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Detection in Vivo of Very Rapid Red Light-Induced Calcium-Sensitive Protein Phosphorylation in Etiolated Wheat (*Triticum aestivum*) Leaf Protoplasts[1]

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Etiolated wheat (*Triticum aestivum* cv Mercia) leaf protoplasts respond to brief red-light irradiation by increasing in volume over a 10-min incubation period (M.E. Bossen, H.A. Dassen, R.E. Kendrick, W.J. Vredenberg [1988] Planta 174: 94–100). When the calcium-sensitive dye Fluo-3 was incorporated into these protoplasts, red-light irradiation initiated calcium transients lasting about 2 min (P.S. Shacklock, N.D. Read, A.J. Trewavas [1992] Nature 358: 153–155). Release of calcium in the protoplasts by photolysis of incorporated 1-{2-amino-5-[1-hydroxy-1-(2-nitro-4, 5-methylenedioxyphenyl)-methyl]-phenoxy}-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid, tetrasodium salt (caged calcium) or caged inositol trisphosphate frequently induced transient increases in intracellular calcium levels, although the kinetics of these changes showed variation between experiments. Upon exposure to red light, a pronounced increase in the phosphorylation of a 70-kD and to a lesser extent a 60-kD peptide was observed, commencing within 15 s and continuing for up to 2 min. Simultaneous far-red and red irradiation attenuated the response. Upon release of incorporated caged calcium by cage photolysis, the labeling of these two peptides was greatly increased. When incorporated caged inositol trisphosphate was photolyzed, only the labeling of the 70-kD peptide was enhanced. Phosphorylation of the 70-kD peptide was also increased when extracellular calcium was elevated, but it decreased with increasing extracellular EGTA. These data thus provide direct evidence for the operation of an in vivo transduction sequence involving red light-dependent, calcium-sensitive protein phosphorylation.

Light initiates a diverse range of phenomena in plants (Smith, 1975). Processes specifically controlled by R include the regulation of growth, reproduction, and time measurement. The effects of R are mediated by the family of photoreceptors designated phytochrome, and the biochemical mechanism of action of R has been of intense interest for many years (Smith, 1975; Kendrick and Kronenberg, 1986). A current hypothesis involves the mediation of R-induced changes in $[Ca^{2+}]_i$, and was first introduced by Haupt and Weisenseel (1976). These authors pointed to the evidence that phytochrome regulated the passage of ions across the plasma membrane and noted that Ca^{2+} had a key role in cell signaling in animal cells. With the detection of calmodulin in plant cells (Anderson and Cormier, 1978) and the discovery by Hetherington and Trewavas (1982) of Ca^{2+}-regulated protein kinases in plants, Roux (1984) and Roux et al. (1986) formulated the modern version of the hypothesis. They proposed that photoactivation of phytochrome leads to elevation of $[Ca^{2+}]_i$, which subsequently activates Ca^{2+}/calmodulin-dependent enzymes, in particular protein kinases.

Direct evidence for the in vivo operation of both parts of the Roux hypothesis has been lacking, although there is considerable circumstantial data, some of which are summarized in Roux et al. (1986).

R-induced changes in $[Ca^{2+}]_i$ have occurred during detected changes in Ca^{2+} influx or efflux (Weisenseel and Ruppert, 1977; Dreyer and Weisenseel, 1979; Hale and Roux, 1980). Using the direct method of fluorimetric measurements with the Ca^{2+}-sensitive dye quin-2, Chae et al. (1990) reported a R-induced increase in $[Ca^{2+}]_i$ in oat leaf protoplasts. Their results may be open to question, however, because the data were not corrected for volume changes. It has been repeatedly shown that etiolated protoplasts from cereal leaves increase their volume after R treatment and decrease their volume after FR treatment (Blakeley et al., 1983; Bossen et al., 1988; Zhou et al., 1990). Volume-induced changes in light scattering of the incident exciting light in a fluorimeter will induce anomalous changes in apparent measured $[Ca^{2+}]_i$. Gilroy showed that these apparent increases in $[Ca^{2+}]_i$ were artifacts resulting from protoplast swelling (Gilroy et al., 1986; Gilroy, 1987).

Direct evidence for in vivo R-induced changes in protein phosphorylation is also lacking, although Roux's laboratory has detected R-induced changes in nuclear protein phosphorylation (Datta et al., 1985) and has isolated a Ca^{2+}-dependent protein kinase (Li et al., 1991) in isolated pea nuclei in vitro. There is evidence that phytochrome is associated with a protein kinase activity (McMichael and Lagarias, 1990), and there are reports of changes in peptide phosphorylation detected on SDS-PAGE gels after R illumination (Otto and Schäfer, 1988; Park and Chae, 1989). However, it is still unclear if phytochrome phosphorylation is related to changes in $[Ca^{2+}]_i$.

