Plant Physiol. (1990) 92, 831–836
0032-0889/90/92/0831/06/$01.00/0

Received for publication June 19, 1989
and in revised form September 27, 1989

Calcium Activation of *Mougeotia* Potassium Channels[1]

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ABSTRACT

Phytochrome mediates chloroplast movement in the alga *Mougeotia*, possibly via changes in cytosolic calcium. It is known to regulate a calcium-activated potassium channel in the algal plasma membrane. As part of a characterization of the potassium channel, we examined the properties of calcium activation. The calcium ionophore A23187 activates the channel at external [Ca^{2+}] as low as 20 micromolar. However, external [Ca^{2+}] is not required for activation of the channel by photoactivated phytochrome. Furthermore, when an inhibitor of calcium release from internal stores, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate, hydrochloride (TMB-8), is present, red light no longer stimulates channel activity. We conclude that phytochrome activates the plasma membrane potassium channel by releasing calcium from intracellular calcium vesicles; the elevated cytosolic calcium then stimulates channel activity by an unknown mechanism. In the presence of TMB-8, red light does induce chloroplast rotation; thus, potassium channel activation may not be coupled to chloroplast rotation.

The alga *Mougeotia* is a classic system for the study of phytochrome since photoactivation of phytochrome regulates the positioning of its single large chloroplast, which is easily monitored (5). It is known that calcium plays a role in the movement of the chloroplast (1–3, 13). Phytochrome may cause cytosolic calcium to increase either by calcium release from internal calcium stores (2) or entry of external calcium into the cell (1), and this in turn results in chloroplast repositioning (13).

We recently discovered a phytochrome-activated potassium channel in *Mougeotia* which is also activated by the calcium ionophore A23187 (8). It is not known what function, if any, the potassium channel has in chloroplast positioning, though we hypothesize that it allows entry of extracellular calcium into the cell. In this paper, we study calcium-activation of the channel, and present evidence that it is activated by internal calcium stores.

[1] Supported by National Science Foundation grants BNS 87-15847 and DCB 87-1558 to C. L. S. and by a grant to B. S. S. from the William and Flora Hewlett Foundation Grant of Research Corporation.

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MATERIALS AND METHODS

Protoplast Production

Mougeotia (UTEX LB 758 *Mougeotia* sp. [15]) was cultured according to Serlin and Ferrell (14). Protoplasts were produced as described by Lew *et al.* (8). Normally, they were stored in osmoticum/buffer solution (0.25 M Mannitol, 0.25 M Sorbitol, 16 mM Mes [pH 5.4 or 6.5 with NaOH], and 2 mM $CaCl_2$) in a refrigerator in the dark. Calcium concentrations were adjusted using the osmoticum/buffer solution without any added $CaCl_2$. Distilled, deionized H_2O was used, so any calcium contamination in the $-CaCl_2$ osmoticum/buffer solution would have come from glassware, reagent-grade chemicals, and the cells themselves.

When the cells were prepared for $-CaCl_2$ treatment (the experiment shown in Fig. 1), they were washed by centrifugation in $-CaCl_2$ solution, typically 2 to 3 times, and stored in $-CaCl_2$ osmoticum/buffer. A small aliquot of cells, typically less than 50 μL, was added to 2.5 mL of $-CaCl_2$ osmoticum/buffer solution prior to patch clamping. The background level of Ca^{2+} was not measured, but maximal contamination can be estimated: Given 30 μm diameter cells, no more than 10^6 cells/mL, and no more that 2 mM [Ca^{2+}] in the cells, complete cell breakage would yield no more than 0.6 μM contamination.

Electrophysiology

Micropipettes were filled with osmoticum/buffer solution plus 100 mM KCl with or without 2 mM $CaCl_2$. All other details are as described by Lew *et al.* and Hamill *et al.* (4). The experiments are shown as chart recordings from a Brush recorder (Clevite Corp., Cleveland, OH 44114).

Analysis

Experiments were stored with a video cassette recorder-based acquisition system (8), then digitized at 200 Hz after filtering at 200 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA 01830). The digitized data were analyzed using pClamp software (Axon Instruments, Burlinghame CA 94010) to obtain measurements of channel amplitudes, open times, and closed times.

