LIGHT-INDUCED Ca^{2+} UPTAKE BY INTACT CHLOROPLASTS

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1. Introduction

When intact chloroplasts are illuminated, H^+/Mg^{2+} exchange [1,2] by the proton-pumping thylakoids causes the stroma to become more alkaline [3] and more enriched with Mg^{2+} [4]. This process is considered prerequisite to CO_2 fixation (review [5]). Associated with this internal ion redistribution is an ion exchange through the envelope: illuminated chloroplasts extrude H^+ [3,6] and take up K^+ (or Na^+ but not Mg^{2+}) though slowly [7]. This trans-envelope H^+/K^+ exchange may be required for the chloroplast stroma to maintain alkalinity and high Mg^{2+} levels in light [8,9].

We report here that illuminated intact chloroplasts take up Ca^{2+}. In wheat chloroplasts (this study) the light-driven Ca^{2+} transport is rather fast (up to 30 μmol $Ca^{2+} \cdot h^{-1} \cdot mg\ chl^{-1}$), more efficient than K^+ transport in terms of the app. K_m (180 μM for Ca^{2+}, 1.7 mM for K^+) and persists even in the presence of relatively high concentrations of K^+. Spinach chloroplasts also take up Ca^{2+} in light, suggesting the generality of this phenomenon. As some of the light-activated chloroplast enzymes have now been recognized as Ca^{2+}-modulated or Ca^{2+}-sensitive enzymes [10–13] (section 4), we suspect that the light-driven Ca^{2+} transport plays an important part in the regulation of chloroplast enzymes.

2. Materials and methods

Wheat (*Triticum aestivum* L.) was grown as in [13]. Spinach (*Spinacia oleracea* L.) was obtained from a local market. Intact chloroplasts were prepared from isolated wheat protoplasts or directly from spinach leaves as in [13]. The ferricyanide reduction test [14] indicated that the final preparations of chloroplasts were >90% intact. Chloroplasts were stored in a thick suspension (1 mg chl/ml) in 0.4 M sorbitol containing 20 mM buffer (Hepes/Tris or Hepes/bis-Tris–propane). The buffers used were from Sigma. Chlorophyll was determined according to [15].

$^{45}Ca^{2+}$ uptake was measured by a modification of the silicon-layer filtering centrifugation method in [16]. After exposure to $^{45}Ca^{2+}$ in the reaction mixture (see figure legends) the chloroplasts were quickly spun down (20 s at 10 000 rev./min) in a microfuge tube through a 100 μl layer of silicon oil (SH 550: SH 556 = 1:1, Torey Silicone Co., Tokyo) to the bottom layer of 1 M sucrose (20 μl) using a Sakuma M-160 refrigerated microfuge (Sakuma Seisakusho Co., Tokyo). The bottom layer was cut off after freezing and counted for radioactivity. $^{45}CaCl_2$ was from Radiochemical Centre, Amersham.

Ca^{2+} uptake was also measured by monitoring the changes in Ca^{2+} level in a continuously magnet-stirred suspension using a Ca^{2+}-sensitive electrode (Radiometer F2112Ca) in combination with a small homemade Ag/AgCl reference electrode. (The Ca^{2+} electrode was also used to detect Mg^{2+} changes, since the electrode responded to Mg^{2+} reasonably well in the absence of Ca^{2+}.) Changes in pH were followed simultaneously using a combination glass electrode (Fujikagaku Seisakusho Co., SE1600GC). Signals from both electrodes were amplified through a pair of pH meters (Toa Electronics Ltd., HTS-10A and Toyo Kagaku Sangyo Co., PT-60D) and recorded on a multi-channel chart recorder. K^+ changes were monitored with a monovalent cation-sensitive electrode (Beckman 39137).

In all experiments, the actinic light used was a broad-band orange light (560–700 nm, ~200 W/m^2) at 25°C. Unless otherwise indicated, the reaction pH was 7.6.

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3. Results

3.1. $^{45}Ca^{2+}$ experiments

Fig.1A presents typical data from time course experiments for $^{45}Ca^{2+}$ uptake by wheat chloroplasts. A greatly enhanced $^{45}Ca^{2+}$ incorporation occurred immediately upon illumination and continued for >5 min. In this experiment the initial slope and the maximum extent of light-induced $^{45}Ca^{2+}$ uptake corresponded to 29 μmol . h^{-1} . mg chl^{-1} and 350 nmol/mg chl, respectively. Spinach chloroplasts (fig.1B) incorporated a considerable amount of $^{45}Ca^{2+}$ in the dark (curve D) but here again illumination caused a large increase in $^{45}Ca^{2+}$ uptake (curve L). The initial rate and the maximum extent of light-dependent Ca^{2+} uptake were estimated from the L minus D curve (inset) to be 6.7 μmol . h^{-1} . mg chl^{-1} and 149 nmol/mg chl, respectively. It seems quite possible that in both wheat and spinach chloroplasts the dark $^{45}Ca^{2+}$ incorporation was due in large part to an exchange reaction with the endogenous Ca^{2+} loosely bound to the chloroplasts. Clearly, however, the light-dependent part of $^{45}Ca^{2+}$ uptake (or most of it) did represent a true uptake, as the electrode experiments showed (see below).