We have recently detected transient increases in $[Ca^{2+}]_i$ levels induced by R in etiolated wheat (*Triticum aestivum*)

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Abbreviations: NITR 5, 1-{2-amino-5-[1-hydroxy-1-(2-nitro-4,5-methylenedioxyphenyl)-methyl]-phenoxy}-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid, tetrasodium salt; NITR 5/AM, NITR 5 acetoxymethyl ester; IP_3, myoinositol 1,4,5-trisphosphate; caged IP_3, myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CLSM, confocal laser scanning microscopy; R, red light; FR, far-red light.

leaf protoplasts using confocal imaging and Fluo-3 (Shacklock et al., 1992), and the use of optically thin confocal sectioning for Ca^{2+} measurement potentially obviates the light-scattering problem involved in earlier fluorimeter measurements. The R-induced $[Ca^{2+}]_i$ transients last no more than 2 min, and, therefore, associated $[Ca^{2+}]_i$-induced changes in phosphorylation must be accomplished in this time period. This study investigates whether apparent protein phosphorylation is altered, and if regulation by $[Ca^{2+}]_i$ is involved.

MATERIALS AND METHODS

Chemicals

Cellulase "Onozuka" RS was obtained from Yakult Pharmaceutical Industrial Co. Ltd. (Tokyo, Japan), ^{32}P-orthophosphate (11 GBq/mL) came from ICN Flow (High Wycombe, Buckinghamshire, UK), and caged IP_3 and caged Ca was from Novabiochem (UK) Ltd., (Nottingham, UK). All other chemicals were standard enzyme grade and were obtained from Sigma London Chemical Co. (Poole, Dorset, UK).

Plant Material and Protoplast Isolation

Wheat seedlings (*Triticum aestivum* L. cv Mercia) were grown in moist vermiculite at 22°C for 8 to 10 d in darkness, harvested under dim (50 nmol m^{-2} s^{-1}) green safe light (primary green filter, Lee Colortran, Wembley, UK, 15-W tungsten bulb), and protoplasts were prepared according to Edwards et al. (1978). Primary leaves were finely chopped (1-mm sections) and incubated in enzyme medium (2% cellulase, 0.2% pectinase, 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM Mes, 5 mM Tris, pH 5.5) for 3.5 h at 22°C. Preliminary experiments suggested that chilling the protoplasts could seriously impair subsequent responsiveness to R and inhibited phosphorylation of proteins carried out at 25°C. Protoplast purification on a Suc cushion was followed by resuspension in incubation medium (0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM Mes, 5 mM Tris, pH 7.0), and determination of viability by trypan blue exclusion or fluorescein diacetate staining (Widholm, 1972). (Trypan blue and fluorescein diacetate techniques gave similar values for viability.)

Light Sources

R was provided by a Rank Aldis 2000 projector equipped with a 150-W bulb and 660-nm (10-nm half band width) interference filter (Glen Spectra, Stanmore, Middlesex, UK), FR was provided by a Liesengang Fantima 250 projector equipped with a 250-W bulb and 730-nm (10-nm half band width) interference filter, and passed through a water filter to reduce heating. Fluence rates at the suspension surface were: R, 74.0 μmol m^{-2} s^{-1}, and FR, 61.8 μmol m^{-2} s^{-1} as determined using a digital quantum photometer (Skye Instruments SKR100).

In Vivo Phosphorylation of Protoplast Protein

Protoplast suspensions were mixed with 74 MBq/mL ^{32}Pi and 50 μM EGTA in incubation medium by drawing them into a wide-orifice micropipet tip and incubated at 22°C in a microcentrifuge tube, before the reaction was terminated by addition of aliquots to an equal volume of SDS-PAGE sample buffer (0.125 M Tris-HCl/5% SDS/10% mercaptoethanol/20% glycerol/0.004% bromphenol blue), followed by heating at 50°C for 15 min.

^{32}Pi Uptake Assay

Protoplasts were labeled with ^{32}Pi as described above, and a sample was taken after 60 s of labeling. After washing in enzyme medium, protoplasts were lysed by osmotic shock and three aliquots were counted in a liquid scintillation counter (Beckman LS5000CE) by Cerenkov counting.