Files containing the compiled channel data were analyzed using SYSTAT statistical software (SYSTAT, Inc. Evanston, IL 60201) to determine the total number of channel opening events and total open time per 5 s interval before and after treatment with either A23187 or red light. Commonly, there

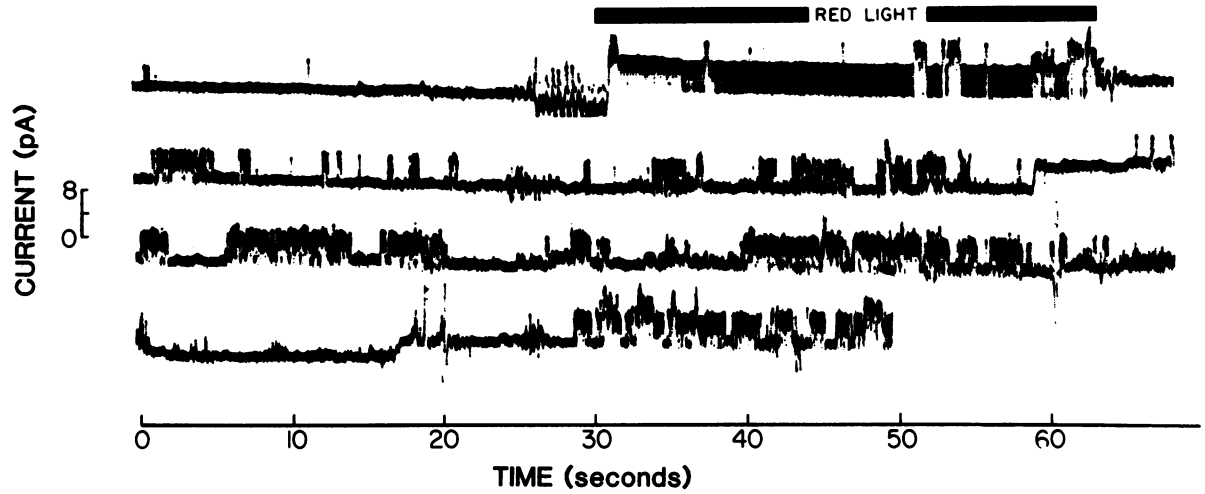

Figure 1. Red light stimulation of ion channel activity in the absence of extracellular [Ca^{2+}]. The cells were held in a $-Ca^{2+}$ stock solution, from which an aliquot was transferred to a $-Ca^{2+}$ solution in the experimental apparatus, and patch clamped. During red light treatment, the record is contaminated with AC interference.

