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Red Light Stimulates an Increase in Intracellular Calcium in the Spores of *Onoclea sensibilis*[1]

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ABSTRACT

Red light (R) stimulates an increase in the total concentration of intracellular calcium in the spores of *Onoclea sensibilis* L. as determined by atomic absorption spectroscopy. Subsequent exposure to far-red light inhibits the R-induced increase in intracellular calcium. The majority of the increase occurs 5 minutes after the onset of irradiation. The calcium antagonist, La^{3+}, inhibits both germination and the R-induced increase in intracellular calcium. The R-induced increase in calcium is sufficient to account for an increase in the concentration of intracellular calcium ions from 0.1 micromolar to 1 to 10 micromolar. Large detectable changes in other elements tested are not required for germination.

Calcium ions function as a second messenger in the coupling of the stimulus to the response in a wide variety of animal cells (3). The primary stimulus interacts with a membrane system and induces a transient increase in the intracellular concentration of Ca ions from 0.1 μM to 1 to 10 μM. In plants, Ca is required for the signal transduction chain in Pfr-stimulated responses (28, 29, 31) and Pfr mediates R^2-induced transplasmalemmal Ca fluxes (6, 11, 23). Haupt and Weisenseel (12) have proposed that Pfr triggers diverse responses by inducing an increase in the intracellular Ca ion concentration.

Micromolar concentrations of external Ca are required for phytochrome-mediated germination of the spores of *Onoclea sensibilis*. The Ca chelator, EGTA, and the Ca antagonists, La^{3+} and Co^{2+}, inhibit germination while the Ca ionophore A 23187 stimulates germination in the dark (29). Here we show that R stimulates a net increase in total intracellular Ca to an extent that is compatible with a transient increase in free intracellular Ca^{2+} from 0.1 μM to 1 to 10 μM. We suggest that Ca ions act as a second messenger in phytochrome-mediated germination of the spores of *Onoclea*.

MATERIALS AND METHODS

Plant Material and Culture. Mature sporophylls of *Onoclea sensibilis* L. were collected in Amherst and Pelham, MA, in January 1981 and February 1982 and stored in plastic bags in the freezer at −15°C. Prior to an experiment, sporangia were wetted with a 0.1% solution of Aerosol O.T. (Fisher Scientific Co.) and sterilized with 1 L of a 20% (v/v) solution of commercial bleach (5.25% NaOCl), and rinsed with 500 ml of sterile water (26). Unless otherwise indicated, 50 mg of sterile spores were sown on 25 ml of 5 mM EGTA (ethyleneglycol-bis(β-amino-ethyl ether)-*N,N,N',N'*-tetraacetic acid, titrated to pH 7.0 with KOH except in the experiment reported in Table V where NaOH was used) in sterile polystyrene Petri dishes, 100 mm in diameter and 20 mm high (Falcon, Oxnard, CA) for 24 h at 23°C. One h prior to the light treatment 100 μmol of $Ca(NO_3)_2$ were added. Lanthanum chloride (300 μmol) was added 5 min prior to the light treatment where indicated. Deionized H_2O (less than 2.5 nM total Ca) was used to mix solutions. Spores were irradiated for 1 to 5 min with R (energy fluence rate = 2.4 J m^{-2} s^{-1}) and/or 1 min FR (energy fluence rate = 430 J m^{-2} s^{-1}); see Reference 29 for a description of the light sources.

