LIGHT-ACTIVATED RHODOPSIN PHOSPHORYLATION MAY CONTROL LIGHT SENSITIVITY IN ISOLATED ROD OUTER SEGMENTS

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1. Introduction

A central problem in understanding visual receptor function has been the lack of data correlating physiological changes related to light with the molecular events which might generate or regulate these changes. The problem arises because physiological studies are usually carried out on living intact receptor cells, where it is difficult to localize chemical or pharmacological effects. Most chemical studies, on the other hand, are performed with isolated or fractionated outer segments which have lost their physiological activity.

To meet this problem we have developed an in vitro assay for rod outer segment function which permits us simultaneously to monitor physiological and chemical changes. In this paper we present a correlation between this in vitro physiology and a light-activated rhodopsin phosphorylation reaction [1–5]. We have observed that phosphorylation inhibitors increase sensitivity, the effectiveness of light in decreasing permeability, but do not influence the maximum change in permeability which can be caused by illumination.

2. Materials and methods

Retinas were dissected from a dark-adapted bullfrog (*Rana catesbeiana*) under dim red light. The rod outer segments were shaken off in 1.2 ml of a Ringer's solution (115 mM NaCl, 2 mM KCl, 10 mM Hepes (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) (CalBiochem, San Diego, California), 10% (v/v) calf serum, 3 mM EGTA (ethylene glycol bis (β-amino ethyl ether) *N,N'*-tetraacetic acid), 1 mM ATP, 0.5 mM $MgCl_2$, and 3 mM dithiothreitol). The material was divided for assay of phosphorylation and for simultaneous measurement of permeability changes, and 0.1 mM papaverine was added to both portions. In some experiments ATP was not included and trace amounts of inorganic ^{32}P were added for phosphorylation (see below).

We and others have presented data [6,7] that indicate the rate of swelling of outer segments in vitro in the dark depends on the permeability of the outer plasma membrane, and thus we take light suppression of swelling to reflect suppression of this permeability. We monitored swelling of rod outer segments in 100 μl samples diluted with 15 ml Ringer's solution containing the indicated additions. The volumes of about 20 000 outer segments were measured for each point by a particle size analysis system [6].

In the dark, outer segments swell (fig.1A). Under light conditions in which at least 5×10^5 rhodopsin molecules are bleached/outer segment/sec (high level of light), the volume of outer segments remains relatively constant (fig.1A).

Low illumination, 5×10^3 rhodopsin molecules bleached/outer segment/sec, caused about half the permeability decrease we observed with high illumination (fig.1A). We examined light sensitivity of the permeability decrease by measuring the amount of swelling suppression during this low level of illumination. An increase in sensitivity was reflected by a greater suppression of swelling.

To measure phosphorylation, trace amounts of [γ-^{32}P] ATP (final specific activity of 6 Ci/mole; carrier-free [γ-^{32}P] ATP from New England Nuclear, Boston) was added to the rod outer segment suspension. Twenty μl samples were removed from the incubation

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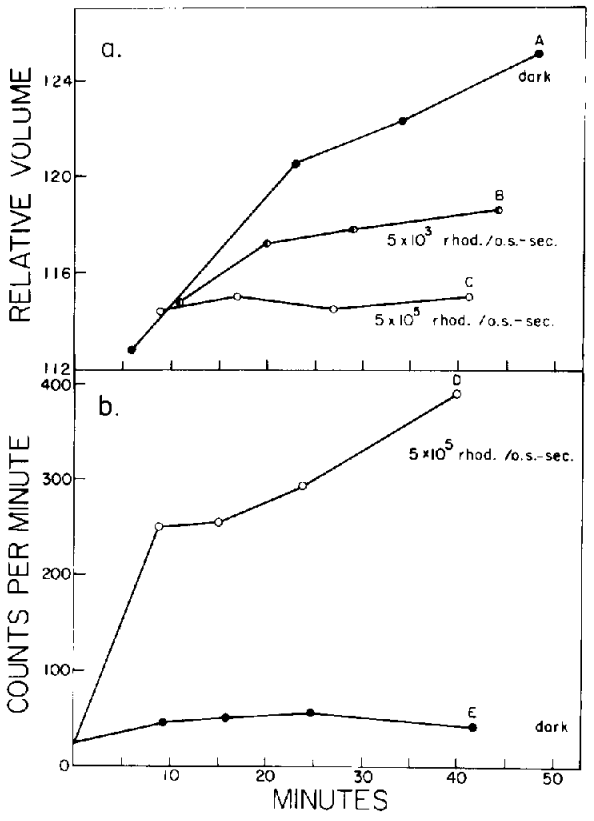

Fig.1. Relation between illumination, swelling and phosphorylation of isolated rod outer segments of bullfrog retinas. (A) Volume changes of isolated rod outer segments. Diluted outer segments kept in the dark swell (•); those illuminated with a high level of light do not swell (○). We define this difference as the permeability change due to high illumination. Outer segments illuminated with a low level of light show partially suppressed swelling (•). This decrease from the dark swelling level is taken as a measure of sensitivity. The lettered points are used in the sample calculations for tables 1 and 2. (B) Light-activated phosphate incorporation from 1 mM ATP (6 Ci/mol [γ-^{32}P] ATP): (○), incorporation in light; (•), dark.

and pipetted into 2 ml of a 10% trichloroacetic acid–50 mM Na_3PO_4 solution. The precipitate was collected and washed on Millipore filters, which were then dissolved in Aquasol (New England Nuclear, Boston) and the radioactivity was measure (fig.1B). Adenosine and β-γ-methylene ATP (both from Sigma, St. Louis, Missouri) were used as inhibitors of phosphorylation.