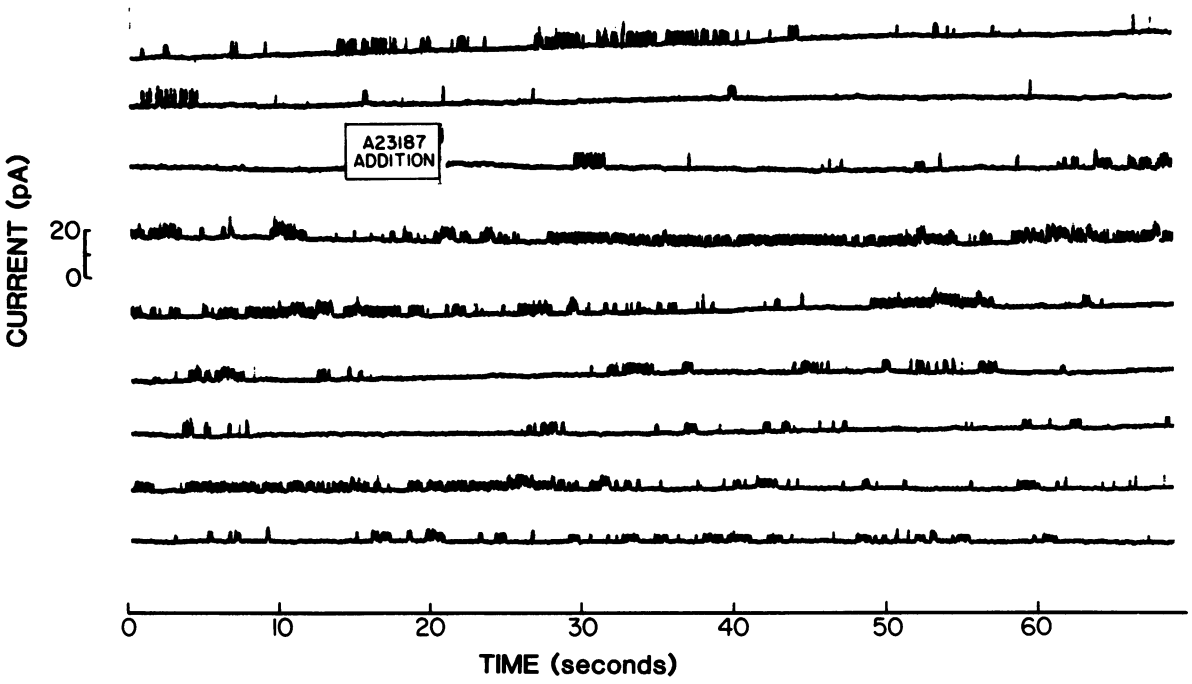

Figure 2. Calcium ionophore stimulation of ion channel activity. A23187 (33 μM) was added as marked on the trace. Note the presence of two different amplitude channels (see ref. 8).

was a base line of about 250 s of recording prior to treatment and about 1000 s of recording afterward. Since activation often took minutes, all 1000 s of posttreatment recording were used in measurements of channel number and open time. Activation was quantified as percentage of pretreatment activity. The percentage of pretreatment activity for both measures of activity, number of channels and channel open time, were averaged together for data presentation. An example of data analysis is shown in Figures 5 and 6 (actual data) and Figure 7 (quantitation).