$[Ca^{2+}]_i$ Measurement in Single Protoplasts

Protoplasts were loaded either with Fluo-3 alone or with Fluo-3 and caged Ca^{2+} by ester loading (Tsien, 1981). Fluo-3 was also loaded in combination with caged ATP, caged IP_3, and caged cGMP by electroporation. Protoplasts were then observed using a Bio-Rad MRC-600 CLSM (Read et al., 1992). The protoplasts were irradiated with either UV light (25 s) to photolyze caged probes, or with R (3 min). Light for both of these irradiation regimens was provided by the microscope 100-W mercury epifluorescence illuminator and interference filters (340 nm, 10-nm half band width, 9 μmol m^{-2} s^{-1}, and 660 nm, 10-nm half band width, 100 μmol m^{-2} s^{-1}, respectively).

Bright-field and fluorescence images were subsequently collected every 3 s and stored on an optical disc for analysis. Image analysis using the histogram command in the CoMoS Confocal Microscope control Software (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) determined the average pixel intensity in the region of the image representing the cytoplasm of protoplasts under examination. Quantification of changes in $[Ca^{2+}]_i$ was performed by fluorescence ratio imaging (Read et al., 1992) using the dual-emission dye Indo-1. Results obtained using this technique were used to give an approximation of the magnitude of changes observed in protoplasts loaded with Fluo-3.

Caged Probe Incorporation and Photolysis

Protoplasts were loaded with caged probes and the Ca^{2+}-indicating dye Fluo-3 by ester loading (Tsien, 1981) (NITR 5/AM, Fluo-3/AM), or electroporation (caged IP_3, Fluo-3, caged cGMP, caged ATP). In the former, protoplasts were incubated with 50 μM NITR 5/AM and 50 μM Fluo-3/AM in incubation medium at 32°C for 2 h. In the latter, protoplasts were electroporated (1200 V/cm field strength, 0.25 μF capacitance, three pulses at 5-s intervals) with 50 μM caged probe using a Gene-Pulser electroporator (Bio-Rad, UK), washed twice in incubation medium, and kept on ice at 4°C for 10 min and 22°C for 90 min before phosphorylation. Loading was confirmed by CLSM as previously described (Shacklock et al., 1992). For caged probe release, protoplast suspensions were irradiated with UV light (5 μmol m^{-2} s^{-1}) using a Blak-Ray model B-100A UV lamp (Ultra-Violet Products Inc., San Gabriel, CA) positioned 5 cm from the suspension, which was incubated in microtiter plate wells. Release of caged Ca^{2+}

was confirmed in solution under similar conditions by fluorimeter measurements using a model LS-5B Luminescence Spectrometer (Perkin-Elmer, Buckinghamshire, UK).

Electrophoresis

SDS-PAGE was performed as described by Laemmli (1970) on a 15 × 17 × 0.1 cm gel apparatus (Gibco BRL, Paisley, UK) with 4% stacking and 12% resolving gel, overnight, with 5 mA/gel at 22°C. After rinsing in distilled water and heating in 10% TCA at 70°C for 1 h, gels were stained, destained, dried, and autoradiographed using preflashed Cronex-4 film and Lightning Plus intensifying screens (Du Pont, Stevenage, Hertfordshire, UK).

RESULTS

Variation in $[Ca^{2+}]_i$ with R, and Release of Ca^{2+} from Caged Ca^{2+} or Caged IP_3

Protoplasts were isolated from wheat leaves that had been grown for 8 to 10 d in total darkness. Isolation was carried out in dim green light and at room temperature, and the protoplasts were washed by Suc gradient fractionation (Edwards et al., 1978). Aliquots of protoplasts were removed for phosphorylation studies and the remainder were kept for $[Ca^{2+}]_i$ measurements. Protoplasts were loaded with Fluo-3/AM ester by incubation for 2 h at 32°C. After two additional washes, individual protoplasts were examined under CLSM and loaded protoplasts were exposed to R on the microscope stage as described in "Materials and Methods." Confocal images of Fluo-3 fluorescence were taken every 3 s (or multiples of 3 s) and total fluorescence of the individual protoplasts was estimated (Fig. 1A).

