Light-Induced Ca^{2+} Influx into Spinach Protoplasts

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Protoplasts from spinach leaves exhibit a light-induced Ca^{2+} influx. The half maximum rate of Ca^{2+} influx is achieved at ~ 5 Wm^{-2}. The action spectrum of this influx is similar to that of photosynthesis. Furthermore, light-induced Ca^{2+} influx is abolished by DCMU ($\geqslant 0.5$ μM) and is sensitive to the uncoupler FCCP. Vanadate up to 3 μM enhances light-induced Ca^{2+} influx. These data indicate that photosynthetic electron transport is involved in light-induced Ca^{2+} influx into spinach protoplasts.

Introduction

In higher plants Ca^{2+} acts as an effector for a variety of physiological processes (for reviews see [1–3]). It may influence cellular processes either directly or, for *e.g.*, through the Ca^{2+}-calmodulin regulatory system [1–5]. As in animal cells, the cytosolic Ca^{2+}-concentration in plant cells is maintained at a low level (10^{-6}–10^{-8} M) against high concentrations of total Ca^{2+} both outside the cell and inside certain cell organelles. It is widely held that Ca^{2+}-sequestration by mitochondria, endoplasmic reticulum, and vacuole as well as extrusion of Ca^{2+} by the plasmamembrane Ca^{2+}-ATPase are the principal mechanisms through which the cytosolic Ca^{2+} concentration in plants is kept at its low level [2]. It has recently become evident, however, that isolated chloroplasts from wheat and spinach leaves accumulate significant amounts of Ca^{2+} from the external medium when illuminated [6, 7]. It thus appears possible that the chloroplast is also involved in the regulation of cytosolic Ca^{2+} levels in the light. In this study we demonstrate a light-induced Ca^{2+} influx into protoplasts from spinach leaves and characterize this process to be linked to photosynthetic electron transport.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; BSA, bovine serum albumine; PIPES, piperazine-N,N′-bis-(2-ethanesulfonic acid); EGTA, ethylene-glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; MES, 2-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ΔpH, transmembrane pH gradient; ΔΨ, transmembrane electrical potential difference.

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Materials and Methods

Chemicals

Arsenazo III, BSA, PIPES, were obtained from Sigma; DCMU, EGTA, FCCP, HEPES, MES, ruthenium red from Serva; ethanol absolute suprapur from Riedel de Haen; vanadate from Aldrich Chemicals Company, Inc.; cellulase "Onuzuka" R-10 from Yakult Biochemicals Co., Ltd.; macerozyme R-10 from Pharmaceutical Industry Co., Ltd.; and Chelex-100 was from BioRad. All other chemicals were from Merck and of analytical grade.

Plant material and protoplasts preparation

Spinach was grown as in [8]. Protoplasts were isolated by a modification of the procedure described in [9]. 6 g tissue of 5–10 weeks old leaves were cut with a razor blade in $\sim$ 1.0 mm thick segments and placed in 40 ml enzyme solution containing 0.5 M sorbitol, 0.5 mM $CaCl_2$, 3% cellulase, 0.5% macerozyme, 0.5% BSA, 5 mM MES-NaOH, pH 5.5. The leaf segments were incubated under illumination for 3 h. The protoplasts were harvested by centrifugation for 5 min at $100 \times g$. The supernatant was discarded and the pellet was resuspended in 0.5 M sorbitol, 0.5 mM $CaCl_2$, 5 mM PIPES-NaOH, pH 6.2. All purification steps were carried out as in [9] except that the gradient media contained 0.5 mM $CaCl_2$, 5 mM PIPES-NaOH, pH 6.2, and centrifugation was carried out at $250 \times g$ for 5 min. The purified protoplasts were collected from the gradient and centrifuged again at $100 \times g$ for 5 min. The pellet was resuspended in a chelex-100 treated medium containing 0.5 M sorbitol, 5 mM PIPES-NaOH, pH 6.2, 0.5 mM $CaCl_2$ (the latter added after chelex-100 treatment).

