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Photoreversible Conductance Changes Induced by Phytochrome in Model Lipid Membranes

(cholesterol/red light/far-red light)

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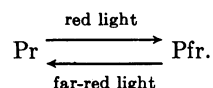
ABSTRACT **The plant protein phytochrome induces photoreversible conductance changes when added to a black lipid membrane made from oxidized cholesterol. The conductance of the phytochrome-modified membrane is increased by red-light illumination but is decreased by illumination with far-red light. Denatured phytochrome does not affect the conductance of the model membrane.**

Phytochrome is a widely distributed plant chromoprotein that mediates many developmental changes triggered by light (1, 2). It has two spectrally different forms that can be reversibly interconverted by irradiation with light of the appropriate wavelength: The red-absorbing form, Pr (λmax 667 nm), and the far-red-absorbing form, Pfr (λmax 725 nm). The interconversion of the two forms can be described by the scheme:



There is some evidence that phytochrome is a membrane protein (3), and that it exerts its effects by regulating the ionic permeability, and, consequently, the electrical potential of some cell membranes (4, 5). We postulated that this regulation could be made possible by differences in membrane conductance induced by the two forms of phytochrome, and thus designed experiments to test this hypothesis (6). Here we present the initial results of our studies in which we have attempted to incorporate phytochrome into a model membrane to determine whether Pr and Pfr can differently affect the ionic permeability of a bimolecular lipid membrane.

The model membrane that we used was developed by Mueller, Rudin, and coworkers (7). It can be made from various lipids, including phospholipids, and has a conformation and other properties similar to the bilayer regions of biological membranes. The membrane is formed between two aqueous compartments, thus allowing its electrical properties to be readily measured by standard techniques of electrophysiology. The effects of proteins on ionic permeability can be tested by incorporating them into the membrane. The very high resistance of the pure lipid membrane (10^9–10^{10} Ω-cm^2) provides an ideal system for detection of small changes in conductance, such as have been reported after the incorporation in the bilayer of low amounts of a protein called excitability-inducing material (EIM) (8).

Abbreviations: Pr, red-absorbing form of phytochrome; Pfr, far-red-absorbing form of phytochrome; EIM, excitability-inducing material; R, resistance.

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MATERIALS AND METHODS

The experimental arrangement for forming and studying the membrane is shown in Fig. 1. The membrane was made across a 0.7-mm hole on the side of a 6-ml Teflon cup that was partially immersed in and filled with a solution of 0.1 M KCl, buffered at pH 7 with 5 mM histidine chloride. The temperature of the system was maintained between 25° and 27°. To form a membrane, a small amount of a solution of 4% lipid in octane was deposited across the hole of the cup by the brush technique (7). Within a minute, excess oil spontaneously migrated toward the wall of the hole, leaving a bimolecular lipid membrane that remained attached to the cup through a torus of oil. The membrane was observed through a 100× stereoscopic microscope. In studies with phytochrome, a membrane was first formed and a solution of the protein was then injected inside the cup and mixed with the electrolyte by a magnetic stirrer. The conductance of the membrane in the presence and absence of phytochrome was monitored with the electrical arrangement of Fig. 1. A potential of 32 mV (positive outside the cup) was normally applied across the membrane during the conductance measurements. In some experiments, the polarity was reversed, but no polarity effects were detected. The calomel electrodes were well shielded from stray light, while electrical noise was minimized by enclosing the membrane, electrodes, and high-impedance amplifier in a Faraday cage. This arrangement allowed the detection of conductance changes of a few percent.

When the effect of injected phytochrome was being tested, the membrane was periodically irradiated with either red (660 nm) or far-red (720 nm) light by means of the optical arrangement shown in Fig. 1. The appropriate wavelength was selected with interference filters that could be readily interchanged. The light flux through the membrane was in the range of 10^{12}–10^{15} photons per sec.

Model membranes were formed from 7-dehydrocholesterol (Mann) and from oxidized cholesterol. Although these lipids are probably not as significant for membranes as are phospholipids, they are easier to handle and thus better suited for initial studies. Oxidized cholesterol was prepared by bubbling oxygen through a refluxing solution of 4% cholesterol in octane, as described by Tien (9). We found, however, that refluxing for 6.5 hr, as recommended, did not always produce a lipid solution that formed membranes sufficiently stable for our studies. Satisfactory preparations were nevertheless obtained by testing the solution every 0.5 hr, after 6 hr of refluxing, until stable membranes could be formed. Refluxing times varied from 7 to 10 hr. Excessive refluxing also produced unsatisfactory solutions.

The phytochrome for these experiments was purified from

Garry or Victory oats (*Avena sativa*) by the method of Roux (2), described in detail in (10). Samples used had an estimated molecular weight of 110,000 on calibrated Bio-Gel P-150 columns and ranged in purity from 15 to 70% (A278/A667 range from 7.0 to 1.5). Though phytochrome samples could affect membrane resistance whether injected into the membrane bathing solution as Pr or Pfr, we standardized these experiments by always injecting phytochrome as Pr under green safe-light illumination. This remained the only illumination, other than the irradiating beam, during the entire course of the experiment. Quantities of phytochrome injected in any one experiment ranged from 5 to 20 μg of total protein in volumes up to 20 μl.