In the first of the examples shown in Figure 1A, $[Ca^{2+}]_i$ increases over a period of about 1 min immediately following R and thereafter decreases to less than half of the initial value. In the second example shown in Figure 1A, $[Ca^{2+}]_i$ is already elevated when measurements begin after R and declines gradually to below-control levels. In approximately 80% of protoplasts studied (18 of 23), R increases cytoplasmic Ca^{2+} but there is considerable variation concerning the time at which the increase commences, the amplitude of the increase, and the decay. Figure 1B shows other examples of changes in $[Ca^{2+}]_i$ observed in this population. Over half of those protoplasts that respond have increased their $[Ca^{2+}]_i$ before the period of R exposure has been completed (Fig. 1A), but the length of the transient seems to be limited to about a total of 2 min. Calibration of this R-induced increase in $[Ca^{2+}]_i$ using the dual-emission ratio dye Indo-1 by fluorescence ratio imaging showed maximal $[Ca^{2+}]_i$ change to be from 250 to 900 nM over this time period in localized regions of the cytosol (Shacklock et al., 1992). We have used the calibrations obtained by fluorescence ratio imaging to provide the $[Ca^{2+}]_i$ values shown in Figure 1.

The origin of the variability observed in Figure 1 may arise from the use of whole leaves for protoplast preparation. These leaves are composed of different cell types, and even among a uniform tissue there are cells of grossly different developmental age. In our previous study (Shacklock et al., 1992), similar variations in $[Ca^{2+}]_i$ change were detected.

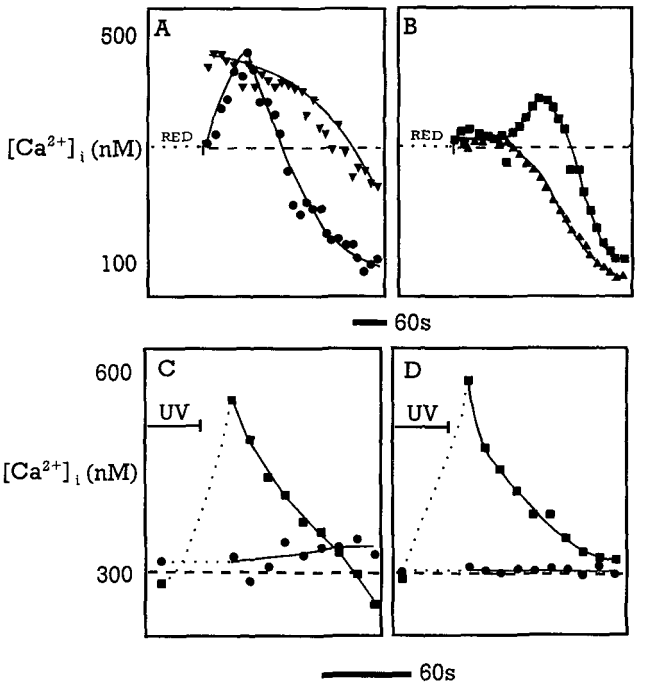

Figure 1. Traces showing the average change in $[Ca^{2+}]_i$ in the cytoplasm of single Fluo-3-loaded wheat protoplasts. A and B, Three minutes R irradiation; C, photolysis of caged Ca^{2+} (■) or caged ATP (●); and D, photolysis of caged IP_3 (■) or caged cGMP (●). Each data point was obtained by calculating the average pixel intensity in the area of the image representing the cytoplasm of the protoplast. The images were obtained by taking optical sections (approximately 0.7 μm thick) through etiolated wheat protoplasts loaded with the Ca^{2+} reporting dye Fluo-3. The average basal level of $[Ca^{2+}]_i$ measured in all protoplasts observed (n = 23, 11, and 13, respectively) is represented by the dashed line. Measurement of $[Ca^{2+}]_i$ began immediately following R or UV irradiation and continued for 10 min, although most changes were complete within 3 to 4 min. In protoplasts irradiated with R, considerable variation was observed between protoplasts, and these are represented in A and B by the main types of $[Ca^{2+}]_i$ change observed.

Transient changes in $[Ca^{2+}]_i$ were also observed when NITR-5 and caged IP_3 were photolyzed in single protoplasts on the stage of the inverted microscope by 25 s of UV light (Fig. 1, C and D). Although the $[Ca^{2+}]_i$ rapidly reaches concentrations over 1 μM in regions of the cytoplasm within 1 or 2 min, the Ca^{2+} concentration returns to basal levels typically within 2 min. Note that the Ca^{2+} transient is not followed in this case by a decline below the initial resting levels. The time length of the R-induced transient may reflect the metabolic capabilities of the protoplast to regulate $[Ca^{2+}]_i$.

Similar kinetics of Ca^{2+} change were observed when IP_3 was released into the cytoplasm from incorporated caged IP_3 (Fig. 1D). In all cases, controls involving release of caged cGMP and caged ATP showed no changes in $[Ca^{2+}]_i$. The characteristics of the Ca^{2+} transients have been observed in 25 experiments using caged Ca^{2+} and caged IP_3, and we have previously observed that all these treatments increase the protoplast volume (Shacklock et al., 1992).