Experimental Procedure and Elemental Analysis. Immediately before or after the light treatments, spores were collected by millipore filtration (5.0 μm pore size; Millipore Corporation) washed for about 1 min with 200 ml of 100 mM EGTA, pH 7.0, then rinsed with 100 ml deionized H_2O. This treatment removes 80% of the total cell calcium (data not shown). Spores were then dried at 105°C for 2 h and weighed immediately. For the determination of Ca, Mg, K, Ca, Fe, Cu, Mn, Ni, Zn, samples were dissolved in hot concentrated H_2SO_4 (1 ml) plus 10 drops of concentrated HNO_3, brought up to a suitable volume (25–50 ml) depending on weight (usually 10 mg), and then analyzed for metals using an Atomic Absorption Spectrophotometer (model 403, Perkin-Elmer). Carbon, H, and N analyses were performed on a Perkin-Elmer 240 Elemental Analyzer according to the modified Pregl-Dumas technique (15). Sulfur analysis was done by classical $BaSO_4$ titration using Thorin indicator after a Schöniger oxygen flask combustion (15). Phosphorous was analyzed by formation of phosphomolybdic acid (molybdenum blue) from Pi after conversion by hot H_2SO_4 and HNO_3 digestion. Molybdenum blue was then measured with a spectrophotometer at a wavelength of 882 nm (15). Oxygen analysis was done by a modified Unterzaucher procedure (15). Chlorine was analyzed

Table I. *Effect of Washing the Spores in EGTA on Bivalent Cation Content*

Spores were either analyzed without any treatment or washed in 5.0 mM EGTA (pH 7.0) for 24 h at 23°C. Samples were then analyzed for Ca or Mg with atomic absorption spectroscopy. Data are presented as mean ± SE (n = 2).

Sample	Ca Content		
	μg/g dry wt	*nmol/mg dry wt*	*% dry spore*
Dry spores	2200 ± 400	55 ± 20	100.00
EGTA-washed spores	265 ± 170	6.6 ± 8.6	12.04
	Mg Content		
Dry spores	3350 ± 700	137.8 ± 20.4	100.00
EGTA-washed spores	2450 ± 100	100.8 ± 4.1	73.15

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[2] Abbreviations: R, red light; FR, far-red light; AAS, atomic absorption spectroscopy.

Table II. *Effect of R on Total Intracellular Ca*

Spores were sown either in the high Ca medium containing 1 mM $Ca(NO_3)_2$, 810 μM $MgSO_4$ or 3.45 mM KNO_3 (pH 5.2) or in 5 mM EGTA (pH 7.0) to which 100 μmol of $Ca(NO_3)_2$ were added 1 h prior to the irradiation. Spores were irradiated with 5 min R, washed with 200 ml of 100 mM EGTA (pH 7.0), and 100 ml deionized H_2O. Spores were then dried and analyzed for Ca by AAS.

Treatment	Total Intracellular Ca in Following Experiments								Germination
	1	2	3	4	5	6	7	Mean	
	nmol/mg dry wt								%
Spores in high CA medium									
Dark	21.1	21.8	19.5					20.8	0.8 ± 1.0
R	24.4	23.8	20.4					22.9	89.0 ± 2.0
Δ Ca	3.3	2.0	0.9					2.1[a]	
Spores in low Ca (Ca-EGTA) medium									
Dark	19.8	5.0	5.6	11.8	21.8	18.8	21.7	14.9	1.5 ± 1.0
R	27.5	10.0	10.8	16.8	28.8	22.5	28.4	20.7	71.0 ± 2.0
Δ Ca	7.7	5.0	5.2	5.0	7.0	3.7	6.7	5.8[b]	

[a] $P < 0.2$ using a two-tailed *t* test for paired samples (21).
[b] $P < 0.001$ using a two-tailed *t* test for paired samples (21).

