotides [1,2,10.11]. Furthermore, rhodopsin phosphorylation is not causally related to the light-induced activation of cyclic GMP phosphodiesterase [12] which may be an essential step in the process of sensory transduction in vertebrate visual cells [13]. Thus, the function of rhodopsin is not yet understood, despite some evidence pointing to an involvement in the control of visual sensitivity at the photoreceptor level [14–16].

In the present paper we have investigated whether or not light-activated phosphorylation of the visual pigment protein occurs in invertebrate photoreceptor membranes. Although the visual pigment systems, as well as the mechanism of visual transduction and adaptation, in invertebrates differ in many respects from that in vertebrates we find that light-activated rhodopsin phosphorylation in octopus photoreceptor membranes proceeds with characteristics similar to that observed in vertebrate rods.

2. Materials and methods

Eyes of the octopus *Eledone aldrovandii* were enucleated under dim red light (> 610 nm) from animals which, prior to the dissection, had been dark-adapted for 12 h. The eyes were frozen immediately to −30°C and stored subsequently at −80°C in the dark. Eyes being used for control experiments with unfrozen material were stored on ice for several hours. The standard procedure for the isolation of photoreceptor membranes included:

(i) Mechanical removal of outer segments from the photoreceptor layer;
(ii) Homogenization of photoreceptor membranes in Hepes buffer (pH 7.0) containing 20 mM Hepes (*N*-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, Serva Heidelberg), 5 mM $MgSO_4$ and 2 mM dithiothreitol and
(iii) Flotation of microvillar membranes by centrifugation (15 000 × *g* for 30 min) on 1.5 M sucrose.

The floated membranes were sedimented by centrifugation in Hepes buffer (12 000 × *g* for 6 min). The resulting membrane pellet was suspended in Hepes buffer to final rhodopsin conc. ~4×10^{-8} mol/ml.

In order to measure rhodopsin phosphorylation 5 mM [γ-^{32}P]ATP (Amersham-Buchler) (spec. act. 2–5 mCi/mmol) was added and the suspension divided into portions for light and dark conditions. Light portions were illuminated 3–5 min after the addition of ATP either with blue flashes or with continuous blue (482 nm) light which established a photostationary state of about 50% rhodopsin and 50% metarhodopsin within 4–8 min. The incubation temperature was 22°C. Sampling of membranes during the incubation period, quenching of probes with 10% trichloroacetic acid and preparation of the membrane material for radioactivity counting and SDS–gel electrophoresis were performed as in [5,6]. Gel electrophoresis was carried out as in [17]. For determination of visual pigment concentrations rhodopsin was extracted with

2% digitonin from membrane portions kept in the dark. The rhodopsin concentration was calculated from the ΔA_{470} (ϵ = 39 500) after rhodopsin had been converted into alkaline metarhodopsin which, in the presence of 0.2 M hydroxylamine, reacted to form retinaloxime.

3. Results and discussion

Illumination with light of a wavelength which converts rhodopsin (λ_{max} 470 nm) into the thermally-stable photoproduct metarhodopsin (λ_{max} 516 nm) [18] markedly increases the incorporation of radioactive [γ-^{32}P]ATP into the photoreceptor membranes. This light-activated phosphorylation of microvillar membranes occurs with material prepared from unfrozen retinas as well as from eyes stored at −80°C (fig.1a,b). SDS–gel electrophoresis of the labeled membranes shows that the light-dependent phosphate incorporation is largely confined to the protein part of rhodopsin (app mol. wt 51 000) and dimers of rhodopsin, respectively (fig.2). Phosphorylation of a further membrane protein (app. mol. wt ~160 000) is not light-dependent. Generally, light induced the incorporation of 0.3–0.8 mol phosphate/mol rhodopsin. In some experiments (table 1) the maximum light-activated phosphorylation was 2 phosphates/rhodopsin present in the membrane. Phosphate incorporation can also be initiated by bright blue flashes. Since the maximum of light-activated phosphate incorporation develops after cessation of the illumination (fig.1b), visual pigment phosphorylation must be a dark reaction following the formation of metarhodopsin.

