Extensor protoplasts of the *Phaseolus* pulvinus: light-induced swelling may require extracellular Ca\(^{2+}\) influx, dark-induced shrinking inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) mobilization

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Abstract

The photonastic upward movement and scotonastic downward movement of the primary leaf of *Phaseolus coccineus* L. depends on ion fluxes across the plasma membrane of extensor and flexor cells of the laminar pulvinus. Extensor protoplasts cultured in 0.4 M mannitol, 10 mM KCl, 1 mM CaCl\(_2\), and 5 mM MES-KOH buffer pH 6 were found to swell upon switching on white light at the end of a 15 h dark period and to shrink upon switching off the light at the end of the following 9 h light period, behaviour consistent with that expected in the cells of intact plants. Light-induced swelling requires Ca\(^{2+}\) in the surrounding medium. Both the Ca\(^{2+}\) channel blocker verapamil and La\(^{3+}\) inhibited this reaction, whereas TMB-8, an inhibitor of intracellular Ca\(^{2+}\) transport, had no effect. When the Ca\(^{2+}\) ionophore A 23187, the Ca\(^{2+}\) channel agonist Bay K-8644, or thapsigargin, an inhibitor of Ca\(^{2+}\)-ATPases at endomembranes, was added to the medium, extensor protoplasts swelled in the dark. These results suggest that in extensor protoplasts light opens Ca\(^{2+}\) channels in the plasma membrane and that the influx of extracellular Ca\(^{2+}\) results in an increased cytoplasmic Ca\(^{2+}\) concentration which is sufficient to mimic the light-on signal in activating or deactivating the ion transporters required for swelling. Dark-induced shrinking occurred in Ca\(^{2+}\)-free medium. It was not inhibited by verapamil, but was by TMB-8. Both neomycin and Li\(^{+}\), substances which are known to inhibit the phosphoinositide pathway of transmembrane signalling, inhibited dark-induced shrinking. Myo-inositol nullified the Li\(^{+}\) inhibition of dark-induced shrinking. Neither A 23187 nor Bay K-8644 induced shrinking in the light, but were able to nullify the inhibitory effect of TMB-8 on dark-induced shrinking. These results suggest that, in extensor protoplasts, the shrinking signal 'light off' is transduced through phosphoinositide hydrolysis and Ca\(^{2+}\) release from internal stores. In addition to the inositol 1,4,5-trisphosphate (IP\(_3\))-induced increase of the cytoplasmic Ca\(^{2+}\) concentration, further events depending on the light-off signal appear to be required for shrinking.

Key words: *Phaseolus* pulvinus, extensor protoplasts, light-induced swelling, dark-induced shrinking, Ca\(^{2+}\), phosphoinositide signalling.

Introduction

Leaf movements can be induced in many plants by transferring them in the morning from darkness to light (photonastic reaction) and in the evening from light to darkness (scotonastic reaction). In the primary leaves of *P. coccineus* L., the dark/light transition induces an upward and the light/dark transition a downward movement of the lamina due to the bending of the laminar pulvinus. The upward movement is brought about by the simultaneous swelling of extensor (cortical cells of the lower half of the pulvinus) and shrinking of flexor cells (cortical cells of the upper half of the pulvinus), the downward movement by inverse volume changes of these motor cells.

Ion and water fluxes across the plasma membrane produce the swelling and shrinking of the pulvinar motor cells. The cells swell when protons are pumped by the
plasma membrane H^+-ATPase into the apoplast and K^+, Cl^- and water are taken up by the cells. K^+ enters the cells through hyperpolarization-activated inward K^+ channels, Cl^- influx presumably occurs through H^+/Cl^- cotransport. The cells shrink when K^+, Cl^-, and water are released by the cells. K^+ and Cl^- are released from the cells through depolarization-activated outward K^+ and Cl^- channels (Mayer and Hampp, 1995).