Transient R-Induced Phosphorylation in Vivo of Etiolated Wheat Leaf Protoplast Protein

The $[Ca^{2+}]_i$ transients in individual protoplasts last no more than 2 min (Fig. 1). For phosphorylation studies, a large number of protoplasts have to be used and in this case the averaged kinetics of $[Ca^{2+}]_i$ change will look rather different. Based on the variations we have observed (Shacklock et al., 1992), $[Ca^{2+}]_i$ should start to increase about 15 to 30 s after R irradiation has commenced. It is more difficult to estimate a return to basal levels because of the R-induced decline below basal levels, but we would expect any increase in population $[Ca^{2+}]_i$ to have disappeared by 4 min. Perhaps more importantly, based on a protoplast population, the increase in $[Ca^{2+}]_i$ does not appear to be significant, thus emphasizing the necessity for measuring $[Ca^{2+}]_i$ on individual cells or protoplasts.

Associated Ca-induced protein phosphorylation should show similar kinetics as a population change in the level of $[Ca^{2+}]_i$. Etiolated wheat leaf protoplasts have been prepared and incubated in ^{32}Pi for periods of 15 min, with R irradiation commencing at the same time as addition of ^{32}Pi. After incubation, the protoplasts were lysed in SDS sample buffer, and the proteins were subjected to SDS electrophoresis and autoradiography (Fig. 2). During these very short labeling periods, only a few phosphorylated peptides were detected and even these were detected as faint bands. The predominant peptide has a mobility consistent with a molecular mass of 70 kD, and the phosphorylation of this peptide is substantially increased within 45 to 60 s of R irradiation and is just detectable at 15 s (Fig. 2, top). Fainter bands running at 60 kD, 100 kD, and at the stacking gel interface were also detected, but effects of R irradiation on the phosphorylation of these polypeptides are less clear. Figure 2 also shows that this increased phosphorylation over the control has disappeared by 5 min of labeling and irradiation (Fig. 2, middle).

To test for possible phytochrome involvement in this R-induced response, protoplasts were also labeled during simultaneous irradiation with approximately equal amounts of R and FR (Fig. 2, bottom). Simultaneous R and FR irradiation appeared to result in a decrease in 70-kD phosphorylation compared with R irradiation alone, and may be an effect of reduced levels of Pfr. It should be noted, however, that the labeling of the 70-kD protein is not eliminated by this treatment, which could be due to the need for higher fluence of FR than of R, since Pr has a greater extinction coefficient than Pfr (Hart, 1988) and a greater quantum yield for Pfr formation. We have not tested the effect of higher FR fluence.

To examine whether the increase in phosphorylation could be affected by R-induced changes in ^{32}Pi uptake, protoplasts were incubated in ^{32}Pi in darkness, or with R or R and FR irradiation. ^{32}Pi uptake into the protoplasts after 60 s of incubation was as follows (cpm ± SD): control, 237,031 ± 13,811; R + FR, 121,091 ± 5,532; R, 133,984 ± 16,541. These data indicate that R or simultaneous R and FR irradiation inhibit ^{32}Pi uptake.

Modification of Protein Phosphorylation by Ca and IP_3

To demonstrate that the acceleration of phosphorylation of the 70-kD peptide is Ca^{2+}-dependent, we have incorporated caged Ca^{2+} into protoplasts by electroporation and then photolyzed the cage to release Ca^{2+} at the same time as ^{32}Pi was added to the protoplast medium. As shown in Figure 3, top, protoplast proteins were subjected to separation of the labeled peptides by SDS-PAGE and autoradiography.