Phosphorylation of the visual pigment protein has no obvious effects on the spectral properties of

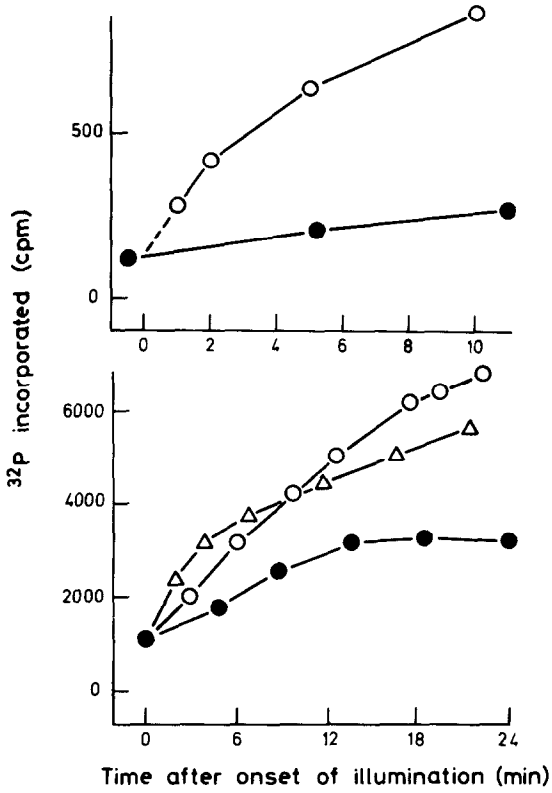

Fig.1. Light-activated phosphate incorporation from 5 mM [γ-^{32}P]ATP into octopus photoreceptor membranes. (a) Phosphate incorporation into membranes from unfrozen retinas suspended in 0.1 M Tris buffer (pH 7.0) containing 4 mM $MgCl_2$ and 2 mM EGTA· (○) during illumination (482 nm); (●) into dark controls. (b) Into membranes obtained from eyes stored at −80°C: (○) during continuous illumination (482 nm); (△) after illumination with 5 flashes applied within the first minute; (●) dark controls. Incubation medium was standard Hepes buffer (pH 7.0).

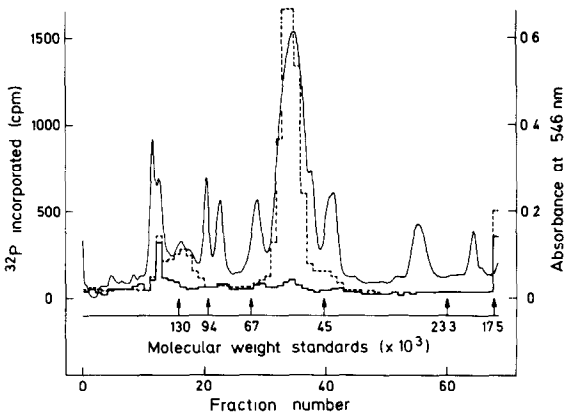

Fig.2. Electrophoretic pattern of incorporation of ^{32}P from [γ-^{32}P]ATP into proteins of octopus photoreceptor membranes. Incubation was carried out in standard Hepes buffer (pH 7.0) containing 5 mM $MgSO_4$ and 2 mM EGTA. Illuminated (continuous light, 482 nm) membrane portions (broken line) and dark controls (solid lines) were dissolved in SDS and subjected to SDS–gel electrophoresis. Radioactivity in gel fractions was measured by liquid scintillation counting. Also shown are the positions of mol. wt markers and the absorbance of the Coomassie blue stained gels.