There is increasing evidence that Ca^{2+} plays an essential role in the transduction pathways mediating the light-on- and light-off-induced activation and deactivation of the ion transporters which produce the volume changes of the motor cells and thus the leaf movements (Mojsses and Simon, 1989, 1990; Roblin et al., 1989, 1990). On the basis of the different effects of substances which influence the Ca^{2+} concentration in the cells (Ca^{2+} channel blockers and activators, Ca^{2+} chelators, Ca^{2+} ionophores) on the photonic opening movement and the scotonic closing movement of the leaflets of *Cassia fasciculata*, Roblin et al. hypothesized that the mobilization of Ca^{2+} from intracellular stores is an essential step in the blue-pigment-mediated photonic opening movement, whereas the influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane is essential for the phytochrome-mediated scotonic closing movement. Consistent with this suggestion is the finding that in whole *Samanea* pulvini a brief exposure to white light at the end of a dark period causes a rapid change in the major components of the photosynthetic pathway (summarized in Crain, 1990), indicating IP_3-mediated Ca^{2+} release from internal stores as an important step in the light-induced photonic opening movement. However, since the light-on signal induces both extensor cell swelling and flexor cell shrinking in the pulvini and the light-off signal the inverse volume changes, these results do not indicate whether hydrolysis of phosphoinositide transduces the light-on signal for extensor cell swelling or for flexor cell shrinking, or whether the mobilization of Ca^{2+} from internal stores is important for the blue-pigment-mediated extensor swelling or flexor shrinking, and the influx of extracellular Ca^{2+} for the phytochrome-mediated extensor shrinking or flexor swelling.

The role of Ca^{2+} is being studied in the transduction pathway mediating light-induced swelling and dark-induced shrinking of protoplasts from the *Phaseolus* pulvinus. Evidence is presented here that the light-induced swelling of extensor protoplasts requires Ca^{2+} influx through Ca^{2+} channels in the plasma membrane, whereas the dark-induced shrinking requires IP_3-mediated Ca^{2+} release from intracellular stores.

**Materials and methods**

**Plant material**

Seedlings of *Phaseolus coccineus* L. cv. Preisgewinner were grown in a greenhouse at 23 ± 1 °C and 9 h light/15 h dark cycles. For the isolation of protoplasts, 3-4-week-old plants were used, each trimmed to the primary leaves.

**Tissue preparation for protoplast isolation**

During the 2nd and 4th hours of the light period, the primary leaves were cut from the plants and the extensor tissue dissected out of 40 laminar pulvini by longitudinal cuts as shown in Erath et al. (1988). Cross-sections about 0.5 mm wide were cut with a razor blade from the extensor pieces and plasmolysed at least 10 min in 4 ml of the wash medium (0.6 M Mannitol, 1 mM CaCl_2 and 5 mM MES-KOH, pH 5.6).

**Extensor protoplast isolation and cultivation**

Protoplasts were isolated as described previously (Erath et al., 1988) with the modifications mentioned below. The preincubated extensor tissue pieces were transferred to 4 ml of cell wall-digesting medium consisting of wash medium supplemented with 0.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium ascorbate and the following enzymes (w/v): 0.7% cellulase TC (Serva, Heidelberg, FRG), 1.2% cellulase from *Penicillium funiculosum* (Sigma, Deisenhofen, FRG), 0.8% pectinase from *Aspergillus niger* (Serva), and 0.12% pectolyase Y23 (Keshin Pharmabio, Japan). Incubation lasted for 1 h 40 min at 30 °C. To collect the protoplasts, the suspension was slightly shaken and filtered through a 100 μm nylon mesh. Incubation flask and nylon mesh were washed with about 4 ml wash medium. The filtrate was centrifuged (150 x g, 2 min) and the floating protoplasts were collected by suctioning off about 2/3 of the supernatant. The supernatant with the protoplasts was divided into two samples and mixed with twice its volume of a raffinose medium (0.5 M raffinose, 1 mM CaCl_2, 10 mM KCl, 5 mM MES-KOH, pH 6), overlaid with 1 ml culture medium (0.5 M mannitol, 10 mM KCl, 1 mM CaCl_2, 5 mM MES-KOH, pH 6), and centrifuged for 3 min at 150 x g. The protoplasts floating throughout the upper phase were gathered by suctioning off this phase. The purification step with the raffinose/mannitol (culture medium)-gradient was repeated once. Afterwards the extensor protoplasts (in about 2 x 1 ml of the culture medium) were transferred to a growth chamber with white light (fluorescent lamps L 40 W/15, Osram, München, FRG; fluorescent light at the protoplast level: 42 μm^-2 s^-1) and constant temperature (23 ± 1 °C). The transfer to the growth chamber normally occurred in the 6th hour of the light period. At the 9th hour of the light period the light was switched off and the protoplasts were maintained in a 15 h dark/9 h light cycle. The protoplast density was about 5 x 10^6 ml^-1, the diameters in the culture medium varied between 38 and 78 μm. Spherical protoplasts with nearly even distribution of chloroplasts at the periphery were used to measure the diameters.