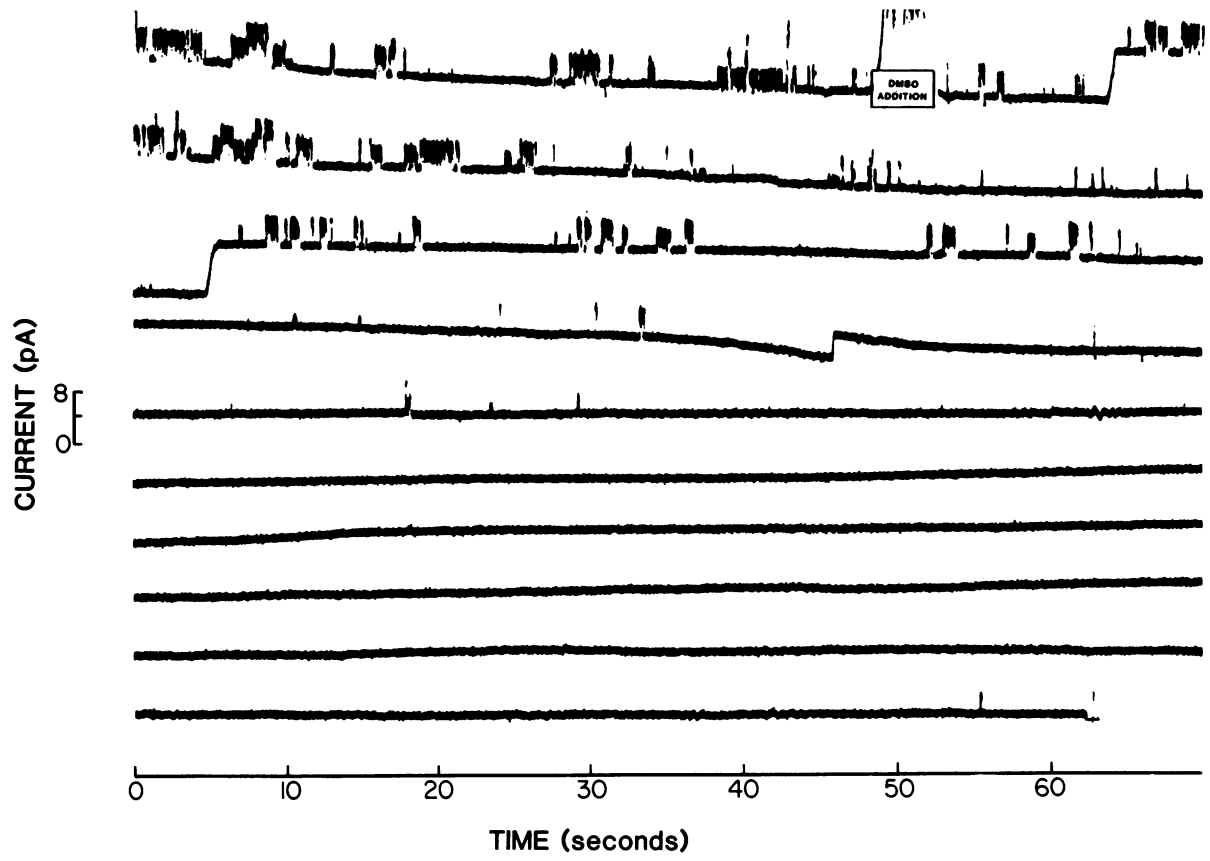

Figure 3. DMSO control—effect on ion channel activity. Since A23187 is added from a 1 mM stock in DMSO, a control with only DMSO (3.3% [v/v] final concentration) added is shown.

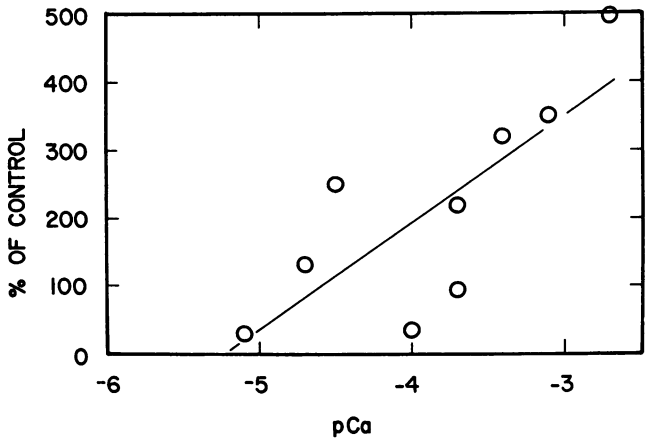

Figure 4. Dependence of calcium ionophore activation on extracellular [Ca^{2+}]. The data are plotted as percentage of activation using the pretreatment period of about 250 s as baseline activity. Measurements of number of channel events per 5 s and channel open time per 5 s were lumped together. The mean of four measurements at 2 mM [Ca^{2+}] is shown. An outlier point (18-fold activation of open time per 5 s at 800 μM) has been omitted.

Chloroplast Rotation

The rotation of the chloroplast after red light irradiation was measured with video microscopy as described in Serlin and Ferrell (14). The filamentous cells, from the same cultures used for protoplast production, were held in osmoticum/buffer solution (pH 6.5; diluted 10-fold to avoid plasmolysis) with added $CaCl_2$.

RESULTS

Red Light Stimulates Channel Activity in the Absence of Extracellular Calcium

The cells were stored as a stock in $-CaCl_2$ osmoticum/buffer solution. From this stock, a small aliquot of cells was added to 2.5 mL of $-CaCl_2$ osmoticum/buffer in the experimental apparatus and then patched. The pipette was filled with $-CaCl_2$ osmoticum/buffer. Under these conditions, red light stimulates ion channel activity (Fig. 1), to the same extent as when 2 mM $CaCl_2$ is present in the extracellular solution (8).

