Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 171-177

EVIDENCE FOR A Ca^{2+} GRADIENT ACROSS THE PLASMA MEMBRANE OF WHEAT PROTOPLASTS

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Received March 22, 1983

Fluxes of Ca^{2+} across the plasma membrane of isolated wheat protoplasts have been measured both as net accumulation and as uptake under steady-state conditions. The ATPase inhibitors, orthovanadate and diethylstibesterol, and the divalent cation ionophore, A23187, were all found to enhance net Ca^{2+} accumulation by protoplasts. The uptake of Ca^{2+} under steady-state conditions was also stimulated by A23187 but relatively unaffected by a range of plant hormones or by red or far red light. Light treatments were compared to dark controls with protoplasts isolated from etiolated wheat.

The results suggest that plant cells maintain a Ca^{2+} gradient across their plasma membrane but it appears not to be under phytochrome control.

In various animal tissues an increase in cytosolic $[Ca^{2+}]$, from 10^{-7}M to about 10^{-6}M, initiates cellular responses including muscle contraction, secretion and cell division (for reviews see 1-3). Recent evidence also suggests that changes in Ca^{2+} concentration may exert such a trigger function in plant cells. In large algae, for example, electrical excitation of the plasma membrane causes an increase in the cytosolic $[Ca^{2+}]$ over that found in resting conditions (4) and certain enzymes, such as the external NADH dehydrogenase of plant mitochondria (5) and NAD kinase (6), are activated by micromolar concentrations of free Ca^{2+}. Furthermore, plant cell microsomes possess a calmodulin activated (Ca^{2+}/Mg^{2+})-ATPase (7), an enzyme involved in Ca^{2+} extrusion from animal cells (3,8,9). It has been suggested,

0006-291X/83 $1.50

therefore, that Ca^{2+} may act as a secondary messenger for primary signals, such as those mediated by plant growth hormones or phytochrome (10-14).

The aim of the present study was, therefore, to investigate whether higher plant cells maintain a Ca^{2+} gradient across the plasma membrane and to determine whether the primary signals mentioned above affect Ca^{2+} influx into such cells.

Materials and Methods

Wheat (Triticum aestivum var.Maris Huntsman) seeds were soaked in running tap water for 24 h (day 1), planted in John Innes No.3 compost and grown in a greenhouse at 25°C. Leaf sections, 5-6 cm above soil level to 1 cm below the tip, were harvested on day 10. Protoplasts were prepared as described by Leegood & Walker (15) with the final pellet being resuspended in basal experimental medium (0.4M sorbitol; 30mM tricine-KOH, pH 7.6; 10mM $NaHCO_3$) at a concentration of 130 µg chlorophyll cm^{-3}. Incubations were carried out at 20°C under illumination similar to that used in protoplast isolation and were initiated by adding 1mM $CaCl_2$ (±5µCi cm^{-3} $^{45}Ca^{2+}$). They were terminated by centrifugating 75 mm^3 samples through 70 mm^3 of silicone oil (AR200: AR20; 5:1 (v/v)) into 20 mm^3 10% (w/v) $HClO_4$ in a Beckman microfuge B. Unless otherwise specified, 75 mm^3 of 4 mM EGTA, in basal medium, overlayed the silicone oil, in order to exclude artifacts due to surface binding of Ca^{2+}. As a relative measure of cell number in each sample, 10 µCi cm^{-3} $[^3H]_2O$ was included in all incubations (16,17). Due to sedimentation and aggregation of cells, this approach has been found to give more reproducible results (17). Following centrifugation, samples were frozen, cut through the oil layer and radioactivity counted by scintillation spectroscopy, using window settings which distinguished $^3H^+$ from $^{45}Ca^{2+}$.

For the experiments on the effects of far red light, wheat was soaked and grown in the dark for 8 days or until day 5 in the light followed by 3 days dark.

The plant hormones, indoleacetic acid, gibberelic acid (GA_3), abscissic acid and kinetin, and A23187 were purchased from Sigma (London) Chemical Co.Ltd., Poole, Dorset, U.K.. Silicone oils were obtained from Wacker-Chemie, Munich, W.Germany, and $^{45}Ca^{2+}$ and $[^3H]_2O$ from Amersham International, Amersham, U.K.. All other reagents were of the highest grade commercially available.