Table 1
Characteristics of phosphate incorporation into octopus photoreceptor membranes

Conditions	Phosphate incorporation from [γ-^{32}P]ATP (ratio to phosphate incorp. into controls)	
	Light-activated[a]	Dark[b]
8.5 mM Mg^{2+}, 0.7 mM EGTA	2.0	1.5
1.3 mM Mg^{2+}, 0.7 mM EDTA	0.2	0.5
5.0 mM Ca^{2+}	1.0	0.55
2.5 mM EGTA	1.2	1.2
100 mM NaCl	2.4	1.1
100 mM KCl	2.3	1.1
0.2 mM cAMP	1.2	1.6
0.2 mM cGMP	1.1	1.7
5 mM [γ-^{32}P]GTP instead of 5 mM [γ-^{32}P]ATP	0.2	0.4

[a] Ratio of light-activated ^{32}P incorporation into test samples to light-activated ^{32}P incorporation into controls

[b] Ratio of ^{32}P incorporation into test samples to ^{32}P incorporation into controls

The standard medium (controls) contains Hepes buffer (pH 7.0), 5 mM $MgSO_4$, 2 mM dithiothreitol and 5 mM [γ-^{32}P]ATP. Values for phosphate incorporation (duplicate determination which agreed within 10%) were taken from time courses for ^{32}P incorporation at 22°C after 20 min of incubation in continuous light (482 nm) and in the dark, respectively

extracted rhodopsin and metarhodopsin. Illumination with light which is absorbed by metarhodopsin, but not by rhodopsin, does not induce phosphorylation. Furthermore, we have found no evidence that photoregeneration of rhodopsin inhibits light-activated phosphorylation or induces dephosphorylation of rhodopsin. Thus, as demonstrated for vertebrate rhodopsin [5,6], the return of visual pigment molecules to a dephosphorylated state in which rhodopsin cannot be phosphorylated, appears to be regulated independently of the regeneration of the spectral properties of rhodopsin.

Phosphorylation of rhodopsin requires the presence of Mg^{2+} (table 1). Further results summarized in table 1 indicate that presence of 5 mM Ca^{2+} primarily decreases dark phosphorylation of photoreceptor membranes. Addition of Na^+ or K^+ increases light-activated phosphate incorporation significantly. As in vertebrates, cyclic nucleotides seem not to activate rhodopsin phosphorylation. The increase in the dark phosphorylation in the presence of cyclic nucleotides is partly due to an increased binding of phosphate to the 160 000 mol. wt protein. Small dark–light differences in the phosphate incorporation are also observed when [γ-^{32}P]GTP is used as a substrate for rhodopsin phosphorylation. Procedures which have been successfully used to extract the rhodopsin phosphorylation catalyzing kinase from vertebrate rod outer segment membranes are here found to reduce phosphate incorporation into octopus photoreceptor membranes. However, so far we have not succeeded in restoring the original light-activated phosphate incorporation by recombining rhodopsin free extracts with the illuminated rhodopsin containing membrane fraction. Therefore, we cannot decide at present that whether rhodopsin phosphorylation is catalyzed by a specific kinase or that the octopus rhodopsin itself displays enzyme activity.

Phosphorylation of octopus rhodopsin, which in vitro appears to be a relatively slow process,

resembles the phosphorylation reaction described for vertebrate rhodopsin. Therefore, further studies on the function of visual pigment phosphorylation should focus on processes which are inherent to both types of photoreceptor cells. Our finding of a light-induced, possibly longer-lasting modification of visual pigment molecules which does not express itself in a change in the spectral properties of rhodopsin or metarhodopsin supports the hypothesis that spectrally identical pigments which have different functional states may exist within the invertebrate photoreceptor membranes. This has been concluded recently from an analysis of the prolonged depolarizing afterpotential [19], which can also be recorded from cephalopod photoreceptors [20]. Our demonstration of a light-activated phosphorylation process in invertebrate photoreceptor membranes may also indicate that light-controlled enzyme reactions are causally involved in the process of visual transduction and adaptation in invertebrates as has been proposed for vertebrate visual cells.

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