**Protoplast measurement**

The light-induced swelling of extensor protoplasts was always measured in the growth chamber at the end of the first 15 h dark period and the dark-induced shrinking at the end of the following 9 h light period. For the light-induced swelling, the first aliquot (20 μl) of the respective protoplasm suspension was taken under a dim green safelight, transferred to a Fuchs-Rosenthal haemocytometer and the diameters of 50 protoplasts floating beneath the cover slip were measured with a Zeiss microscope with a green filter (Leitz) inserted into the light path. During these manipulations, the fluorescent rate was below 1 μm^-2 s^-1. The following aliquots were taken after switching on the light. In the experiments in which the effect of the various substances on light-induced swelling was tested, the
protoplast suspension was divided into two samples under a dim green safelight 1 h before switching on the light. One served as the test sample, the other as the control. To achieve the final substance concentration, aliquots from stock solutions were injected into the test samples. The resulting volume increase of the test samples was for water-soluble substances below 0.8% and for ethanol-solubilized substances (Bay K-8644, A 23187, thapsigargin) below 0.2%. In the experiments with the ethanol-solubilized substances, the same volume of ethanol was added to the control. All substances were added 1 h before the light was switched on except for Bay K-8644, ionophore A 23187, and thapsigargin, which were added immediately before the measurements started. To be sure that the protoplasts for which the effect of a substance was tested are physiologically intact, the effects of light on a control sample were examined in nearly every experiment. In some experiments the light was switched on at the same time for the control protoplasts and the protoplasts with the test substance, while in other experiments the light was switched on for the control protoplasts 1 h after illuminating the protoplasts with the test substance. A significant difference in light-induced swelling was not found when the light was switched on 15 h or 16 h after the onset of darkness. For dark-induced shrinking the protoplasts were handled as described for light-induced swelling except that the manipulations of the protoplast samples and the first diameter measurement occurred in the light and the following measurements under a dim green safelight. All of the experiments were repeated at least twice and in most cases three times with qualitatively similar results. Representative individual results are shown.

For the experiments with the Ca^{2+}-free medium, 1 mM MgCl₂ instead of 1 mM CaCl₂ was added to the raffinose and culture medium. In this medium the viability of the protoplasts was lower than in the Ca^{2+}-containing medium, but the number of vital protoplasts was sufficient to measure the effects of light at the end of the 15 h dark period or the following 9 h light period. CaCl₂ was added to the medium of the control protoplasts to a final concentration of 1 mM 1 h before light was switched on or off.

Chemicals
Bay K-8644 and thapsigargin were from Calbiochem, Bad Soden, FRG. Neomycin sulphate, sodium orthovanadate, sodium ascorbate, TMB-8, A 23187, and verapamil were purchased from Sigma, Deisenhofen, FRG. LiCl, LaCl₃, myo-inositol, and TEA were from Merck, Darmstadt, FRG.

Results
Light-induced swelling and dark-induced shrinking of extensor protoplasts
Extensor protoplasts were isolated in the light (between 2 and 4 h in the 9 h light period) and maintained in the culture medium (0.5 M mannitol, 10 mM KCl, 1 mM CaCl₂, 5 mM MES-KOH, pH 6) in the same 9 h light/15 h dark cycle as were the intact plants. Measurements of light-induced swelling were made after the first 15 h dark period and measurements of dark-induced shrinking at the end of the following 9 h light period.