RESULTS AND DISCUSSION

Our experiments with oxidized cholesterol membranes indicate that phytochrome can *spontaneously enter and induce photoreversible conductance changes in these membranes*. Fig. 2 shows the results of one of these experiments. As shown, addition of 6 μg of phytochrome decreased, within a few minutes, the membrane resistance R by about a factor of five from its initial value of 10^{10} Ω. Irradiation with red light accelerated the drop in resistance, causing R to decrease by almost 10 times in about 1 min. This decrease, however, was reversed by subsequent far-red light irradiation, which increased the resistance almost to the level before red-light illumination. Renewed irradiation with red light again dropped the resistance, but this time the membrane became quite unstable and unresponsive to light, and finally broke at 30 min.

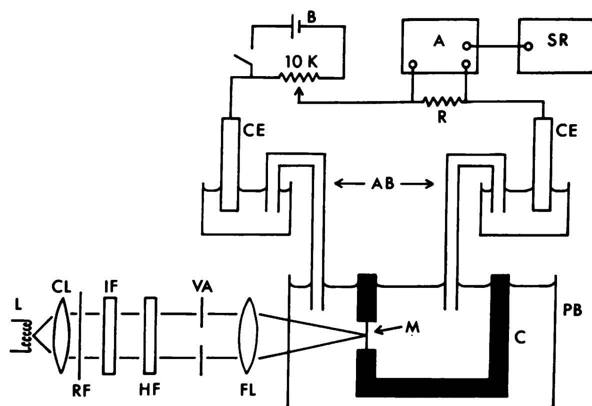

FIG. 1. Schematic diagram of the experimental arrangement for forming and studying the electrical properties of model membranes in the presence and absence of phytochrome. The membrane, *M*, is made across a 0.7-mm hole in the side of a 6-ml Teflon cup, *C*, which sits inside a small transparent plastic box, *PB*. Voltage in the range 0–300 mV is applied continuously or in pulses from a 10-KΩ potentiometer connected to a battery, *B*. Contact with the membrane-forming solution is made through calomel electrodes *CE*, and salt-agar bridges, *AB*. Current through the membrane is monitored by a high-impedance amplifier, *A*, which detects the voltage across a dropping resistor, *R*, and displays it on a strip chart recorder, *SR*. The magnitude of *R* can be adjusted in the range 10^5–10^{10} Ω. The optical system for illuminating the membrane with red or far-red light consists of a tungsten filament lamp, *L;* red cellophane filter, *RF;* collimating lens, *CL;* interchangeable interference filter, *IF;* heat filter, *HF;* variable aperture, *VA*, for regulating light intensity; and focusing lens, *FL*. The interchangeable interference filters had maximum transmission at 660 nm for red light and 720 nm for far-red light.

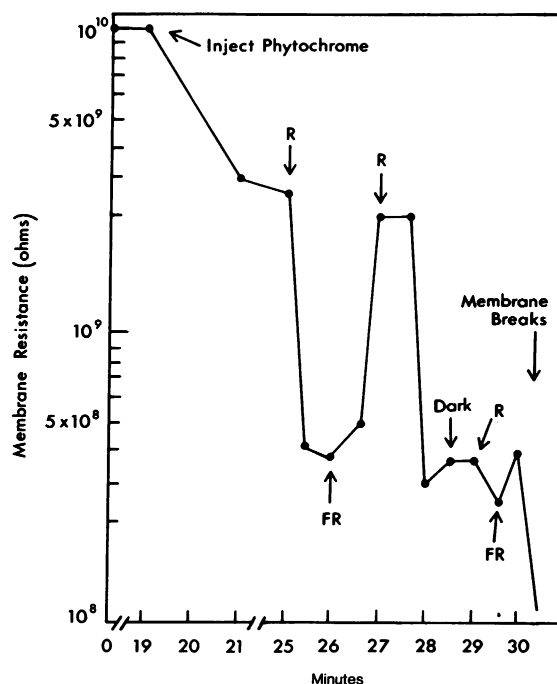

FIG. 2. Time course of the resistance of an oxidized cholesterol membrane on addition of 6 μg of phytochrome (20 μg of total protein, 20-μl volume) to the bathing solution and illumination with red (660 nm) and far-red (720 nm) light. The light flux in this experiment was 10^{15} photons per sec. The resistance was monitored continuously, but only a few points connected by straight lines are shown in this figure. The arrows point to the times the membrane was irradiated with red (*R*) or far-red (*FR*) light.