Results

Wheat protoplasts incubated in the light at 20°C in the presence of 1 mM Ca^{2+} demonstrate an initial increase in the net accumulation of this cation until a steady-state level is achieved, normally within 30-40 min. (Fig.1A). Both initial rates of accumulation and the final steady-state level of Ca^{2+} were enhanced by the ATPase inhibitors orthovanadate (18,19) and diethylstibesterol (19,20). Fig.1B compares the effect of these compounds to that seen for A23187, a Ca^{2+} ionophore known to equilibrate Ca^{2+} gradients across membranes (21). Addition of 2 µM A23187 caused the steady-state Ca^{2+}

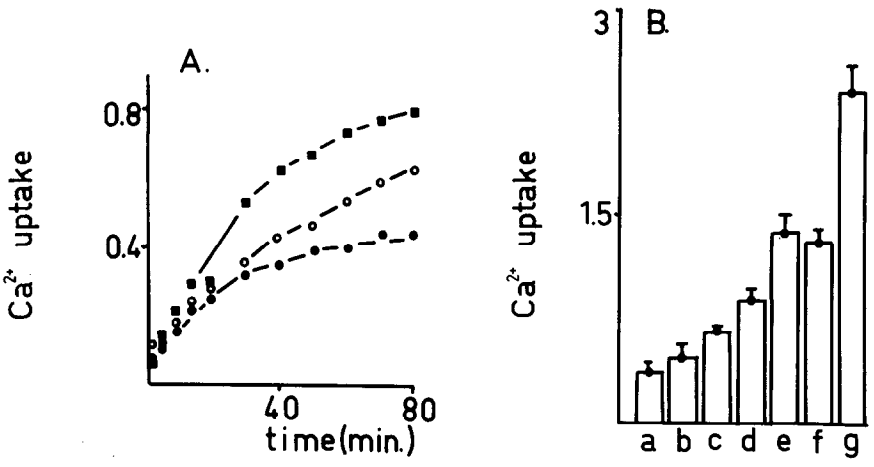

Figure 1. Effect of vanadate, diethylstibesterol and A23187 on net Ca^{2+} accumulation by wheat protoplasts

Protoplasts were suspended in the basal experimental medium, at 20°C, to a final concentration of 130 µg chlorophyll cm^{-3}.

A. Incubations were started (t=0) by the addition of 1mM $CaCl_2$, 5 µCi cm^{-3} $^{45}Ca^{2+}$ and 10 µCi cm^{-3} $[^3H]_2O$ alone (●) or plus 2mM vanadate (○) or 0.2mM diethylstibesterol (■) and samples taken at the times indicated.

B. Experimental conditions were as in A and samples were taken at t=60 min after starting the reaction with (a) $^{45}Ca^{2+}$ alone, (b) $^{45}Ca^{2+}$ + 2mM vanadate or (c) $^{45}Ca^{2+}$ + 0.2mM diethylstibesterol. When present, 2µM (d) or 4µM (e) A23187 was added 5 min prior to sampling (t=55 min). Protoplasts were also pelleted directly through the oil layer without an EGTA quench in the absence (f) or presence (g) of 4µM A23187.

Results are expressed as nmol Ca^{2+} per mm^3 pellet H_2O ± SEM (n=4).

level to increase by a factor of 2.4 and 4 µM A23187 by a factor of 3.7, suggesting that, in the absence of ionophore, it is the ATP-dependent efflux of Ca^{2+} which determines the level of Ca^{2+} in the cell.

Since the plasma membrane is highly permeable to Ca^{2+} in the presence of A23187 the measured accumulation could be an underestimation because there may be some release of Ca^{2+} during the EGTA treatment. As Fig.1B (f and g) shows,however, the stimulation by A23187 is about the same without EGTA treatment, suggesting that no redistribution of Ca^{2+} occurs.