After the light-on signal, the diameter of the protoplasts typically increased within the first 50–60 min by about 3–6 μm, corresponding to a volume increase of about 20–40% and decreased somewhat thereafter (Fig. 1A). The volume decrease varied in repeated experiments as can be seen from the control measurements in Figs 2, 3, 6, and 8. After the light-off signal the diameter of the protoplasts typically decreased within the first 60 to 80 min by about 3–5 μm, corresponding to a volume decrease of about 15–30% (Fig. 1B).

The observed light- and dark-induced volume changes of the extensor protoplasts are in agreement with what would be predicted on the basis of the photonastic and scotonomastic leaf movement of Phaseolus. The dark/light transition induces in all leaves an immediate upward movement of the lamina (corresponding to extensor cell swelling) lasting about 50 min. Thereafter the movement varies between leaves in that some remain in the upward leaf position, whereas others move downward and exhibit afterwards more or less regular short period movements. Similarly, the light/dark transition induces in
all leaves an immediate downward movement (corresponding to extensor cell shrinking) lasting about 60 min. Thereafter the lamina moves more or less irregularly up and down.

To demonstrate that the plasma membrane H+-ATPase and the K+ influx through K+ channels is involved in the observed light-induced swelling, the effects of vanadate, known as an inhibitor of the plasma membrane H+-ATPase, and of TEA, a K+ channel blocker, on the light-induced swelling of the extensor protoplasts were examined. When 50 µM vanadate or 20 mM TEA was added 1 h before turning on the light, the light-induced swelling of the extensor protoplasts was inhibited (Fig. 2A, B).

**Extracellular Ca2+ is required for light-induced swelling but not for dark-induced shrinking**

To investigate a possible role of Ca2+ in the light-induced swelling or in the dark-induced shrinking response, Ca2+ was replaced in the washing and culture medium of the protoplasts by 1 mM MgCl2. In the absence of Ca2+, no swelling occurred after the light-on signal (Fig. 3A), however, the light-off signal induced shrinking of the protoplasts 9 h later (Fig. 4A). When Ca2+ was added 1 h before the light was turned on, the light-on signal induced swelling (Fig. 3A, control). This, as well as the observed shrinking reaction in the Ca2+-free medium, clearly indicate that the protoplasts were functional and not damaged during culture in the Ca2+-free medium.

**The Ca2+ channel antagonist verapamil inhibits light-induced swelling but not dark-induced shrinking**

The requirement for Ca2+ in the medium indicates that the influx of Ca2+ is a prerequisite for light-induced swelling. This was confirmed by experiments with the Ca2+ channel blockers verapamil and La3+. The light-induced swelling of the protoplasts was inhibited when 100 µM verapamil (Fig. 3B) or 50 µM LaCl3 (Fig. 3C) was added to the culture medium containing 1 mM CaCl2.
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Fig. 4. Effect of a Ca$^{2+}$-free medium (A) or 100 μM verapamil (B) on the dark-induced shrinking of extensor protoplasts (filled circles). Open symbols, controls. Otherwise as in Fig. 3.

Verapamil, however, failed to inhibit the dark-induced shrinking of the protoplasts (Fig. 4B).

Substances that probably increase the cytoplasmic Ca$^{2+}$ concentration induce swelling in darkness

If an influx of extracellular Ca$^{2+}$ is a prerequisite for light-induced swelling, an experimentally induced increase of the cytoplasmic Ca$^{2+}$ concentration may substitute the light-on signal and induce swelling in darkness. The effects were examined of Bay K-8644, an agonist of Ca$^{2+}$ channels in animal cells (Tsien and Tsien, 1990), of the Ca$^{2+}$-ionophore A 23187, and of thapsigargin, which is known to increase the cytoplasmic Ca$^{2+}$ concentration by inhibiting Ca$^{2+}$-ATPases localized in endomembranes (Inesi and Sagara, 1994). When 0.1 μM Bay K-8644, 10 μM A 23187, or 1 μM thapsigargin was added to the culture medium (containing 1 mM CaCl$_2$) at the end of the 15 h dark period, the protoplasts swelled in darkness (Fig. 5).