The labeling of the 70-kD peptide is increased by this

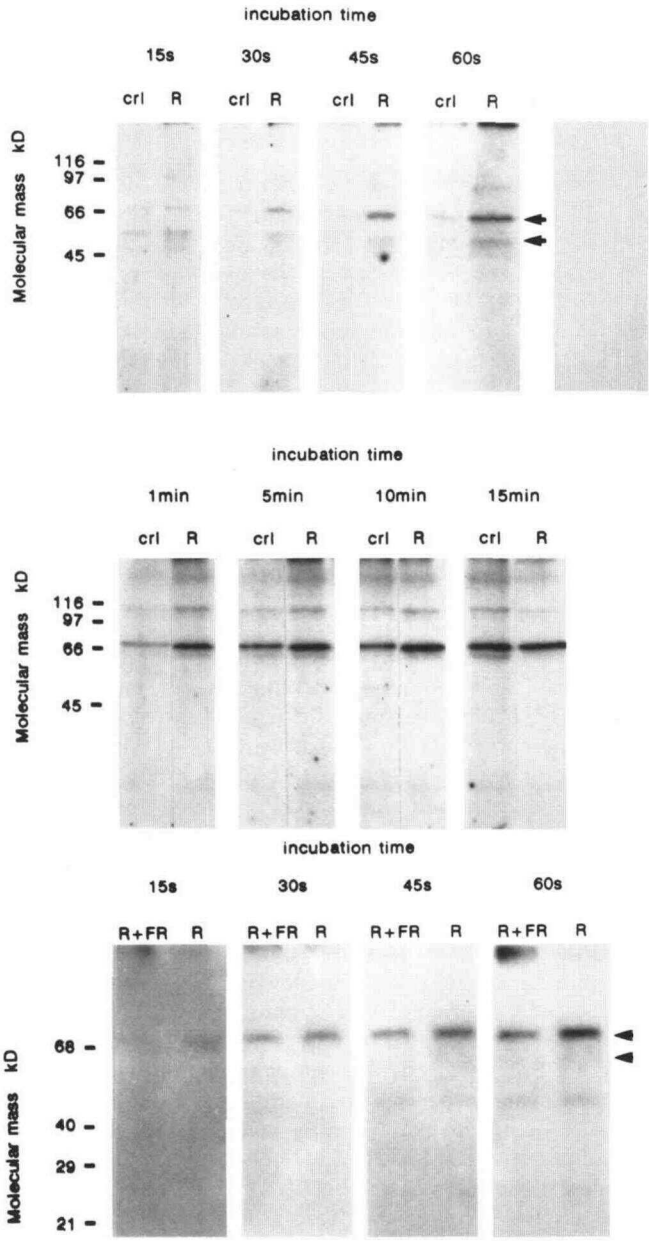

Figure 2. Phosphorylation of etiolated wheat leaf protoplast protein in the presence and absence of R and R and FR. Etiolated wheat leaf protoplasts were incubated with ^{32}P-orthophosphate for 15, 30, 45, and 60 s (top), 1, 5, 10, and 15 min (middle), or 15, 30, 45, and 60 s (bottom) with R irradiation (R), simultaneous R and FR irradiation (R+FR), or without irradiation (crl). The reactions were terminated by addition of Laemmli (1970) buffer, samples were separated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography.

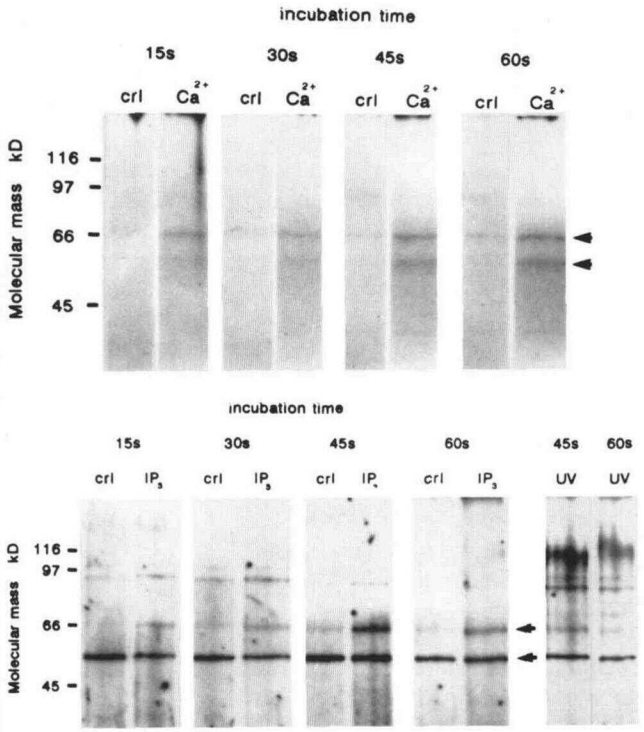

Figure 3. Phosphorylation of etiolated wheat leaf protoplast protein upon photolysis of incorporated caged Ca^{2+} and caged IP_3. Etiolated wheat leaf protoplasts were loaded with caged Ca^{2+} (top) or caged IP_3 (bottom), ^{32}Pi-orthophosphate was added at the same time as exposure to UV light, and samples were taken at 15, 30, 45, and 60 s. Controls (crl) were incubated in dim green light, and UV-irradiated controls (UV) were irradiated during incubation. The reaction was terminated by addition of Laemmli (1970) buffer, samples were separated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography.

treatment (Fig. 3, top), and with time kinetics very similar to those shown for R-induced phosphorylation of this protein (Fig. 2). There is also an easily detectable increase in the labeling of the 60-kD band (Fig. 3, top), which could only be faintly observed to increase with R (Fig. 2).