In order to assess the unidirectional influx of Ca^{2+} across the plasma membrane $^{45}Ca^{2+}$ was added to the protoplasts when net accumulation had reached a steady-state (t=60 min). As reported previously for mammalian cells (17) in wheat protoplasts a fast initial rate of uptake is observed, followed by a much slower phase of Ca^{2+} influx (Fig.2). When A23187 and $^{45}Ca^{2+}$

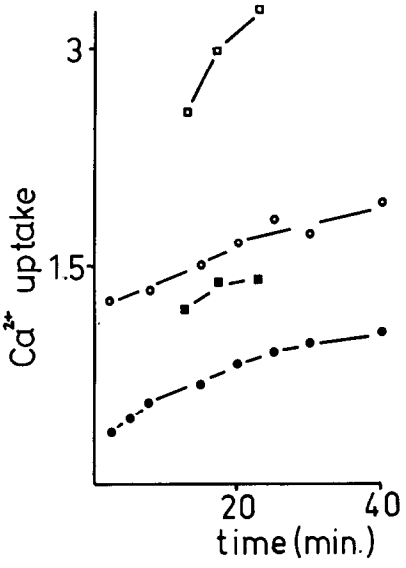

Figure 2. Effect of A23187 on Ca^{2+} uptake by wheat protoplasts

Protoplasts, 130µg chlorophyll cm^{-3}, were incubated for 50 min at 20°C in the presence of 1mM $CaCl_2$ and 10µCi cm^{-3} $[^3H]_2O$. Reactions were initiated by the addition of 5µCi cm^{-3} $^{45}Ca^{2+}$ (t=0) in the absence (●,■) or presence (○,□) of 4µM A23187. Samples were also centrifuged without an EGTA quench layer (■,□). Results are expressed as nmoles Ca^{2+} per mm^3 pellet H_2O.

were added together the initial rate of influx was enhanced 3-fold within 2.5 min. Controls without an EGTA quench layer again showed that the A23187 effect was significant but not enhanced.

Results in Fig.3 show that, when a similar experiment was carried out using the plant hormones gibberelic acid, indoleacetic acid, abscissic acid or kinetin, all at 10^{-6}M, no significant effect was detectable whereas A23187 still caused an increase in Ca^{2+} influx.

Illuminating protoplasts isolated from dark grown wheat with red (659 nm) or far red (729 nm) light for the duration of a Ca^{2+} pulse under steady-state conditions, was also found to have no long-term influence on uptake of this cation (Fig.4). A small decrease in the rate of Ca^{2+} influx was seen, however, in the 5 min incubation for both tissues.

Discussion

The results of the present study suggest that, as recently found in giant algae (4), protoplasts isolated from higher plants maintain an inwardly

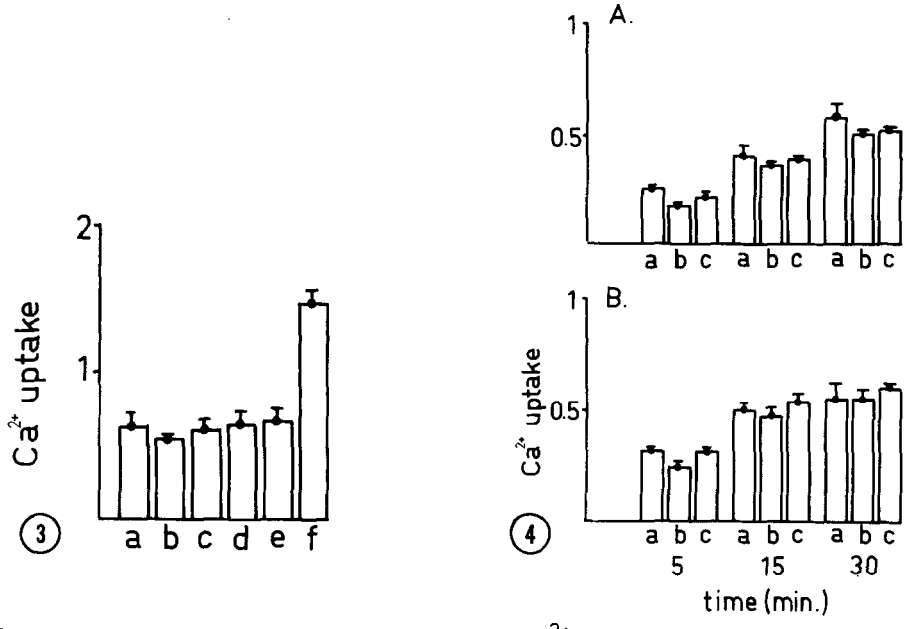

Figure 3. Effect of various plant hormones on Ca^{2+} influx into wheat protoplasts

Protoplasts were incubated for 50 min as outlined in Figure 2. Pre-mixed solutions of $^{45}Ca^{2+}$ (5 μCi cm^{-3}) ± hormones (10^{-6}M) or A23187 (4μM) were added to start the reactions and uptake was measured in samples taken 10 min later. Incubations were Ca^{2+} (a) alone, (b) + gibberelic acid, (c) + indoleacetic acid, (d) + abscissic acid, (e) + kinetin, (f) + A23187. Results are expressed as nmoles Ca^{2+} per mm^3 pellet H_2O ± SEM (n=3).