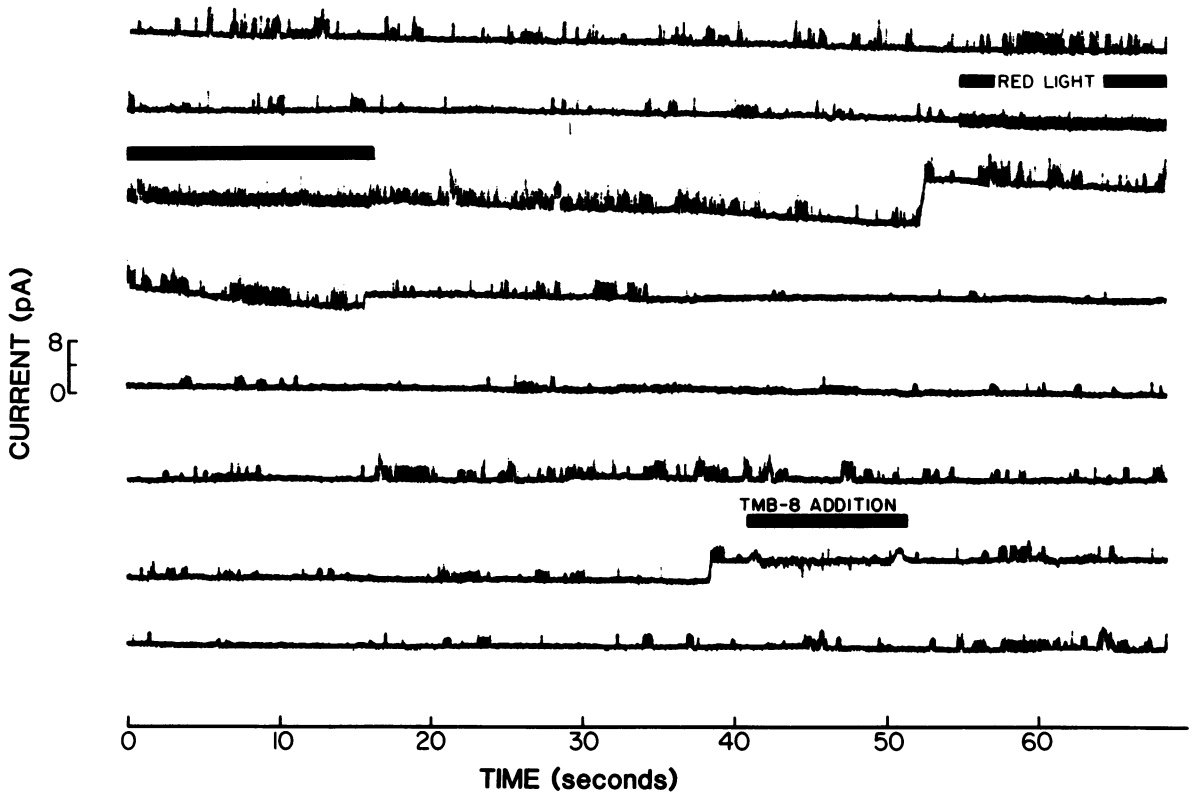

Figure 5. Red light/TMB-8 effect on ion channel activity. Red light treatment commenced as shown on the trace. TMB-8 (18.5 μM) was added as shown after channel activity had declined to baseline levels. This trace is continued in Figure 6.