In order to demonstrate that it is not the osmotic effect of the Bay K-8644-induced Ca$^{2+}$ influx, but the regulatory effect of the increased cytoplasmic Ca$^{2+}$ concentration that is important for swelling, the effect of Bay K-8644 in a K$^+$-free culture medium was examined. In this medium 0.1 μM Bay K-8644 induced no swelling in darkness (results not shown).

Is the phosphoinositide pathway involved in the light-induced swelling or dark-induced shrinking of extensor protoplasts?

To investigate the possibility that the phosphoinositide pathway is part of the signal transduction for light-on-induced swelling or for the light-off-induced shrinking, the effects of inhibitors of this pathway were studied. Neomycin, which is known to inhibit phospholipase C (PLC) action (Quarmby et al., 1992) by binding to the substrate phosphatidylinositol 4,5-bisphosphate (PIP$_2$), had no effect on light-induced swelling (Fig. 6A), but inhibited dark-induced shrinking completely at 10 μM (Fig. 7A). Lithium ions are known to inhibit inositol-1-phosphatase and could therefore diminish the availability of PIP$_2$ (Berridge and Irvine, 1989). The addition of 1 mM LiCl likewise failed to inhibit light-induced swelling (Fig. 6B), but did inhibit dark-induced shrinking (Fig. 7A). The inhibitory effect of 1 mM LiCl on dark-induced shrinking was overcome when 0.4 mM myo-inositol was applied simultaneously with LiCl (Fig. 7B), while the same treatment had no effect on light-induced swelling (Fig. 6C).
Fig. 6. The effect of 10 μM neomycin sulphate (A), 1 mM LiCl (B) or 1 mM LiCl+0.4 mM myo-inositol (C) on the light-induced swelling of the extensor protoplasts (filled circles). Open circles, controls.

TMB-8, an inhibitor of intracellular Ca\(^{2+}\) transport, inhibits dark-induced shrinking but not light-induced swelling

TMB-8 is known as an inhibitor of intracellular Ca\(^{2+}\) transport (Chiou and Malagodi, 1975) and is shown to block IP\(_3\)-induced Ca\(^{2+}\) release from vacuolar membrane vesicles in oat roots (Schumaker and Sze, 1987). Figure 8 shows that 50 μM TMB-8 had no effect on light-induced swelling, but completely suppressed dark-induced shrinking.

Substances that probably increase the cytoplasmic Ca\(^{2+}\) concentration induce no shrinking in the light

If IP\(_3\)-induced mobilization of Ca\(^{2+}\) from intracellular stores is a prerequisite for dark-induced shrinking, an experimentally induced increase of the cytoplasmic Ca\(^{2+}\) concentration may fulfil the function of the light-off signal and induce shrinking in light. However, Bay K-8644, A 23187, and thapsigargin, when examined at the end of the light period at the same concentrations which induced swelling in the dark (Fig. 5), did not induce shrinking in the light (Fig. 9).

To examine whether dark-induced shrinking requires further dark-induced steps besides an increased cytoplasmic Ca\(^{2+}\) concentration, it was tested whether substances which probably increase the cytoplasmic Ca\(^{2+}\) concentration nullify the inhibitory effect of TMB-8 on dark-induced shrinking. Figure 10 shows that Bay K-8644 (0.1 μM) and A 23187 (10 μM) were both able to overcome the TMB-8 inhibition of dark-induced shrinking.

Discussion

Light-induced swelling and dark-induced shrinking of extensor protoplasts

The observed light-induced swelling and dark-induced shrinking of extensor protoplasts from the Phaseolus
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Fig. 8. The effect of 50 μM TMB-8 on the light-induced swelling (A) or dark-induced shrinking (B) of extensor protoplasts (filled circles). Open circles, controls.