Figure 4. Effect of red and far red light on Ca^{2+} influx into etiolated wheat protoplasts

Protoplasts, isolated from wheat grown until day 5 in the light followed by 3 days dark (A) and 8 days in the dark (B), were incubated for 50 min in the dark as outlined in Figure 2. Reactions were started by addition of 5 μCi cm^{-3} $^{45}Ca^{2+}$ (t=0) and samples taken at the indicated times. Conditions of incubation were (a) in the dark throughout, (b) red light from t=0, (c) red light from t=-5 to t=0 followed by far red light. Each incubation contained about 0.5 x 10^6 cells cm^{-3} and results are expressed as nmoles Ca^{2+} per mm^3 pellet H_2O ± SEM (n=3).

directed Ca^{2+} gradient across the plasma membrane. For this reason the Ca^{2+} ionophore A23187 causes a considerable increase in net accumulation of Ca^{2+} and in $^{45}Ca^{2+}$ uptake, under steady-state conditions, into wheat protoplasts. In mammalian cells the Ca^{2+} gradient across the plasma membrane is maintained by the aid of a (Ca^{2+}/Mg^{2+})-ATPase, which extrudes Ca^{2+} ions from the cytosol and requires calmodulin for maximal activity (3,8,9). Our finding that orthovanadate and diethylstibesterol, inhibitors of plasma membrane associated ATPases (18-20), caused an increase in the steady-state

level of Ca^{2+} in these protoplasts would suggest that an ATP-linked Ca^{2+} extrusion pump is also present in the plasma membrane of higher plants. Vanadate has also been found to block the (Ca^{2+}/Mg^{2+})-ATPase in the erythrocyte plasma membrane, decreasing Ca^{2+} efflux (22-24) and, at high concentrations, increasing net Ca^{2+} uptake (25).

Changes in cytosolic Ca^{2+} concentrations are known to trigger responses in animal (1-3) and plant (4-6) cells, these often being calmodulin mediated (3,7-9). In animal cells it is the primary signal information of a hormone or an electrical excitation which causes an increase in Ca^{2+} influx and the subsequent increase in the cytosolic [Ca^{2+}]. The data presented here indicate that for plant cells there is no direct affect on Ca^{2+} uptake by the hormones tested. This agrees with a recent finding that the plant hormones, indoleacetic acid and zeatin, do not influence an ATP-dependent Ca^{2+} movement into plasmalemma vesicles, prepared from etiolated, elongating soya bean hypocotyls (10). Only when whole tissue sections were exposed to the hormones for 1 h was any increase in Ca^{2+} uptake by the subsequently isolated vesicles detectable.

It has been suggested that activation/inactivation of phytochrome by red/far red light, respectively, may lead to changes in cytosolic [Ca^{2+}], possibly mediated via calmodulin (11-14). Variable results have, however, been published. Using the metallochromic indicator murexide, Roux and coworkers (13,14) reported that red light caused a Ca^{2+} release from plant cells and an increased efflux and decreased influx of Ca^{2+} into isolated mitochondria. These effects were reversed by far red light. The method they used, however, is relatively insensitive and artifacts due to nonspecific absorbance changes are possible (26,27). It should also be noted that by using $^{45}Ca^{2+}$ to determine Ca^{2+} measurements in isolated mitochondria, other workers have observed no effects of red or far red light (12). As shown in this study neither red nor far red light appear to have any significant, long-term effect on $^{45}Ca^{2+}$ influx into wheat protoplasts. This would suggest that photoactivation of phytochrome by red light does not influence Ca^{2+} uptake

across the plasma membrane. The results do not, however, exclude the possibility that there is a transient red light effect, seen as a slight decrease in Ca^{2+} uptake after 5 min, or that red light induces Ca^{2+} release from internal storage sites, analogous to the initiation of muscle contraction (28). These areas are currently under investigation.

Acknowledgements

This work was supported by grants from the ARC and SERC (A.L.M) and the Sigrid Juselius Foundation (K.E.O.Å). We would like to thank Mr. S.D. Blakeley for help in preparing the etiolated protoplasts.

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