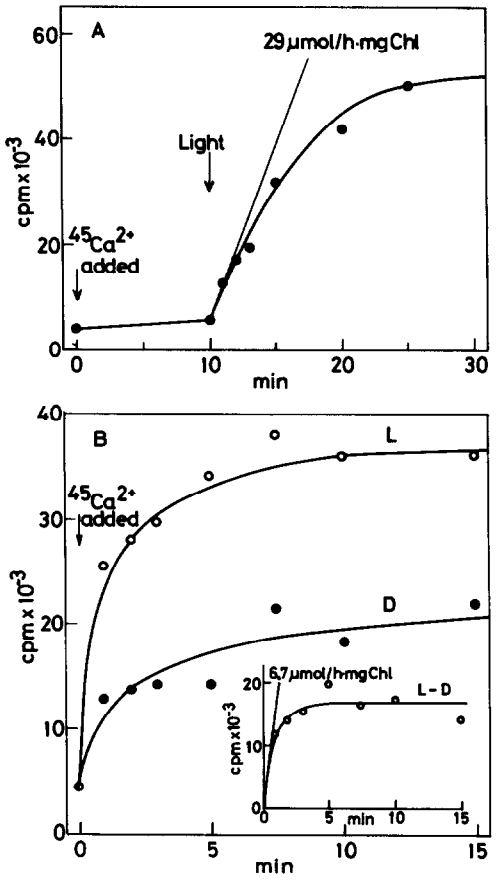

Fig.1. Light-induced $^{45}Ca^{2+}$ uptake in wheat (A) and spinach (B) chloroplasts. (A) 1.5 ml chloroplast suspension (0.33 M sorbitol, 25 mM Hepes/Tris (pH 7.6), 50 μg chl/ml) in a small test tube were incubated for 2 min in the dark before 75 μl 10 mM $^{45}CaCl_2$ (spec. act. 19 mCi/mmol, final conc. 476 μM) were added at t = 0. At each of the indicated times, a 100 μl sample was taken from the mixture and processed as in section 2. (B) Conditions as in (A) except that spinach chloroplasts were used and that in 'L', illumination started 5 s before t = 0, when $^{45}CaCl_2$ was added. 'D' represents a dark control.

Shown in fig.2–4 are some of the characteristics of light-induced Ca^{2+} transport in wheat chloroplasts. In these experiments, amounts (in cpm) of $^{45}Ca^{2+}$ take up in the initial 1 min of illumination were taken as relative rates of Ca^{2+} uptake. Ca^{2+} uptake thus measured exhibited an optimum between pH 7.6–8 (slightly dependent on the buffer used) as most chloroplast reactions did (fig.2). At pH 7.6, the app. K_m

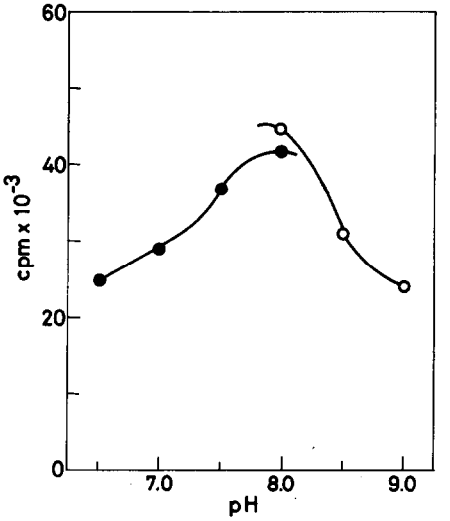

Fig.2. Effect of pH on light-induced $^{45}Ca^{2+}$ uptake in wheat chloroplasts. To 100 μl chloroplast suspension in the buffer containing 0.33 M sorbitol (50 μg chl/ml) which had been layered upon silicon oil in a microfuge tube, were added 5 μl $^{45}CaCl_2$ (10 mM; spec. act. 19 mCi/mmol). The buffers used were Hepes/bis-Tris–propane (20 mM) for pH 6.5–8 and TAPS/lysine (20 mM) for pH 8–9. After 1 min incubation under illumination or in the dark, the chloroplasts were spun down for radioactivity assay (section 2). The radioactivities shown are those corrected for dark $^{45}Ca^{2+}$ incorporation. The highest activity obtained corresponds to 340 μmol Ca^{2+} . h^{-1} . mg chl^{-1}.