Fig. 9. Effect of 0.1 μM Bay K-8644 (circles), 10 μM A 23187 (triangles) or 1 μM thapsigargin (rectangles) on the protoplast diameters at the end of the 9 h light period in the light. The substances were added to the culture medium at the time when the light was normally switched off for the dark-induced shrinking. The diameters were determined immediately before (t=0) and after adding the substances. The controls are not shown.

Fig. 10. Bay K-8644 or A 23187 rescue of the TMB-8 inhibition of the dark-induced shrinking of extensor protoplasts. After measuring the protoplast diameters in the light at the end of the 9 h light period (t=0), 0.1 μM Bay K-8644 + 50 μM TMB-8 (filled circles) or 10 μM A 23187 + 50 μM TMB-8 (triangles) or 50 μM TMB-8 alone (open circles) was added to the medium bathing the protoplasts and then the light was switched off.

by activation of the plasma membrane H⁺-ATPase and an opening of inward K⁺ channels was found, while a transfer to darkness in the second half of a 16 h light period closed the TEA-sensitive inward K⁺ channels. In Phaseolus vanadate or TEA also inhibited the light-induced swelling of the extensor protoplasts (Fig. 2).

Signal transduction for light-induced swelling of extensor protoplasts

The results indicate that an increase of the cytoplasmic Ca²⁺ concentration via an influx of extracellular Ca²⁺ through Ca²⁺ channels in the plasma membrane is an essential step in the signal transduction events coupling light perception to extensor protoplast swelling, that is, to the activation of the plasma membrane H⁺-ATPase and opening of inward K⁺ channels (Kim et al., 1992, 1993) and to the closing of the Cl⁻ and K⁺ outward channels.

No swelling after light-on occurred when the medium bathing the protoplasts was without Ca²⁺ (Fig. 3A) or when the Ca²⁺ channel blockers verapamil or La³⁺ (Fig. 3) were added to the protoplast bathing medium. In plants La³⁺ and verapamil block Ca²⁺ influx across the plasma membrane (summarized in Bush, 1995) and block a voltage-dependent Ca²⁺-selective channel in wheat roots (Piñeros and Tester, 1995). Also the ability of verapamil to bind to membrane proteins has been used to identify components of putative plasma membrane Ca²⁺ channels (summarized in Bush, 1995). However, both La³⁺ and verapamil have also been shown to block K⁺ channels in plants (Terry et al., 1992; Thomine et al., 1994). Therefore, conclusions about the mode of action of these substances are not definite. The conclusion that verapamil inhibits light-induced swelling of the extensor

pulvinus are not only in agreement with the leaf movement of Phaseolus, but also with results obtained for the regulation of ion transporters by light in extensor protoplasts of the Samanea pulvinus (Kim et al., 1992, 1993). In response to white or blue light at the end of an 8 h dark period a vanadate-sensitive hyperpolarization caused
protoplasts via inhibition of Ca\textsuperscript{2+} influx across Ca\textsuperscript{2+} channels, however, is given credence by the findings that extracellular Ca\textsuperscript{2+} is required for light-induced swelling and that dark-induced shrinking, for which K\textsuperscript{+} efflux through K\textsuperscript{+} channels, but not the influx of extracellular Ca\textsuperscript{2+} is required, is not affected by verapamil (Fig. 4B). In addition, the swelling of extensor protoplasts could be induced in darkness by the addition of the Ca\textsuperscript{2+} channel agonist Bay K-8644, the Ca\textsuperscript{2+} ionophore A 23187 or thapsigargin (Fig. 5) to the Ca\textsuperscript{2+}-containing medium. These substances are thought to increase the cytosolic Ca\textsuperscript{2+} concentration by different mechanisms. The Ca\textsuperscript{2+} channel agonist Bay K-8644 opens voltage-gated 'L-type' Ca\textsuperscript{2+} channels in animals (Tsien and Tsien, 1990), A 23187 permeabilizes the lipid bilayer of membranes for Ca\textsuperscript{2+}, and thapsigargin is known to increase the cytoplasmic Ca\textsuperscript{2+} concentration by inhibiting Ca\textsuperscript{2+}-ATPases at endomembranes (Ghosh et al., 1991; Inesi and Sagara, 1994). Because it is unlikely that these three substances have the same side-effects their light-mimicking effect in triggering extensor swelling is most likely due to an increase of the cytoplasmic Ca\textsuperscript{2+} concentration.