The intactness of the protoplasts was evaluated by light microscopy. The number of intact, broken protoplasts and free chloroplasts was determined in a counting chamber (0.1 mm depth). 125 chloroplasts were assumed as an average chloroplast number per protoplast [10]. The average intactness determined by this method was 90%. After 3 h of storage at 4 °C intactness was still 78% (n = 6). In addition the integrity of the plasmamembrane was evaluated by exclusion of Evan's blue [11]. Photosynthetic activity was determined by CO_2-dependent oxygen evolution as in [9] except that the assay medium contained 50 mM HEPES-Tris, pH 7.0, 400 mM sorbitol, 5 mM $NaHCO_3$, 20 mM KCl, 50 μM $CaCl_2$, 10 μg chlorophyll. The assay conditions were 25 °C, preincubation in the dark for 2–4 min, unless otherwise stated, followed by illumination (120 Wm^{-2}). The average rate of CO_2-dependent oxygen evolution was 100 $\mu mol \cdot mg \cdot Chl^{-1} \cdot h^{-1}$. Chlorophyll was determined as in [12].

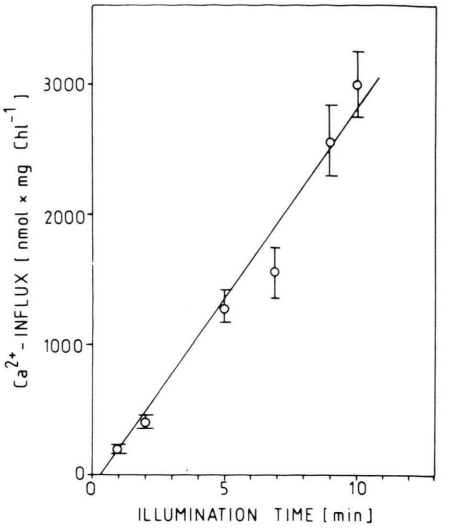

Fig. 1. Time course of light-induced Ca^{2+} influx into intact spinach protoplasts. Assay conditions compare "measurement of Ca^{2+} influx". Results are expressed as mean ± standard error. n = 10 different protoplast preparations.

Measurement of Ca^{2+} influx

Ca^{2+} influx was measured by following changes in the free Ca^{2+} in the medium, using the metallochromic indicator arsenazo III according to [7]. The standard assay medium, however, contained 400 mM sorbitol, 50 mM HEPES-Tris (pH 7.0), 20 mM KCl, 10 μM arsenazo III, 5 mM $NaHCO_3$, 10 μM ruthenium red, 20 μM $CaCl_2$ and 4 μg chlorophyll. The standard conditions were 25 °C, 5 min preincubation in the dark, unless otherwise stated, with the different additions, then 5 min illumination with 25 Wm^{-2} in a stirred and cooled cuvette. Ruthenium red was added to exclude Ca^{2+} influx by contaminating chloroplasts [7] and mitochondria [13]. The used concentrations of ruthenium red, $CaCl_2$, EGTA and arsenazo III did not affect photosynthetic oxygen evolution.

Results

Intact spinach protoplasts exhibit a light-induced Ca^{2+} influx. The observed Ca^{2+} influx is linearly dependent on illumination time (Fig. 1). Saturation of uptake, however, is not observed within 10 min of illumination. The total amount of Ca^{2+} uptake by the protoplasts after 10 min of illumination is 3 $\mu mol \cdot mg \cdot Chl^{-1}$ (n = 10 different preparations) and corresponds to 60% of the total available Ca^{2+} in the used assay medium. Under our conditions no measurable Ca^{2+} influx is observed with dark kept protoplasts. Ca^{2+} influx into spinach protoplasts is already induced by low energy fluence rates (Fig. 2).

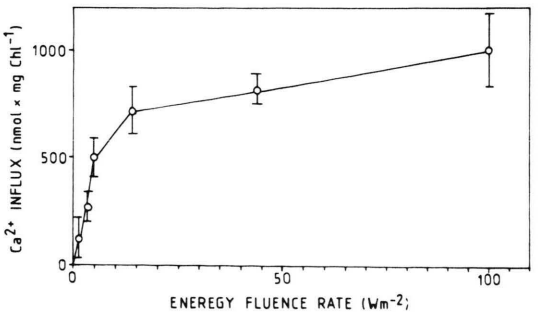

Fig. 2. Influx of Ca^{2+} into intact spinach protoplasts as a function of energy fluence rate. Assay conditions compare "measurement of Ca^{2+} influx". The experiments were carried out under safe-green light. Different energy fluence rates were obtained using neutral density filters (Schott & Gen., Mainz, FRG). Results are expressed as mean ± standard error. n = 4 different protoplast preparations.