3. Results and discussion

The maximum permeability change of the rod outer segments and the light-activated phosphorylation reaction can be inhibited independently. Table 1 compares the effects of cyanide and adenosine on the light-dependent processes. Inhibition by cyanide of the permeability decrease observed at high light levels did not correlate with a decrease in phosphorylation. Conversely, inhibition by adenosine of phosphorylation did not alter the permeability decrease due to a high level of light. In other experiments, in vitro dark adaptation (recovery of permeability in the dark after illumination [6]) was also unaltered by the presence of adenosine.

However, the permeability decrease caused by a low light level was significantly enhanced by adenosine (1–10 mM) (table 2). No change resulted from 0.1 mM

Table 1
Inhibition of light-dependent processes

	% of control	
	Permeability decrease	Phosphorylation
5–10 mM NaCN	53 ± 10 (3)[a]	106 ± 8 (6)
1–10 mM Adenosine	117 ± 14 (4)	7 ± 3 (2)[a]

The extent of each light-dependent process in the presence and absence of the indicated addition is expressed as a ratio. We define permeability decrease as the difference between the volumes of unilluminated rod outer segments and of illuminated rod outer segments (e.g., A minus C for the indicated points in fig.1A). The data in the first column are the ration of permeability decrease in a high level of light with cyanide or adenosine to the decrease without that addition (control). We define light-activated phosphorylation as the difference between counts of ^{32}P incorporated in the illuminated sample and in the unilluminated sample (e.g., D minus E for the indicated points in fig.1B). The data in the second column are the ratios of light-activated phosphorylation with the addition, to that without the addition. The tabulated numbers are the mean of the ration ± sample estimate of standard error of the mean ($S/\sqrt{n-T}$). The number of experiments is in parentheses. The probability that the experimental mean is not less than the control mean (tabulated ratio < 100%) was determined by Student's t-test.
[a] $p < 0.005$

Table 2
Effect of phosphorylation inhibitors on sensitivity

Condition	Sensitivity (% maximum permeability decrease)
I control	61 ± 12 (2)
0.1 mM adenosine	62 ± 7 (2)
II control	56 ± 2 (7)
1–10 mM adenosine	71 ± 3 (7)[a]
III control (no ATP)	35 ± 2 (2)
1–10 mM adenosine	51 ± 1 (2)[a]
IV control (no ATP)	32 ± 12 (3)
5 mM β-γ-methylene ATP	107 ± 20 (3)[a]

The data are expressed as ratios of permeability decrease in low light to that in high light (fig.1A: $\frac{A-B}{A-C}$). The tabulated numbers are the mean of the ratios ± sample estimate of the standard error of the mean. The number of experiments is in parentheses. The probability that the mean for the incubations with an inhibitor is not different from the control mean was determined by Student's t-test.
[a] $p < 0.05$.

adenosine. Phosphorylation was completely inhibited by 1–10 mM adenosine, but not measurably inhibited by 0.1 mM adenosine. Thus, adenosine affected sensitivity and phosphorylation in the same concentration range.

When rod outer segments were incubated in a solution prepared without ATP, adenosine caused a 93% (±3%, five experiments) inhibition of the light-dependent phosphate incorporation measured with inorganic ^{32}P. Under these conditions phosphorylation must be using endogenous trinucleotides as substrate [8]. When sensitivity was measured in the absence of added ATP, we again observed that the presence of adenosine enhanced the permeability decrease by low light (table 2).

Another inhibitor of rhodopsin phosphorylation is β-γ-methylene ATP. With no ATP in the incubation solution, 5 mM β-γ-methylene ATP inhibited phosphate incorporation up to 80% during illumination, which bleached 5×10^5 rhodopsin molecules/outer segment/sec. Like adenosine, β-γ-methylene ATP enhanced the decrease in permeability due to low light (table 2). Although the β-γ-methylene ATP had a greater effect on sensitivity than adenosine, its high cost limited our experimentation.

In separate experiments we have measured suppression of swelling (in the absence of adenosine) as a function of light intensity [9]. Low light (5×10^3 rhodopsins bleached/outer segment/sec) in the presence of adenosine caused the same suppression of swelling as 10-fold greater illumination without adenosine. In β-γ-methylene ATP, the suppression is approximately equivalent to that of a 100-fold increase in light intensity.

Thus, these data suggest a link between rhodopsin phosphorylation and control of photoreceptor light sensitivity.

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