Similar experiments have been carried out with caged IP_3 incorporated into the protoplasts. Upon release of IP_3 by UV photolysis, Ca^{2+} is elevated, possibly by mobilization from internal reserves that have the appropriate IP_3 binding protein (Fig. 1). As above, IP_3 was released at the same time as ^{32}Pi was added to the protoplasts. A clear increase in labeling of the 70-kD peptide is seen (Fig. 3, bottom), but the primary difference compared with the release of Ca^{2+} from caged Ca^{2+} is in the labeling of the 60-kD peptide, which at this time shows no increased labeling.

The release of Ca^{2+} from caged Ca^{2+} is likely to be relatively uniform throughout the cytoplasm, whereas IP_3-mobilized Ca^{2+} is likely to be localized to the region bordering IP_3-sensitive stores (e.g. the vacuole). The 60- and the 70-kD proteins may thus be located in different cell compartments or membranes. Other protein bands can be faintly detected in Figure 3, bottom, but there are no obvious effects of IP_3-mobilized Ca^{2+} on them. To test for any UV-dependent changes in phosphorylation, protoplasts not loaded with caged probes were illuminated with UV light as described above. No increases in phosphorylation were detected (Fig. 3, bottom).

The Effects of Extracellular Ca^{2+} and Extracellular EGTA on Protoplast Protein Phosphorylation

Further evidence that the phosphorylation of the 70-kD peptide is Ca^{2+} dependent is shown in Figure 4. In Figure 4, top, protoplasts have been incubated in different extracellular Ca^{2+} concentrations simultaneously with ^{32}Pi. Increased labeling of the 70-kD peptide is clearly visible when the extracellular Ca^{2+} concentration is increased from 0.1 to 1 and 10 mM. It has been previously shown that an increasing extracellular Ca^{2+} concentration can increase $[Ca^{2+}]_i$ (Gilroy et al., 1991).

Figure 4, bottom, shows that increasing the extracellular

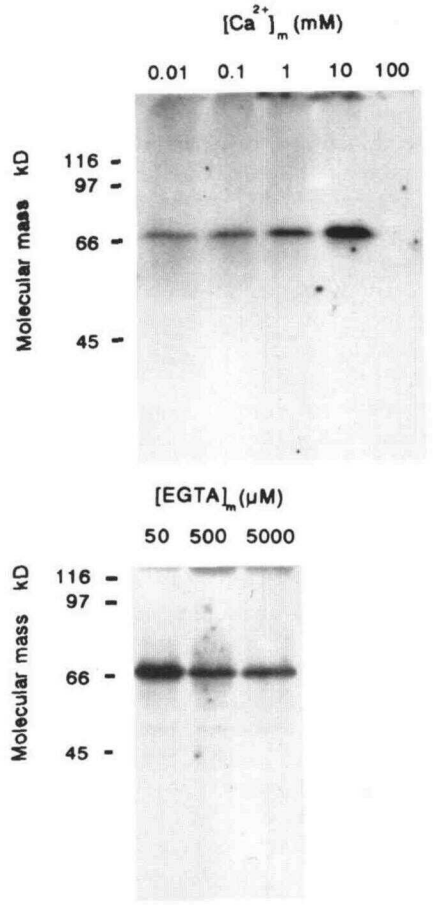

Figure 4. Effect of changes in extracellular Ca^{2+} or extracellular EGTA on phosphorylation of etiolated wheat leaf protoplast protein. Etiolated wheat leaf protoplast suspensions were incubated in ^{32}Pi-orthophosphate, in dim green light, in (top) medium supplemented with 0.01, 0.1, 1, 10, or 100 mM $CaCl_2$ for 30 min; or (bottom) medium supplemented with 1 mM $CaCl_2$ and 50 (7.5), 500 (0.75), or 5000 (0.075) μM EGTA ($[Ca^{2+}]_{free}$). Incubations were terminated by Laemmli (1970) buffer, and SDS-PAGE phosphorylated proteins were detected by autoradiography.

EGTA concentration in the presence of a constant Ca^{2+} concentration diminishes the 70-kD protein phosphorylation. It has also been shown that such treatments decrease $[Ca^{2+}]_i$ (Gilroy, 1987).