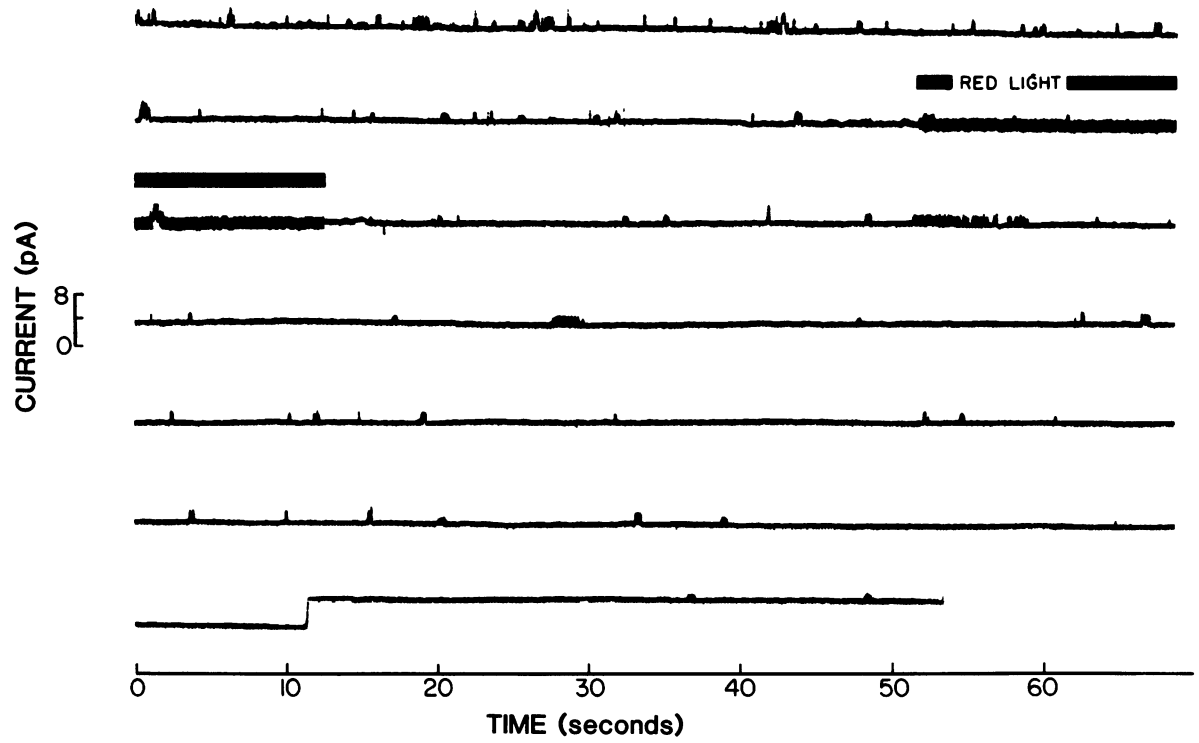

Figure 6. TMB-8/red light effect on ion channel activity. With TMB-8 (18.5 μM) present (note: this trace is a continuation of the trace in Fig. 5), red light treatment (shown on the trace) did not activate the ion channels.

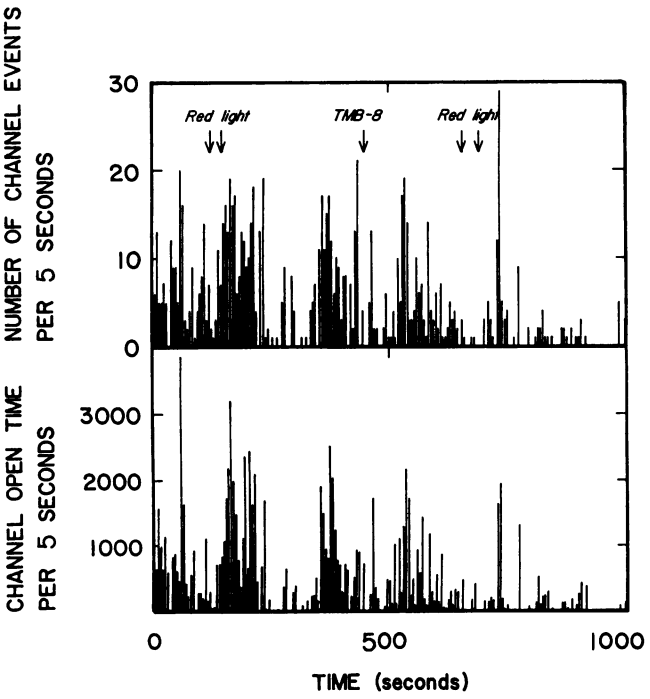

Figure 7. Synopsis of red light/TMB-8/red light experiment. Data have been quantified as number of channel openings and channel open time per 5 s interval. For the 250 s prior to the first red light pulse, the total and mean number of channel openings per 5 s interval were 173 and 7.5, while total and mean channel open time were 18.38 and 0.8 s. During the 250 s after the red light pulse, the total number of channel openings and mean number both increased 142%, while total channel open time and mean open time both increased 155%. For the 250 s prior to the second red light pulse, the total and mean number of channel openings per 10 s interval were 108 and 4.7, while total and mean channel open time were 108.35 and 0.47 s. During the 250 s after the red light pulse, the total number of channel openings and mean number changed by 71% and 126% of control, respectively, while total channel open time and mean open time changed 61% and 108%, respectively.

A23187 Activates the Channel

In osmoticum/buffer solution containing 200 μM $CaCl_2$, the addition of 33 μM A23187 (from a stock in DMSO, final DMSO concentration of 3.3% [v/v]) stimulated channel activity (Fig. 2). For this experiment, channel open time per 5 s interval increased 2.48-fold and number of channel events per 5 s interval increased 1.86-fold. DMSO (3.3% [v/v]) by itself appears to inhibit channel activity (Fig. 3). Buffering of calcium with the calcium chelator EGTA (pH was adjusted after EGTA addition) was not possible, since EGTA, even in the presence of 30 μM free Ca^{2+}, caused cellular rupture (seen as vacuole release from the cells). So the final $[Ca^{2+}]$ concentration was adjusted by adding in varying amounts of cells from a cell stock containing 2 mM $CaCl_2$. The Ca^{2+} concentration dependence of A23187-stimulation of ion channel activity is shown in Figure 4. The data are variable, but there is a clear trend of A23187-induced channel stimulation as extracellular $[Ca^{2+}]$ increases.