Half maximum Ca^{2+} influx is achieved at ~ 5 Wm^{-2}. The action spectrum of light-induced Ca^{2+} influx into spinach protoplasts exhibits enhanced influx in the red region and in the blue region of the spectrum (Fig. 3).

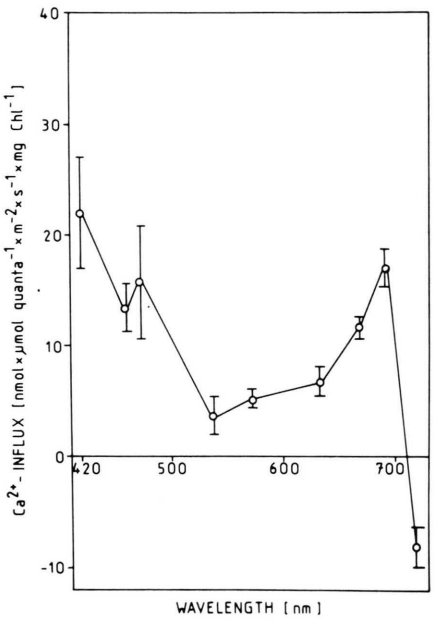

Fig. 3. Action spectrum of Ca^{2+} influx into intact spinach protoplasts. Assay conditions compare "measurement of Ca^{2+} influx". The various wavelengths were obtained using interference filters (Schott & Gen.; type AL). Results are expressed as mean ± standard error. n = 4 different protoplast preparations.

Both light-induced Ca^{2+} influx and CO_2-dependent oxygen evolution are abolished by DCMU-concentrations ≥ 0.5 µM (Fig. 4). Low concentrations of FCCP (0.5 µM), which are sufficient to uncouple electron transport, stimulate light-induced Ca^{2+} influx into spinach protoplasts, whereas higher concentrations of FCCP, known to inhibit photosynthetic electron transport [14], suppress light-induced Ca^{2+} influx (Fig. 5). Vanadate up to 3 µM enhances light-induced Ca^{2+} influx into spinach protoplasts (Fig. 6). Concentrations greater than 6 µM vanadate cause inhibition of Ca^{2+} influx. In contrast CO_2-dependent oxygen evolution is already suppressed by low vanadate concentrations.

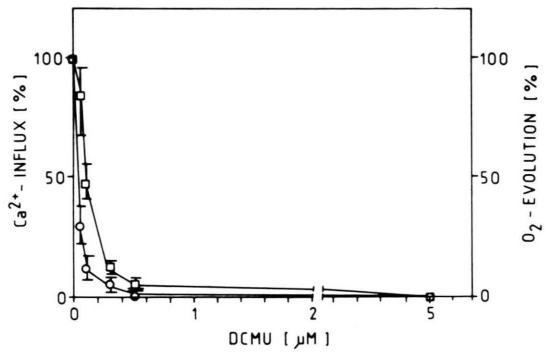

Fig. 4. Inhibition of light-induced Ca^{2+} influx into intact spinach protoplasts by DCMU. Assay conditions compare "measurement of Ca^{2+} influx". DCMU was dissolved in ethanol absolute suprapure. Ca^{2+} influx (○), results are expressed as mean ± standard error. n = 5 different protoplast preparations; CO_2-dependent oxygen evolution (□), assay conditions compare "plant material and protoplasts preparation" except that preincubation in the dark was 5 min, results are expressed as mean ± deviation. n = 3 different protoplast preparations.

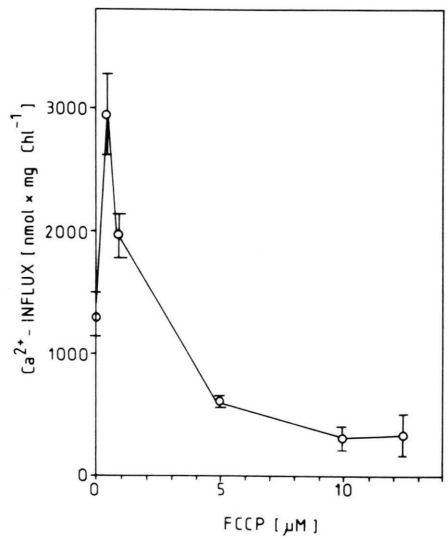

Fig. 5. Effect of FCCP on light-induced Ca^{2+} influx into intact spinach protoplasts. Assay conditions compare "measurement of Ca^{2+} influx". FCCP was dissolved in ethanol absolute suprapure. Results are expressed as ± mean standard error. n = 3 different protoplast preparations.