That the increase of the cytoplasmic Ca\textsuperscript{2+} concentration via influx of extracellular Ca\textsuperscript{2+} mediates cell swelling has been postulated recently for extensor tissue of P. coccineus (Bialczyk and Lechowski, 1992) and is now proven for the phytochrome-controlled swelling of etiolated wheat leaf protoplasts. Consistent with our results, Bossen et al. (1988, 1990) and Tretyn et al. (1990) showed that the red-induced swelling depends on the presence of Ca\textsuperscript{2+} in the protoplast medium and that the Ca\textsuperscript{2+} channel blockers verapamil, nifedipine and La\textsuperscript{3+} inhibit this reaction, while swelling is induced in darkness by Bay K-8644 or A 23187. The conclusion that red causes protoplast swelling by an increase of the cytoplasmic Ca\textsuperscript{2+} concentration via opening of Ca\textsuperscript{2+} channels in the plasma membrane has been confirmed by Shacklock et al. (1992) by directly measuring the red-induced transient increase of the cytoplasmic Ca\textsuperscript{2+} concentration with Ca\textsuperscript{2+}-sensitive fluorescent dyes and by the fact that Mn\textsuperscript{2+} added to the protoplast medium quenched this Ca\textsuperscript{2+}-signal.

There are, however, also differences between the phytochrome-controlled swelling of wheat protoplasts and the white-light-induced swelling of the extensor protoplasts of Phaseolus.

Firstly, neomycin as well as LiCl, both inhibitors of the phosphoinositide pathway, prevented the red-induced swelling of wheat protoplasts (Bossen et al., 1990), while neither these substances nor TMB-8, an inhibitor of intracellular Ca\textsuperscript{2+} transport (Chiu and Malagodi, 1975; Shumaker and Sze, 1987) had any effect on the light-induced swelling of extensor protoplasts (Figs 6, 8A). The findings that in Samanea extensor protoplasts blue-light did not increase the IP\textsubscript{3} level and that neomycin did not inhibit the blue-light-induced opening of K\textsuperscript{+} channels are consistent with these results and, additionally, support the conclusion that light-induced extensor swelling is not caused by IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from intracellular stores (Kim et al., 1996).

Secondly, for Phaseolus, it is not definitely known whether the white-light-induced swelling of extensor cells is blue-pigment-mediated (Kim et al., 1993; Roblin et al., 1989, 1990) or mediated by both phytochrome and the blue-absorbing-pigment (Bialczyk and Lechowski, 1990). However, in addition to the phytochrome-mediated swelling of wheat leaf protoplasts, blue-pigment-mediated swelling of guard cell protoplasts has also been reported (Zeiger and Heppler, 1977; Kearns and Assman, 1993).

In the extensor protoplasts the chain of events from light perception to the opening of Ca\textsuperscript{2+} channels and increase of cytoplasmic Ca\textsuperscript{2+} concentration, and the subsequent activation of the H\textsuperscript{+}-ATPase and the opening of K\textsuperscript{+} inward channels is still unknown (Cote, 1995).

Results from other plant systems indicate that G-proteins (Bossen et al., 1990; Bowler et al., 1994), the cAMP pathway (Robin et al., 1990; Kurosaki and Nishi, 1993; Assmann, 1995) and Ca\textsuperscript{2+}/calmodulin activation of protein kinases and phosphorylation of the H\textsuperscript{+}-ATPase (Robin et al., 1990; Schaller and Sussman, 1988; Fallon et al., 1993) could be involved in this signal transduction chain.