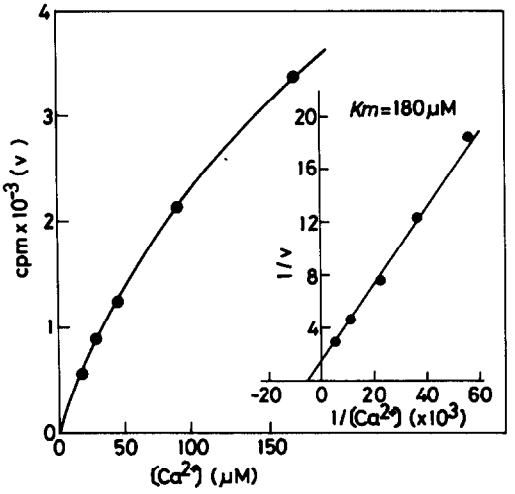

Fig.3. Effect of [Ca^{2+}] on light-induced Ca^{2+} ($^{45}Ca^{2+}$) uptake in wheat chloroplasts. Conditions and procedures used were as in fig.2 except for the use of 20 mM Hepes/bis-Tris–propane (pH 7.6) containing 0.33 M sorbitol, and the additions of various concentrations of $^{45}CaCl_2$ to chloroplast suspension.

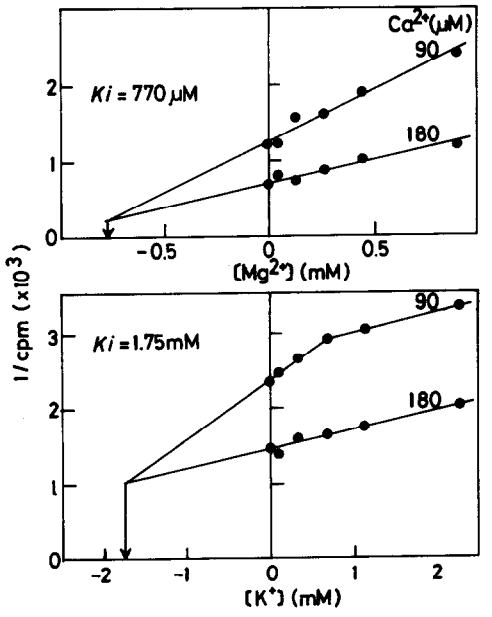

Fig.4. Effect of KCl and $MgCl_2$ on light-induced Ca^{2+} uptake in wheat chloroplasts. Conditions and procedures used were as in fig.2 except for the inclusion of KCl and $MgCl_2$ in the reaction medium and the use of 90 or 180 μM $^{45}CaCl_2$ (spec. act. 19 mCi/mmol). In the Dixon plots shown, the vertical arrows indicate K_i values.

for Ca^{2+} was 180 μM (fig.3) and the rate-saturating concentration ≧500 μM (not shown). With 500 μM Ca^{2+} in the medium, the coexistence of relatively high levels of other salts such as KCl (10 mM), NaCl (10 mM) or $MgCl_2$ (1 mM) only partially inhibited Ca^{2+} uptake (50–60%, not shown). Analysis of the effects of KCl and $MgCl_2$ at two different rate-limiting concentrations of $CaCl_2$ (90 and 190 μM) indicated that K^+ and Mg^{2+} (<1 mM) acted as weak competitive inhibitors of Ca^{2+} uptake with relatively high K_i values of 1.75 mM and 770 μM, respectively (fig.4).

3.2. *Electrode experiments*

Fig.5 represents experiments in which ion-specific electrodes were used to monitor Ca^{2+} and H^+ changes