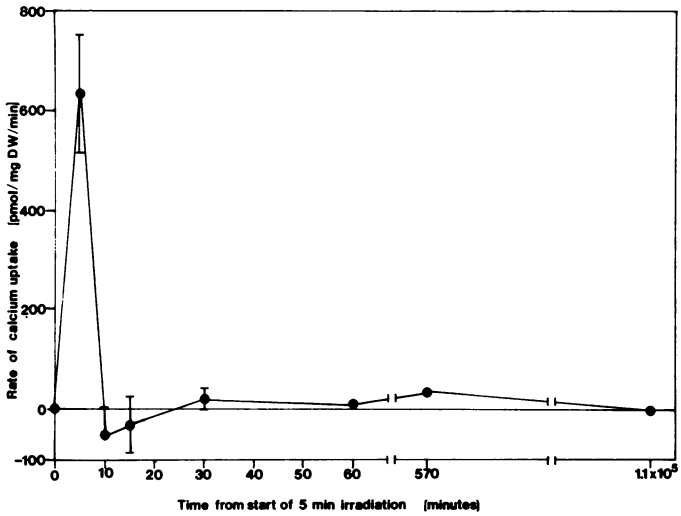

FIG. 1. The change in the rate of net Ca uptake with respect to time following R irradiation. Spores were sown in 5 mM K-EGTA to which 100 μmol of $Ca(NO_3)_2$ were added 1 h prior to irradiation. Spores were then collected at times indicated following R irradiation and analyzed for Ca by AAS. Rates were calculated by dividing the difference of the Ca contents of consecutive samples by the time interval between them. Error bars represent two SE.

Table III. *Effect of R and FR on Total Intracellular Ca*

Spores were sown in 5 mM EGTA (pH 7.0) to which 100 μmol of $Ca(NO_3)_2$ were added 1 h prior to irradiation. Spores were irradiated with 1 min R and/or FR, washed with 200 ml of 100 mM EGTA (pH 7.0), and 100 ml deionized H_2O. Spores were then dried and analyzed for Ca by AAS. Data are presented as mean ± 2 SE ($n = 2$).

Treatment	Total Intracellular Ca	Germination
	nmol/mg dry wt	%
Dark	12.6 ± 1.8	1
FR	11.7 ± 0.2	3
R	17.1 ± 0.8	60
R-FR	13.5 ± 1.0	18

Table IV. *Effect of La^{3+} on Total Intracellular Ca*

Spores were sown in 5 mM EGTA (pH 7.0) to which 100 μmol of $Ca(NO_3)_2$ were added 1 h prior to irradiation. Three hundred μmol of $LaCl_3$ were added 5 min prior to the light treatment. Spores were irradiated with 5 min R, then washed with 200 ml of 100 mM EGTA (pH 7.0), and 100 ml deionized H_2O. Spores were then dried and analyzed for Ca by AAS. Data are presented as mean ± 2 SE ($n = 2$).

Treatment	Total Intracellular Ca	Germination
	nmol/mg dry wt	%
Dark		
0 μmol $LaCl_3$	21.1 ± 1.3	0.0 ± 0.0
300 μmol $LaCl_3$	23.5 ± 0.4	0.0 ± 0.0
R		
0 μmol $LaCl_3$	28.5 ± 0.1	93.0 ± 6.0
300 μmol $LaCl_3$	21.3 ± 0.1	0.0 ± 0.0

as Cl^- after a Schöniger oxygen flask combustion followed by a coulometric titration with silver (15).

Per cent germination was determined 48 h after irradiation by the acetocarmine-chloral hydrate method (8). Data are expressed as the mean ± two SE. All experiments included two replicates and were repeated at least twice with similar results. Results from independent experiments are not grouped together except for the experiment reported in Table II, because the dark Ca content in spores varies drastically from batch to batch. This variation is shown in Table II. This variation can be accounted for by the variation found in dry spores collected from natural populations (30).

RESULTS AND DISCUSSION

Ca accounts for an average of 0.22% of the dry weight of the unhydrated spore (Table I). This can vary from 0.1 to 0.4% depending on the batch of spores (30). Approximately 90% of the total Ca can be removed by soaking the spores in the Ca chelator EGTA. We assume that, in agreement with Ca distributions in cells of other plants (14), the EGTA-accessible Ca is external to the plasmalemma and that the remaining is intracellular. Furthermore, the amount of Ca remaining after the EGTA/H_2O wash is approximately the same as is found in animal cells stripped of their glycocalyx (2). By contrast, only 26.85% of the