**Signal transduction for dark-induced shrinking of extensor protoplasts**

An influx of extracellular Ca\textsuperscript{2+} is not required for dark-induced shrinking of extensor protoplasts because this shrinking occurred in Ca\textsuperscript{2+}-free medium (Fig. 4A) and was not inhibited by verapamil in Ca\textsuperscript{2+}-containing medium (Fig. 4B). While having no effect on light-induced swelling, however, neomycin, known as an inhibitor of PIP\textsubscript{2} hydrolysis by PLC (Quarmby et al., 1992), and Li\textsuperscript{+}, known as an inhibitor of inositol-phosphatases (Berridge and Irvine, 1989), inhibited the dark-induced shrinking of the extensor protoplasts. In addition, the inhibitory effect of Li\textsuperscript{+} overcame by myo-inositol (Fig. 7B), as might be expected if the Li\textsuperscript{+} effect results from Li\textsuperscript{+} inhibition of inositol-phosphatases. The same simultaneous application of the two substances had no effect on light-induced swelling (Fig. 6C). It is concluded from these results that the light-off signal for the shrinking of extensor protoplasts is transduced by the phosphoinositide pathway. Inhibition of dark-induced shrinking (Fig. 5B), but not of light-induced swelling (Fig. 8A), by TMB-8 also supports this conclusion and, in addition, indicates that IP\textsubscript{3}-induced mobilization of Ca\textsuperscript{2+} from intracellular stores is an essential step in the dark-induced shrinking of extensor protoplasts.

Recent results obtained for extensor protoplasts of the Samanea pulvinus strongly support this conclusion. Kim
et al. (1996) showed that transfer to darkness during the light period increased IP$_3$ levels and closed inward K$^+$ channels. Furthermore, neomycin inhibited both IP$_3$ production and K$^+$ channel closure induced by this signal. Based on the findings in guard cells that ABA-treatment increased IP$_3$ levels (Coté and Crain, 1994), that microsomal Ca$_{2+}$ levels inhibited the inward K$^+$ channels (Schroeder and Hagiwara, 1989) and stimulated anion channels (Hedrich et al., 1990), the authors proposed that for shrinking signals IP$_3$ is the second messenger that mobilizes intracellular Ca$_{2+}$, which inhibits K$^+$ influx and activates Cl$^-$ efflux.

In extensor protoplasts of Phaseolus an A 23187- or Bay K-8644-mediated elevation of the cytosolic Ca$_{2+}$ concentration was not sufficient to cause extensor protoplasts to shrink in the light (Fig. 9). However, both substances nullified the inhibitory effect of TMB-8 (an inhibitor of intracellular Ca$_{2+}$ transport) on dark-induced shrinking (Fig. 10). It is concluded that, in addition to the IP$_3$-induced increase in the cytoplasmic Ca$_{2+}$ concentration, another intracellular mediator has to be involved in this transduction pathway.

Conclusions

The presented evidence for extensor protoplasts that light-induced swelling requires influx of extracellular Ca$_{2+}$, dark-induced shrinking IP$_3$-mediated mobilization of Ca$_{2+}$ from intracellular stores seem to contradict the proposal of Roblin et al. (1989, 1990) that the blue-pigment-mediated opening movement of Cassia leaflets (corresponding to extensor swelling) requires the mobilization of Ca$_{2+}$ from intracellular stores and acceleration of phospholipid turnover, but that the phytochrome-mediated closing movement (corresponding to extensor shrinking) requires the mobilization of extracellular Ca$_{3+}$. The result are not contradictory, however, if one assumes that the light-off and light-on responses of the flexor cells dominate the movement of whole Cassia pulvin. The result that blue-light (which induces an opening movement of the leaflets corresponding to shrinking of flexor cells) increased the IP$_3$ level in Samanea flexor protoplasts (Kim et al., 1996) supports this assumption. Future studies must show whether dark-induced swelling of the flexor cells (corresponding to the closing movement of the leaflets) is mediated by the influx of extracellular Ca$_{2+}$ as found for the light-induced swelling of the extensor protoplasts.

References


