THE ROLE OF BLEACHING AND PHOSPHORYLATION OF RHODOPSIN IN THE
CONTROL OF Ca²⁺ PERMEABILITY

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(Received September 5, 1979)

Summary

Samples of rod outer segments have been extracted with low concentrations of sodium dodecyl sulphate to yield a vesicular preparation which consists almost entirely of rhodopsin and lipids. Exposure of such preparations to light increases their rate of calcium uptake while exposure of calcium loaded preparations to light increases their rate of calcium efflux. It is thus concluded that bleaching of rhodopsin increases the calcium permeability of the preparations by opening a calcium pore which is part of the rhodopsin molecule itself.

Previous work has demonstrated that if preparations of rod outer segment discs are incubated with ATP under conditions where rhodopsin will be phosphorylated the rate of both calcium uptake and efflux are lowered. It was concluded that phosphorylation of rhodopsin lowers calcium permeability of the discs overcoming the effect of light. It could, however, be argued that the effect was not due to phosphorylation of rhodopsin but to binding of ATP. Experiments were thus carried out with the vesicular preparation of rhodopsin to test this point. It was found that the calcium permeability of the vesicles was only lowered when they had been preincubated with ATP and opsin kinase. Incubation with ATP or opsin kinase alone had no effect. The suggestion that phosphorylation of rhodopsin lowers calcium permeability is thus confirmed. Phosphorylation presumably blocks a calcium channel in the rhodopsin molecule itself.

Introduction

It is widely believed that changes in the concentration of intracellular Ca²⁺ have an important role in the physiological response of photoreceptors to light. It has been suggested that the mechanism by which light causes the hyperpolarisation of vertebrate rod outer segments (ROS) is that photons are absorbed by the rhodopsin of the ROS discs causing a release of Ca²⁺ which interacts with the outer membranes of the ROS lowering their permeability to Na³⁺. In support of this hypothesis several groups have reported that exposure of intact ROS discs to light causes an efflux of Ca²⁺ though other workers find difficulty in confirming these observations (see 10 for references). We have shown that exposure of ROS discs to light causes an increase in Ca²⁺ permeability (an opening of Ca²⁺ pores) since the rate of entry of ⁴⁵Ca into light exposed discs is increased, as is the rate of efflux of ⁴⁵Ca from preloaded material, while exposure to light does not affect the binding of ⁴⁵Ca to the discs at equilibrium. Despite these observations several authors have expressed considerable doubt about the hypothesis that liberation of Ca²⁺ by light is the mechanism by which the photoresponse is generated. Even if the hypothesis is incorrect, however, there is universal agreement that Ca²⁺ must play an important role in regulating the sensitivity of ROS to light.

It is now well established that rhodopsin from frog or cattle ROS can be phosphorylated in a reaction which is stimulated by light, due to the presence of an enzyme in the ROS
opsin kinase) which acts specifically on bleached, but not unbleached, rhodopsin\textsuperscript{12,21}. We have shown that incubation of light-exposed ROS discs, with ATP and Mg\textsuperscript{2+}, reduces the Ca\textsuperscript{2+} permeability to a value similar to that found in the dark-kept material\textsuperscript{11,13} and have postulated that this is due to phosphorylation of rhodopsin. The reaction may play an important role in dark and light adaptation\textsuperscript{22} though it is too slow by several orders of magnitude\textsuperscript{23} to be directly involved in visual excitation. It could, however, be argued that the effect of incubating the ROS discs with ATP and Mg\textsuperscript{2+} was not due to phosphorylation of rhodopsin but to binding of ATP. Experiments were thus designed to overcome this criticism.

It is possible to extract non-rhodopsin proteins and bleached rhodopsin from ROS with low concentrations of sodium dodecyl sulphate\textsuperscript{24}; the remaining material consists almost entirely of rhodopsin and lipids and is in the form of vesicles\textsuperscript{25,26}. The rhodopsin in such preparations can only be phosphorylated on incubation with ATP and Mg\textsuperscript{2+} in the presence of an extract from ROS which contains opsin kinase activity\textsuperscript{12}. It is thus possible to measure the effect of incubation with ATP under phosphorylating and non-phosphorylating conditions.

**Methods**

**Rod Outer Segments (ROS)**

Were prepared by the method of Virmaux et al.\textsuperscript{27}.

**Tris Extract from Rod Outer Segments**

Samples of rod outer segments were homogenised at a protein concentration of about 0.5 mg/ml in dim red light in 10 mM tris HCl pH 7.0 and centrifuged at 100,000 g for 60 min.

**Rhodopsin rich Vesicles**

were prepared by twice extracting the pellet remaining after extraction of ROS with tris HCl with 0.1% sodium dodecyl sulphate in 10 mM tris HCl (pH 7.0). The insoluble material was washed three times with 66 mM Na phosphate buffer (pH 7.0) and twice with 10 mM tris HCl (pH 7.0)). This procedure removes not only non-rhodopsin proteins but also bleached rhodopsin\textsuperscript{24}.

**Phosphorylation of Rhodopsin**

The amount of phosphate transferred to rhodopsin from \[^{32}P\] ATP was determined as previously described\textsuperscript{12}.

**Determination of Calcium uptake**

Samples of rhodopsin rich vesicles were divided into two parts, one of which was kept in dim red light while the other was exposed to white light for 2 min (all subsequent operations being carried out in dim red light). Samples of the light exposed material were either not treated further or incubated for 10 min at a protein concentration of about 0.5 mg/ml with 50 mM tris HCl pH 7.4, 1 mM MgCl\textsubscript{2}, 1 mM ATP and 80 \(\mu\)g (protein)/ml of a tris extract prepared from ROS which contains opsin kinase activity (Weller, Virmaux and Mandel (1974)). Samples of the light exposed material were also incubated under the same conditions but in the absence of ATP or in the absence of the opsin kinase. Separate experiments demonstrated that incubation of the rhodopsin rich vesicles with opsin kinase and ATP under the above conditions results in maximal phosphorylation, 16 nMoles of phosphate being incorporated/mg of protein. Incubation with ATP in the absence of opsin kinase, however, gave no detectable phosphorylation. All samples were centrifuged for 15 min at 100,000 \(\times\) g; the pellet washed once by resuspension and centrifugation from 10 mM tris HCl pH 7.4, suspended in 10 mM tris HCl pH 7.4, 0.3 M sucrose at a protein concentration of about 0.3 mg/ml, and 100 \(\mu\)M \[^{45}\text{Ca}\text{Cl}\text{]}\text{ (specific radioactivity about }1 \times 10^7 \text{ cpm/\mu mol)}\text{ added to a concentration of }10 \mu\text{M. Samples (0.5 ml) were filtered at stated times through millipore filters (0.45 \(\mu\) pore size). The filters were then washed twice with 5 ml lots of 10 mM tris HCl (pH 7.4), dried, and the amount of bound radioactive calcium counted as previously described (Weller, Virmaux and Mandel (1975)). Samples were also saved to determine the amount of protein in the suspensions.

**Determination of Calcium Efflux**

Samples of rhodopsin rich vesicles were divided into two parts one of which was kept in dim red light while the other was exposed to white light for 2 min (all subsequent operations were carried out in dim red light). Samples of the light exposed material were either not treated further