DISCUSSION

The results described here show that the R-induced phosphorylation of a 70-kD protein broadly concurs with a R-induced change in $[Ca^{2+}]_i$. Furthermore, there is strong evidence that the phosphorylation is Ca^{2+} dependent and results from the R-induced activation of a Ca^{2+}-sensitive protein kinase, helping to confirm the notion that R induces increases in $[Ca^{2+}]_i$. Because R and the release of Ca^{2+} from caged Ca^{2+}, or by mobilization triggered by released IP_3, all increase swelling of the protoplasts (Shacklock et al., 1992), the change in phosphorylation of this 70-kD protein and perhaps the 60-kD protein is well placed to be involved in this physiological event.

We have used very short labeling periods to detect these phosphorylation changes. The 70-kD peptide is clearly a rapidly labeled protein, and saturation of its phosphorylation within 10 min (Fig. 2) suggests a metabolic difference from other phosphorylated peptides in the protoplasts. Previously, we have observed a similar variation in the kinetics of labeling of different proteins (Blowers and Trewavas, 1987). When purified pea plasma membrane was incubated in vitro with [^{32}P]ATP, we observed one protein (in this example with a molecular mass of 18 kD) to complete its phosphorylation within 15 s, whereas other proteins required 5 to 10 min before phosphorylation was apparently completed. We were able to demonstrate subsequently (Blowers and Trewavas, 1989) that this very rapid phosphorylation was the result of autophosphorylation of a protein kinase. The 70- and the 60-kD peptides may simply be protein kinases that autophosphorylate in a Ca^{2+}-dependent manner, a possibility we are testing at present (Grimm et al., 1989).

We have detected slight differences in the phosphorylation patterns induced by release of Ca^{2+} from caged Ca^{2+} or by mobilization of Ca^{2+} by release of IP_3 (Fig. 3). Both 60- and 70-kD proteins increase their phosphorylation with caged Ca^{2+} photolysis, whereas only the 70-kD protein is increased by IP_3-mobilized Ca^{2+}. We suggest that this difference can be explained as resulting from different cellular locales of the two proteins. The 70-kD protein should be at or near an IP_3-mobilizable source of Ca^{2+} (such as the vacuole or rough ER), whereas the 60-kD protein could be elsewhere in the cell.

We detected variation in the labeling intensity of the 60-kD protein (Figs. 3 and 4). We have noticed that the phosphorylation of the 60-kD protein is increased in protoplasts from older leaves. One additional reason for the variation in the labeling of this protein might simply result from different preparations of protoplasts containing differing proportions of protoplasts from cells of different ages in the leaf. The R effects on the phosphorylation of the 70-kD protein seem largely independent of age, being detectable in leaves anywhere from 8 to 12 d old. Much higher protoplast yields can be obtained from older leaves.

Otto and Schäfer (1988) and Park and Chae (1989) have reported previously that R irradiation of tissues or protoplasts alters protein phosphorylation. Otto and Schäfer (1988) carried out measurements in *Avena* coleoptiles and reported rapid changes in the phosphorylation of three proteins, two of which decreased their phosphorylation. Park and Chae (1989), using etiolated oat protoplasts, detected rather small changes in the phosphorylation of two proteins. Both of these groups used lengthy tissue incubation in ^{32}Pi (2–5 h) and thus are looking at proteins that are different from ours.

After 3 h of labeling of wheat leaf protoplasts with ^{32}Pi, we were unable to detect the 70-kD protein at all by SDS-PAGE and autoradiography because the labeling of other proteins is so much more intense. We have carried out many experiments in which wheat leaf protoplasts were labeled for 2 to 5 h and then irradiated with R. In marked contrast with the claims of Park and Chae (1989), we were unable to detect changes in the phosphorylation of any cellular proteins using short- or long-term R irradiations. Neither could we repeat their observations on the effects of added EGTA. We further fractionated these labeled R-irradiated protoplasts and separated the labeled proteins by SDS-PAGE, but again, we could detect no significant changes in phosphorylation. At the present time we have detected only very rapid transient changes in phosphorylation, which are in agreement with our measurements of $[Ca^{2+}]_i$ (Fig. 1).

The data described in this paper indicate that R irradiation of etiolated wheat leaf protoplasts increases $[Ca^{2+}]_i$ and that there is an associated alteration in phosphorylation that in part results from the change in Ca^{2+} levels. These data are thus consistent with our previously published investigations (Shacklock et al., 1992), and the hypothesis proposed by Roux and collaborators concerning the mechanism of action of phytochrome or R.

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