Red Light Does Not Activate the Channel in the Presence of the Calcium-Release Inhibitor, TMB-8[3]

As a control, red light stimulation of channel activity is shown (Fig. 5). Subsequent addition of TMB-8 (18.5 μM) had no effect on channel activity. In the presence of TMB-8, a second treatment with red light did not activate the channel (Fig. 6). This is shown quantitatively in Figure 7. In one out of five experiments with TMB-8 present, there was a slight red light-stimulation of channel activity, otherwise there was no stimulation by red light.

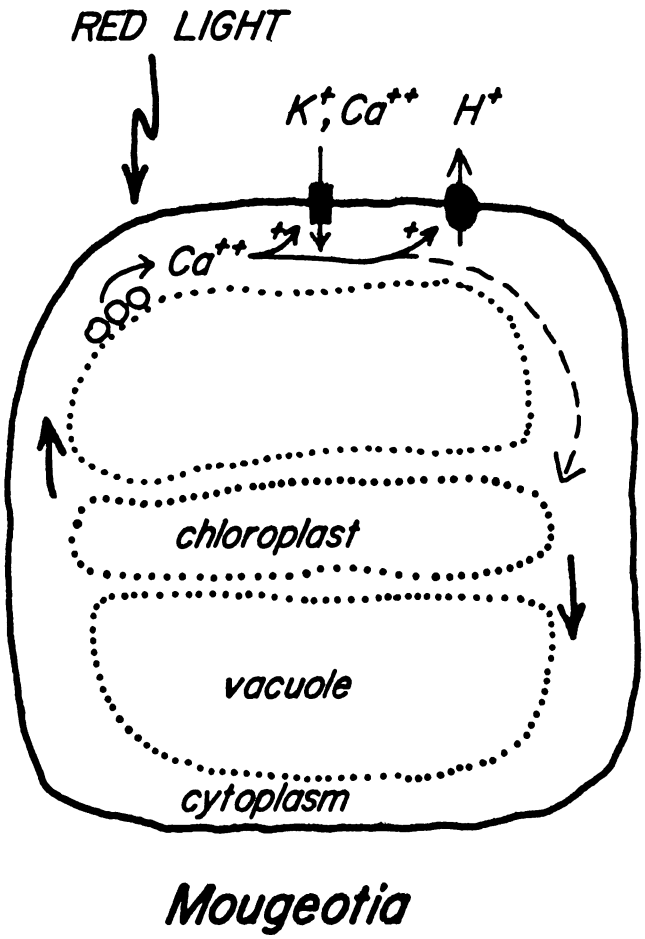

Figure 8. Model of phytochrome effects. Initially, red light activates phytochrome, which causes the release of calcium from internal stores. The increase in cytosolic calcium activates the potassium channel, causing calcium entry from the extracellular solution. Increased cytosolic calcium may also activate the plasma membrane proton pump (7) and vacuolar channels (6). It is not known how increased calcium levels cause chloroplast rotation, but it is unlikely that channel activation is a part of the mechanism leading to chloroplast rotation since TMB-8 inhibits channel activation but not chloroplast rotation.

[3] Abbreviation: TMB-8, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate, hydrochloride.

With TMB-8 Present, Red Light Does Cause Chloroplast Rotation

In the presence of 25 μM TMB-8, red light induces chloroplast rotation in the algal filaments when the cells were kept in 10-fold diluted osmoticum/buffer solution with added $CaCl_2$ (data not shown).

DISCUSSION

The ion channels shown in this paper have been identified as potassium channels on the basis of inhibition by a quaternary ammonium derivative, tetrapentylammonium chloride (8). Their role in chloroplast movement is not known. Once activated, they could clamp the algal transmembrane potential to the Nernst potential for potassium, and may allow a significant amount of Ca^{2+} to enter the cell (8).

The channels are clearly activated by A23187 (Fig. 2) and exhibit some dependence on extracellular [Ca^{2+}] with A23187 present (Fig. 4). Thus, the A23187 effect is due to its activity as a calcium ionophore. In the presence of A23187, the cytosolic [Ca^{2+}] must be less than the extracellular [Ca^{2+}] because 2 mM extracellular [Ca^{2+}] causes no visually deleterious effects on the cells when A23187 is added to the medium.