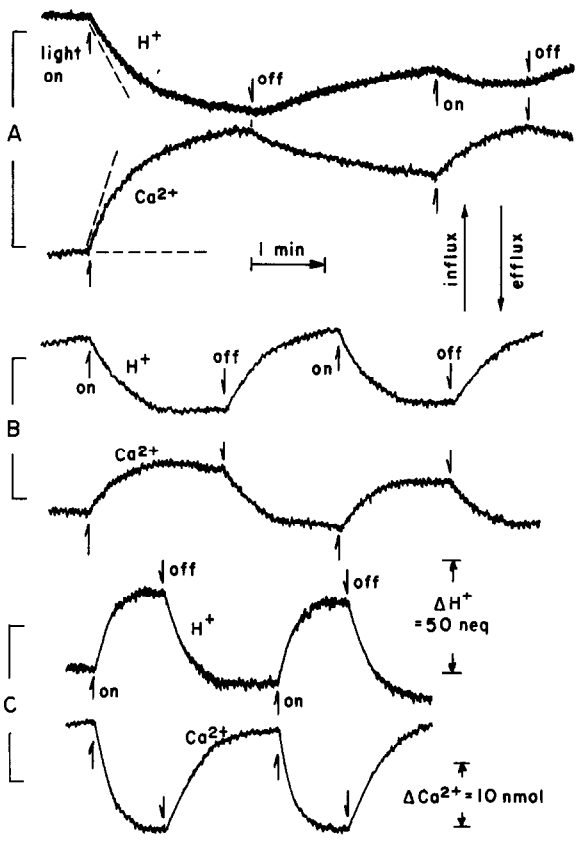

Fig.5. Light-induced Ca^{2+} and H^+ changes in suspensions of wheat chloroplasts as measured with ion-selective electrodes. (A) Reaction mixture (3 ml) contained 0.33 M sorbitol, 200 μM $CaCl_2$, 1 mM Hepes/bis-Tris–propane (pH 7.6), and chloroplasts equivalent to 40 μg chl/ml. (B) 10 mM KCl was added to the mixture. (C) Sorbitol was omitted from the reaction medium used in (A).

simultaneously in weakly buffered suspensions of wheat chloroplasts. As traces A and B show, light-induced Ca^{2+} uptake was accompanied by a release of H^+ from the chloroplasts. In A, the initial slope (21 μmol . h^{-1} . mg chl^{-1}) and the maximum extent of Ca^{2+} change (205 nmol/mg chl) observed in the first light cycle were quite close, in equivalents, to those for H^+ (38 μequiv . h^{-1} . mg chl^{-1} and 390 nequiv./mg chl). The slow dark decay kinetics and incomplete reversibility of Ca^{2+} and H^+ changes were apparently related to the low salt conditions used (200 μM $CaCl_2$ and 1 mM buffer only). When 10 mM KCl was present (traces B) the kinetics of Ca^{2+} and H^+ changes became faster and both processes became completely or almost completely reversible. However, the amplitude of Ca^{2+} changes (but not that of H^+ changes) was decreased by ~60%, thus yielding a decreased Ca^{2+}/H^+ ratio (0.5). The initial slope of Ca^{2+} influx was also decreased substantially (~50%). When intact wheat chloroplasts were ruptured osmotically in a hypotonic reaction medium, the directions of Ca^{2+} and H^+ changes were totally reversed (traces C), thus confirming that the light-induced Ca^{2+} uptake and H^+ extrusion observed were indeed manifestations of intact, enveloped chloroplasts. Ca^{2+} is known to serve as an excellent exchange cation for the proton pump of naked thylakoids [2].

The partial inhibition of Ca^{2+} uptake by KCl shown above (see also fig.4) seemed to be due to competition by K^+ transport, since illuminated wheat chloroplasts did take up K^+ at measurable rates (17 μmol . h^{-1} . mg chl^{-1}) when the reaction medium contained KCl (1 mM) instead of Ca^{2+}. As predicted by its relatively high K_m (K_i in fig.4), K^+ uptake at 1 mM K^+ was strongly inhibited by 200 μM Ca^{2+} (fig.6). No Mg^{2+} uptake was detected. In fact, wheat chloroplasts extruded Mg^{2+}, irreversibly, when illuminated in a medium in which $CaCl_2$ was replaced by $MgCl_2$. A reversible H^+ efflux was still observed (fig.6). Whether or not this Mg^{2+} efflux occurs in the presence of Ca^{2+} is not known.