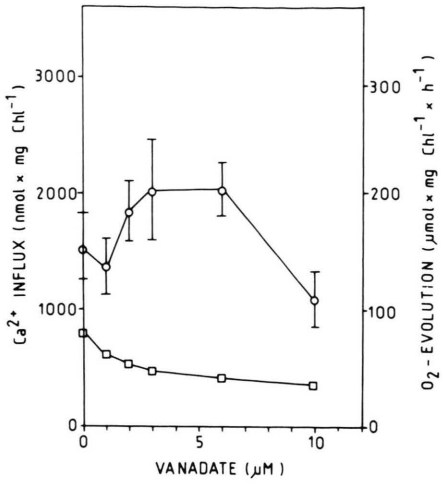

Fig. 6. Effects of vanadate on the light-induced Ca^{2+} influx into intact spinach protoplasts. Assay conditions compare "plant material and protoplasts preparation" and "measurement of Ca^{2+} influx" except that preincubation in the dark was 9 min. Ca^{2+} influx (○), results are expressed as ± mean standard error, $n = 3$ different protoplast preparations; CO_2-dependent oxygen evolution (□).

Discussion

It has previously been shown that isolated chloroplasts exhibit a light-induced Ca^{2+} influx [6, 7, 15]. The observations made in this study demonstrate that light triggers Ca^{2+} influx into spinach protoplasts as well. The inhibition of Ca^{2+} influx by DCMU and the action spectrum of Ca^{2+} influx indicate that photosynthetic electron transport, as shown for isolated spinach chloroplasts [7, 15], is involved in Ca^{2+} influx into spinach protoplasts. This conclusion is further supported by the effects of the uncoupler FCCP on light-induced Ca^{2+} influx. Similar results using FCCP and other uncoupling agents have been obtained for the Ca^{2+} uptake by isolated spinach chloroplasts [7, 15]. Since the standard assay contained 10 μM ruthenium red, a potent inhibitor of Ca^{2+} uptake by chloroplasts and mitochondria [7, 13], it is quite unlikely that the observed effects of DCMU and FCCP are due to their action on contaminating chloroplasts.

Since light-induced signal transduction from the thylakoids over the chloroplast envelope to the plasmamembrane might occur electrically [16, 17], it seems possible that the $\Delta\Psi$ at the plasmamembrane serves as the driving force for light-induced Ca^{2+} influx into spinach protoplasts. This proposal receives support from the observation that low energy fluence rates, which are sufficient to build up the proton motive force over the thylakoids and the chloroplast envelope [18, 19], induce Ca^{2+} influx, as in isolated spinach chloroplasts [15]. Light-induced Ca^{2+} influx into spinach protoplasts is probably not driven by ΔpH, since it was not inhibited by 0.5 μM of the protonophore FCCP. These data and the inhibition by DCMU provide evidence that light-induced Ca^{2+} influx into spinach protoplasts is not directly driven by ATP.

Vanadate, which inhibits ion-transport ATPases of the plasmamembrane [20–22] is not effective on redox-driven H^+-ATPases of chloroplasts and mitochondria [21, 23, 24]. However, it inhibits Ca^{2+}-ATPases, which are believed to be the major Ca^{2+} efflux system at the plasmamembrane [1, 3, 24, 25]. Our data and those previously obtained with wheat protoplasts [25] indicate that some of the entered Ca^{2+} is removed from the cytosol by the activity of a Ca^{2+} efflux pump. Nevertheless, light induces a significant net Ca^{2+} influx into spinach protoplasts. We therefore suggest that light at least transiently increases cytosolic free Ca^{2+}. Cytosolic functions, however, require the maintenance and fine regulation of low free Ca^{2+} levels [2, 3]. In this context it is often proposed that the endoplasmic reticulum, the vacuole and mitochondria are the main organelles involved in the regulation of cytosolic free Ca^{2+} [1–3]. However, about 40% of the accumulated Ca^{2+} by isolated spinach protoplasts is taken up by isolated spinach chloroplasts under similar experimental conditions [7]. We therefore suggest that chloroplasts may be important in the maintenance of low cytosolic free Ca^{2+} in the light.

Acknowledgement

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