We have done over 40 experiments related to the effects of phytochrome on the ionic conductance of oxidized cholesterol membranes. Some of these were control or exploratory experiments to find the conditions that optimized reproducibility. We found that stability and freshness, respectively, of the oxidized cholesterol and phytochrome preparations were the most critical variables. With these conditions satisfied, we found the results quite reproducible, and in one set of 15 experiments we observed photoreversible conductance changes of the type shown in Fig. 2 twelve times. In 8 of these 12 experiments, the conductance change was reversed once by irradiation with far-red light before the membrane became unstable and broke. In the other four cases, more than one reversal in resistance was observed before the membrane broke. *The striking feature of the experiments is the increase in conductance produced by red-light illumination.* A common result, however, is that the membranes become unstable and usually break within 30 min after addition of phytochrome. Instability first appears as random fluctuations in membrane resistance (not shown in Fig. 2) that are superimposed on the much larger resistance changes that are produced by irradiation with light of different wavelengths. The short duration of oxidized cholesterol membranes in the presence of phytochrome and red light limited our experiments to measuring photoreversible conductance changes.

In control experiments, addition of large amounts of boiled or aged phytochrome, which had lost its spectral photo-

reversibility, had no significant effect on membrane resistance over a period of one hour in the presence or absence of red or far-red light. The effects described above were thus characteristic of fresh phytochrome preparations.

Although some of the changes in membrane resistance described above may be due to impurities in our partially pure preparation (15–70% phytochrome), it seems certain that the photoreversible resistance changes are associated with phytochrome. The experiments, however, do not reveal the mechanism by which phytochrome changes membrane resistance on irradiation with light of different wavelengths. Several mechanisms can be postulated, including one in which phytochrome enters and leaves the membrane, depending on whether it is in the Pr or Pfr form. The simplest explanation of our results, however, is that *the membrane-bound Pfr form of phytochrome induces a higher conductance than the bound Pr form.*

Addition of fresh phytochrome to membranes made from 7-dehydrocholesterol also decreased the membrane resistance by 10 times. However, the decreased resistance R_c was very stable and unaffected by light, in contrast to the results with oxidized cholesterol membranes. Since the resistance changes are not sensitive to light, it is difficult in this case to decide whether the decrease in R_c is due to phytochrome or to some other protein component in our phytochrome preparation. However, if phytochrome is indeed responsible for the effects on resistance, then the different responses to light of phytochrome in 7-dehydrocholesterol and oxidized cholesterol membranes indicate differences in protein–lipid interactions. Such differences in the function of a membrane additive with varying lipid composition are not unexpected and have already been observed in other studies with bilayer model membranes. Thus, for example, the resistance changes induced by EIM are strongly dependent on voltage for membranes made from oxidized cholesterol or sphingomyelin, but are only weakly dependent for lecithin membranes (11).

It is of interest to determine whether the magnitude of the changes in membrane resistance detected in our experiments on illumination with red light are physically realistic, in view of the small amount of protein injected into the solution bathing the membrane. An estimate of the expected changes can be made on the basis of the known conductance of other systems. Thus, a single voltage-dependent Na^+ channel in a nerve membrane has a conductance of $4.4 \times 10^{-10}\ \Omega^{-1}$ (12), whereas an EIM molecule in an oxidized cholesterol membrane gives a conductance of $3 \times 10^{-10}\ \Omega^{-1}$ (8). If we assume a value of $10^{-10}\ \Omega^{-1}$ for the conductance of a membrane-bound phytochrome molecule in its presumably high-conductance Pfr state, then 10^3 bound molecules in that state would be sufficient to change the membrane resistance from 10^{10} to $10^7\ \Omega$. With the light intensities used in our experiments, this number of Pfr molecules can easily be produced within a few seconds from a population of 10^4 Pr phytochrome molecules bound to the membrane. An injection of 10 μg of phytochrome in the Pr form contains about 10^{14} phytochrome molecules and produces a phytochrome concentration of about 10^{-8} M in the 6-ml cup. Of course, not all of the added phytochrome molecules are accessible to the membrane, but it can be shown by calculation that a sufficient number reach the membrane during the time of our experiment to easily produce the 10^4 Pr membrane-bound molecules required by the calculation above. Both the magnitude and the speed of the resistance changes seen in our experiments are thus well within physical expectation.

The fact that phytochrome is a water-soluble protein with a considerable polar amino-acid content would seem to designate it as a poor candidate for incorporation into artificial lipid bilayers. But it has an even higher nonpolar amino-acid content; and, by criteria described by Capaldi and Vanderkooi (13), has a low polarity index, a feature common to most known membrane proteins. Counting Asp, Asn, Glu, Gln, Lys, Ser, Arg, Thr, and His as polar residues and the remaining amino acids as nonpolar (13), current (10, 14) analyses estimate the composition of oat phytochrome as having about 206 polar residues and 278 nonpolar residues per 60,000-dalton molecule, giving phytochrome a low polarity index of *40.5*. These data say nothing about whether phytochrome is in fact a membrane protein, but only suggest the likelihood that there may be considerable regions of nonpolarity in phytochrome capable of interacting with the hydrophobic environment of lipid bilayers.

A major question these results raise is whether the photoreversible conductance changes reported here involve selective permeability to specific ions. To answer this question it will be necessary to find a lipid or lipid combination that forms model membranes that, in the presence of phytochrome, gives responses to red and far-red irradiation that are more stable than those found with oxidized cholesterol membranes. This and other refinements of the model membrane system should contribute to our understanding of the possible modes of action of phytochrome *in vivo.*

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