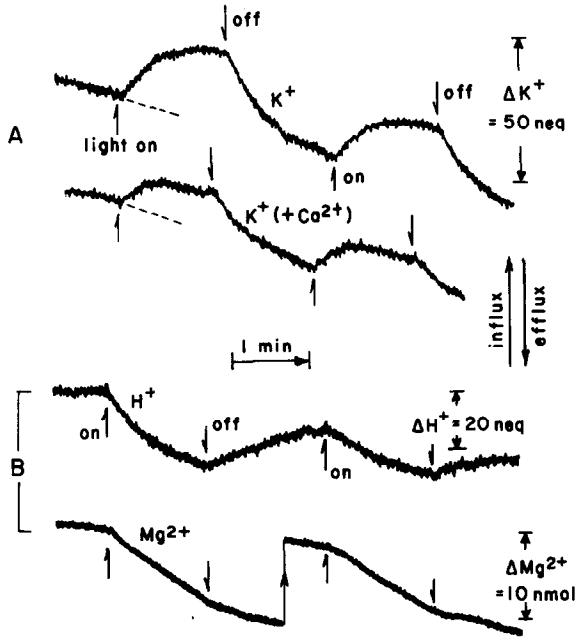

Fig.6. Light-induced K^+ and Mg^{2+} changes in suspensions of wheat chloroplasts as measured with electrodes. (A) the reaction mixture (3 ml) contained 0.33 mM sorbitol, 1 mM KCl, 8 mM Hepes/bis-Tris–propane (pH 7.6) and chloroplasts equivalent to 40 μg chl/ml. When added, $CaCl_2$ was 200 μM. (B) the mixture was as in A except that 200 μM $MgCl_2$ was substituted for KCl and the buffer was 1 mM. No Ca^{2+} was added.

4. Discussion

The possibility that the light-induced Ca^{2+} transport we observed plays a role in the regulation of chloroplast enzyme is suggested by the high Ca^{2+}-sensitivity which some of the light-regulated chloroplast enzymes exhibit in solution. For instance, chloroplast fructose bisphosphatase is activated by preincubation with Ca^{2+} and/or Mg^{2+} in the presence of fructose bisphosphate and reduced thioredoxin ($A_{0.5}$ for Ca^{2+} = 55 μM) [10,17], while the activated enzyme is inhibited by Ca^{2+} (K_i for Ca^{2+} = 7–40 μM) [10,11,17]. Sedoheptulose bisphosphatase has also been shown sensitive to Ca^{2+} [11]. Chloroplast NAD kinase represents an interesting case of Ca^{2+}-modulation in that its activator protein [18] is a Ca^{2+}-binding regulatory protein known as calmodulin ($A_{0.5}$ for Ca^{2+} = 70 μM with excess calmodulin) (submitted). Although only a small photoactivation of NAD kinase has been shown [13], its activity in vivo (NAD to NADP conversion in green cells and chloroplasts) is definitively light-dependent [13,19–21] and it is entirely possible that the function of light here includes something in addition to providing the necessary $MgATP^{2-}$ and alkalinization in the stroma required for the reaction.

In line with other evidence for low Ca^{2+} in the stroma [22,23], the enzyme data cited above suggest that stromal free Ca^{2+} is regulated within the 10^{-7}–

10^{-5} M range, just as is the cytoplasmic Ca^{2+} of most eukaryotic cells [24]. (A large amount of Ca^{2+} usually found with isolated intact chloroplasts is said to be surfacebound [23].) Note that if the number of Ca^{2+} binding sites within the chloroplast is small enough, an inflow of only 1 or 2 nmol Ca^{2+}/mg chl could raise the stromal Ca^{2+} level from 0–30 μM (assuming a stromal space of 30–50 μl/mg chl).

If indeed the light-driven Ca^{2+} transport plays an essential part in the light activation of chloroplast enzymes, then under proper experimental conditions, photosynthesis or some of its partial reactions in isolated intact chloroplasts should show a definite requirement for external Ca^{2+}. We are currently trying to demonstrate this. Inhibition of some of the key enzymes by the excess Ca^{2+} uptake in chloroplast was suggested by the experiments in [4] which showed that the addition of 1 mM Ca^{2+} to photosynthesizing spinach chloroplasts caused a partial (30%) inhibition of photosynthesis and nearly complete inhibition in the presence of the divalent ionophore A23187. They suggested fructose bisphosphatase to be the target of Ca^{2+} inhibition.

The Ca^{2+}/H^+ ratio of $\leqq 1$ (eq/eq) and the ability of K^+ to compete, if weakly, with Ca^{2+} seem consistent with Ca^{2+} influx being a counterion movement to light-induced H^+ efflux, as suggested for K^+ influx [7–9]. The mechanism of light-driven H^+ export by intact chloroplasts has been discussed [25] (see also [9]). The relatively low K_m for Ca^{2+} and the absence of light-dependent Mg^{2+} uptake may indicate a specific Ca^{2+} carrier. Qualitatively, the low K_m and the lack of severe interference by K^+ and Mg^{2+} are also necessary conditions for the Ca^{2+}-transport mechanism to be operative in the cytosol where the K^+/Ca^{2+} and Mg^{2+}/Ca^{2+} ratios are high [26].

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