Table V. *The Effect of R on Atomic Content of Onoclea Spores*

Spores (350 mg) were sown in 7 Petri dishes 5 mM Na-EGTA (pH 7.0) to which 100 μmol of $Ca(NO_3)_2$ were added to each dish 1 h prior to irradiation. Spores were irradiated with 5 min R, then washed with 1000 ml of 100 mM Na-EGTA (pH 7.0), and 500 ml deionized H_2O. Spores were then dried and subjected to elemental analysis. Data are presented as mean ± 2 SE; $n = 2$ except in the case of P, S, Mg, Ca, and Fe where $n = 3$, 3, 6, 7, and 8, respectively.

Element	Atomic Content		$\%\Delta\left[\frac{(R - dark)}{dark} \times 100\%\right]$
	Dark	R	
	nmol/mg dry wt		
C	48397.3 ± 1765.0	49071.7 ± 1032.4	1.4
O	15000.6 ± 125.0	13906.8 ± 1437.5	−7.3
H	81555.7 ± 3373.4	81605.3 ± 1488.2	0.1
N	3666.1 ± 221.4	3748.3 ± 271.3	2.2
P	259.9 ± 3.2	263.1 ± 3.2	1.2
S	152.9 ± 9.5	154.6 ± 9.7	1.1
K	131.7 ± 12.8	139.4 ± 2.6	5.8
Mg	100.8 ± 4.2	102.9 ± 0.0	2.1
Cl	28.2 ± 0.0	28.2 ± 0.0	0.0
Ca	13.8 ± 1.4	19.4 ± 2.5	40.6
Fe	7.09 ± 1.63	6.54 ± 2.33	−7.8
Cu	1.42 ± 1.26	1.26 ± 0.32	−11.2
Mn	0.55 ± 0.00	0.54 ± 0.36	−1.8
Zn	0.51 ± 0.26	0.51 ± 0.20	0.0
Ni	<0.51 ± 0.00	<0.51 ± 0.00	0.0
Co	<0.34 ± 0.00	<0.34 ± 0.00	0.0
% Germination	0.0 ± 0.0	95.0 ± 2.0	

total Mg content is accessible to an EGTA/H_2O wash (Table I).

R stimulates an increase in total intracellular Ca (Table II). The magnitude of the increase is dependent on the composition of the medium. In media containing 1 mM Ca $(NO_3)_2$, 0.81 mM $MgSO_4$, and 3.45 mM KNO_3 (pH 5.2, free $Ca^{2+} \sim 1$ mM), spores take up an average of 2.1 nmol Ca/mg dry weight. In media containing 5.0 mM K^+-EGTA (pH 7.0) plus 100 μmol of $Ca(NO_3)_2$ (free $Ca^{2+} \sim 3$ μM), the spores take up an average of 5.6 nmol calcium/mg dry weight.

The greater uptake of Ca from external solutions of low Ca concentration at first seems perplexing but similar results are seen in *Pelvetia* eggs (22) and soybean roots (9). These results may be explained by a general increase in membrane permeability that occurs in the presence of low external Ca (16, 19) which could cause more Ca to be transported down its electrochemical gradient in response to R. Alternatively, there may be an unmasking of latent transport sites, or a change in specificity of existing sites so that Ca can enter instead of or in addition to other ions (22). Any of these hypotheses would explain an increase in the observed membrane permeability to Ca in the presence of low external Ca (16). The difference in uptake between spores in the two media may also be due to the competitive inhibition of Ca uptake by Mg ions (10) and/or by H^+ acting through a pH -dependent inhibition of the uptake mechanism (2). The latter explanation is likely given the large difference in pH between the two treatments.