The channels are activated by red light in the absence of extracellular calcium to the same extent as when 2 mM $CaCl_2$ is present (8) even though the driving force for Ca^{2+} movement into the cell would be at least three orders of magnitude less ("Materials and Methods"). This suggests that, if the channels are activated by an increase in cytosolic calcium *in vivo*, the calcium may be released, by photoactivated phytochrome, from previously reported internal calcium stores (2). To test this, we used TMB-8, an inhibitor of calcium release from internal stores (9–11). In the presence of TMB-8, channel activity is unaffected, but red light no longer stimulates channel activity. Since we are not measuring the changes in cytosolic [Ca^{2+}], we cannot exclude the possibility that TMB-8 is acting at some other point in the transduction pathway between red light and channel activation, although inhibition of calcium release is the simplest explanation.

Since chloroplast rotation is induced by red light in the presence of TMB-8 under conditions where the potassium channel is not activated, there may be no obligatory coupling between potassium channel activation and chloroplast rotation. We can speculate that the threshold of increased intracellular [Ca^{2+}] required to activate chloroplast rotation is lower than the threshold for channel activation, and that TMB-8 is not inhibiting release of calcium from all possible sources (vacuole, endoplasmic reticulum, and calcium-containing vesicles). If this is the case, then our results are consistent with the report that internal calcium-containing vesicles are involved in chloroplast movement (2, 3).

These results lead us to the following conclusion. Phytochrome initially causes release of calcium from internal stores (Fig. 8). Whether this is by a direct effect, or by a second messenger such as inositol 1,4,5-trisphosphate (12) is not known. The released stores of calcium in turn activate the potassium channel in the algal plasma membrane. The ion channel will act as a voltage clamp, and will allow the entry of more calcium from the extracellular solution. The increased cytosolic calcium may stimulate the proton pump in the plasma membrane (7). The model is summarized in Figure 8. This role for the ion channel is consistent with the report of Dreyer and Weisenseel (1) that red light causes uptake of $^{45}Ca^{2+}$ from the external solution.

LITERATURE CITED

1. **Dreyer EM, Weisenseel MH** (1979) Phytochrome-mediated uptake of calcium in *Mougeotia* cells. Planta **146:** 31–39
2. **Grolig F, Wagner G** (1987) Vital staining permits isolation of calcium vesicles from the green alga *Mougeotia*. Planta **171:** 433–437
3. **Grolig F, Wagner G** (1989) Characterization of the isolated calcium-binding vesicles from the green alga *Mougeotia scalaris*, and their relevance to chloroplast movement. Planta **177:** 169–177
4. **Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ** (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch **391:** 85–100
5. **Haupt W** (1983) Light-mediated movement of chloroplasts. Annu Rev Plant Physiol **33:** 205–233
6. **Hedrich R, Neher E** (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833–835
7. **Lew RR** (1989) Calcium activates an electrogenic proton pump in *Neurospora* plasma membrane. Plant Physiol **91:** 213–216
8. **Lew RR, Serlin BS, Schauf CL, Stockton ME** (1989) Red light regulates calcium-activated potassium channels in *Mougeotia* plasma membrane. Plant Physiol **92:** 0000–0000
9. **Owen NE, Villereal ML** (1982) Effect of the intracellular Ca^{2+} antagonist TMB-8 on serum-stimulated Na^+ influx in human fibroblasts. Biochem Biophys Res Commun **109:** 762–768
10. **Saunders MJ, Hepler PK** (1983) Calcium antagonists and calmodulin inhibitors block cytokinin-induced bud formation in *Funaria*. Dev Biol **99:** 41–49
11. **Saunders MJ, Jones KJ** (1988) Distortion of plant cell plate formation by the intracellular antagonist TMB-8. Protoplasma **144:** 92–100
12. **Schumacher KS, Sze H** (1987) Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. J Biol Chem **262:** 3944–3946
13. **Serlin BS, Roux SJ** (1984) Modulation of chloroplast movement in the green alga *Mougeotia* by the Ca^{2+} ionophore A23187 and by calmodulin antagonists. Proc Natl Acad Sci USA **81:** 6368–6372
14. **Serlin BS, Ferrell S** (1989) The involvement of microtubules with chloroplast rotation in the alga *Mougeotia*. Plant Sci **60:** 1–8
15. **Starr R** (1978) The culture collection of algae at the University of Texas at Austin. J Phycol **14:** 47–100