A comparison of the average value of Ca content in dark spores in the presence of Ca (Table II) with the value of the Ca content in EGTA-washed spores (Table I) indicates that there is a dark uptake of Ca without a subsequent stimulation of germination. The average rate of dark net Ca uptake is 168 pmol/mg dry weight·min; whereas the average R-stimulated net Ca uptake is 930 pmol/mg dry weight·min. The increased rate of uptake may momentarily overcome the mechanisms that maintain Ca homeostasis and thus cause an increase in the concentration of intracellular Ca ions (2, 3). R causes a greater than 5-fold increase in the rate of Ca uptake.

The large increase in the rate of net Ca uptake stimulated by R occurs during the first 5 min following the onset of irradiation (Fig. 1). A transient, small, net Ca efflux follows which is not statistically significant, but may be real, resulting from an overshoot by the plasmalemmal Ca pumps. A small, sustained, net uptake of Ca then continues for the next 9 h and finally falls to zero. Ca is taken up over this time, however, since the gametophytes are assimilating carbon and the Ca content is expressed relative to the dry weight. Ca is needed later for rhizoid growth (18) and protonema development (4). The R-stimulated increase in total intracellular Ca can be reversed by a subsequent irradiation with FR indicating that the increase in intracellular Ca is mediated through phytochrome (Table III). Phytochrome also mediates fluxes of Ca in *Mougeotia* (6), corn microsomes (5), oat coleoptile cells (11), and isolated mitochondria (24).

Lanthanum inhibits R-stimulated germination; when given prior to the R irradiation, the inhibition is complete but becomes less effective when given during the irradiation and ineffective when given at the end of a 5 min R irradiation (29). We assume that La^{3+} inhibits germination by preventing a Ca influx since it blocks Ca influx in animal (10, 27) and *Nitella* (31) cells. Here we show (Table IV) that La^{3+} inhibits the R-stimulated increase in total intracellular calcium on *Onoclea* spores. The period of time when spores lose their sensitivity to La^{3+} occurs during the time of maximal R-stimulated calcium uptake.

Onoclea spores germinate maximally in a medium that contains only $Ca(NO_3)_2$ and either K-EGTA or Na-EGTA. Under these conditions, Ca is the only element to appreciably change its concentration after a 5 min irradiation with R (Table V). However, the procedure used optimizes the chance of seeing R-induced changes in Ca at the expense of detecting changes in other elements. When K is present in the medium, we only see an 8.6% increase in K (data not shown) whereas the Ca change is 40%.

The average increase of Ca in response to 5 min R in the high Ca and in the low Ca medium corresponds to an increase of 465 and 1288 μM total Ca, respectively (density of spores equals 222

mg/ml). A similar increase in total Ca occurs in smooth muscle in response to various stimuli that induce contraction (27) and is close to the increase in total Ca (~450 μM) that occurs in sea urchin eggs immediately following fertilization (1). Why is a large increase in total intracellular Ca needed for only a small rise in free intracellular Ca? Significant amounts of Ca are sequestered into nonionic stores like the mitochondria (2, 13, 17) and the ER (2, 17) or bound to natural ligands (*e.g.* phospholipids, inorganic phosphates, etc.; 3, 7). Further, sufficient Ca must be bound to regulatory proteins such as calmodulin in order for them to be activated. All these exchangable sites must be filled before the intracellular free concentration of Ca can rise from 0.1 to 1 to 10 μM (20, 21).

We emphasize that R stimulates an increase in total intracellular Ca which is sufficient to account for an increase in intracellular free Ca from 0.1 to 1 to 10 μM; we have not demonstrated, however, an increase in intracellular free Ca *per se*. We are presently unable to measure changes in free Ca using fluorescent indicators due to spore autofluorescence. Detection of free Ca changes with absorption dyes or photoproteins will have to await our gaining further experience in microinjection and low level quantitative light detection.

Our previous results indicate that external Ca is required for R-activated germination (29). Here we present direct evidence that phytochrome mediates an increase in total intracellular Ca. The inward flux of Ca that gives rise to the observed increase strongly suggests that, at least transiently, the intracellular Ca ion concentration has also risen and that this rise activates processes essential for germination.

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