Volume 212, number 1, 133–137 FEB 04436 February 1987

Calmodulin antagonists increase free cytosolic calcium levels in plant protoplasts in vivo

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Received 9 December 1986

Inhibition by calmodulin antagonists has often been used to identify the involvement of calmodulin in physiological processes in plants. Therefore the role of calmodulin in the maintenance of cytosolic calcium levels was investigated using these drugs on carrot cell protoplasts loaded with the $[Ca^{2+}]$ indicator, quin 2. Resting $[Ca^{2+}]$ was 361 ± 47 nM ($n = 30$). On incubation with the anticalmodulins, W7, TFP and tetracaine, calcium levels increased, over 15 min, to $> 10^{-6}$ M (500 μM extracellular Ca^{2+}) or 500 nM (1 mM extracellular EGTA). This rise in calcium level provides an alternative explanation for some of the effects of calmodulin antagonists on the direct involvement of calmodulin in plant processes.

Calmodulin; cytosolic Ca^{2+}; Protoplast

1. INTRODUCTION

A variety of physiological processes, in the plant, have been inferred to involve calmodulin from the inhibitory effects of phenothiazine (e.g. trifluoperazine) and sulphonamide (W-series) calmodulin antagonising drugs or local anaesthetics that inhibit calmodulin activity (e.g. tetracaine) [1]. Processes as diverse as gravitropism [2], cytokinin-induced pigment accumulation [3], secretion [4], mitotic progression [5] and auxin transport [6] have been shown to be affected by calmodulin antagonists at concentrations of 10^{-3}–10^{-6} M. The precise nature of the involvement of calmodulin in these processes is unknown though several calcium-calmodulin-dependent enzymes do occur in plants [7], including the Ca^{2+}-ATPase Ca^{2+} efflux pump of the plasmalemma and the Ca^{2+}-sequestering activity of the endoplasmic reticulum [8,9], thought to be responsible for maintaining cytosolic calcium levels below 10^{-6} M [9].

We report here an investigation of the effects of anticalmodulin agents on the maintenance of free cytosolic calcium levels in carrot cell protoplasts. At low concentrations, specific for calmodulin antagonism (10 μM or less), these drugs cause an increase in cytosolic calcium levels which itself could modulate those processes previously inferred to involve calmodulin more directly [2–6].

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Abbreviations: quin 2, methoxyquinolinebis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; TFP, trifluoperazine; FDA, fluorescein diacetate; Ca_i^{2+}, cytosolic free calcium

2. MATERIALS AND METHODS

2.1. *Protoplast isolation*

Protoplasts were prepared from carrot cells maintained in Murashige and Skoog's medium (Flow Labs), 2% sucrose, 0.1 mg/l 2,4-D, 0.1 mg/l zeatin, pH 5.6 (adjusted with 1 N KOH), at 26°C and 120 rpm orbital shaking. Cells were

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00145793/87/$3.50

preplasmolysed in culture medium supplemented with 250 mM sorbitol for 15 min and then digested for 2 h in preplasmolysis solution with 2% (w/v) driselase (Fluka) and 1% (w/v) rhozyme HP150 (Genencor). The protoplasts released over this period were sedimented (50 × *g* for 10 min) and resuspended to 10^6 ml^{-1} in fresh growth medium. Cells 6–8 days after subculture (mid culture cycle) proved optimal for both protoplast isolation and electroporation (see below). All protoplast suspensions used showed >80% viability as assessed by FDA staining [10].

2.2. *Electroporation*

The electroporation procedure for carrot protoplasts was modified from that reported for mung bean [11]. After isolation protoplasts were washed twice in electroporation buffer (250 mM sorbitol adjusted to pH 7.1 with 1 N KOH) by sedimentation (50 × *g* 10 min) and resuspension to fresh buffer (final concentration 10^7 protoplasts/ml). Protoplasts were incubated at 4°C for 10 min and permeabilised with a single 6–8 kV/cm d.c. pulse, 15 μs duration (time constant), using equipment as in [11]. These conditions were optimal for permeabilisation and subsequent membrane resealing. Immediately after permeabilisation 12 mM quin 2, 1 mM $MgSO_4$, 1% sucrose and $CaCl_2$ to bring free Ca^{2+} to 1 μM (calculated by reiterative computer program) was added and protoplasts were incubated at 4°C for 10 min, to slow membrane resealing. Protoplasts were then resealed over 90 min at 26°C and washed 4 times in 50 vols fresh buffer with 500 μM $CaCl_2$, to stabilise resealed membranes. After this procedure no quin 2 fluorescence could be detected in the supernatant of the final wash.

2.3. *Permeabilisation assays*

Permeabilisation and resealing were assessed by the loss and reattainment of the ability to exclude ethidium bromide (0.01% solution) for 1–2 min, the conditions being as in [11]. In unelectroporated or resealed protoplasts ethidium bromide uptake took 10 min; on permeabilisation it was instantaneous.

2.4. *Fluorimetric assays*

Fluorimetric assays, calculation of $[Ca^{2+}]$ and quin 2 content of loaded protoplasts were carried out in a Baird Nova spectrofluorimeter [11]; assuming 1 mM intracellular Mg^{2+}, pH 7.1, and Ca^{2+}-quin 2 K_d of 115 nM [12].

3. RESULTS

3.1. *Quin 2 uptake on electroporation*

The ability of the carrot protoplasts to exclude ethidium bromide decreased from 83 ± 6 to 17 ± 14% on electroporation (n = 6) indicating that most protoplasts had been permeabilised. After resealing for 90 min ethidium bromide exclusion recovered to 71 ± 9% (n = 6) showing that the plasmalemma had resealed. Electroporation and resealing in 12 mM quin 2 allowed the intracellular trapping of 128 ± 41 pmol quin 2/10^6 protoplasts (n = 10). This is equivalent to an intracellular [quin 2] of 10^{-4} M assuming a mean protoplast volume of 0.5 pl (average diameter 10.6 ± 0.6 μm, n = 45). At such levels of uptake quin 2 fluorescence represented 10–40% of the total fluorescence at 490 nm.

3.2. *Basal calcium levels*

Resealed protoplasts maintained a steady resting $[Ca^{2+}]_i$ of 361 ± 47 nM, n = 30 (500 μM extracellular calcium) over a 15 min period (fig.1). After this period $[Ca^{2+}]_i$ began to rise, probably due to the onset of anoxic conditions in the sample cuvette (not shown). Therefore continuous $[Ca^{2+}]_i$ measurements were limited to the initial 15 min of each treatment.

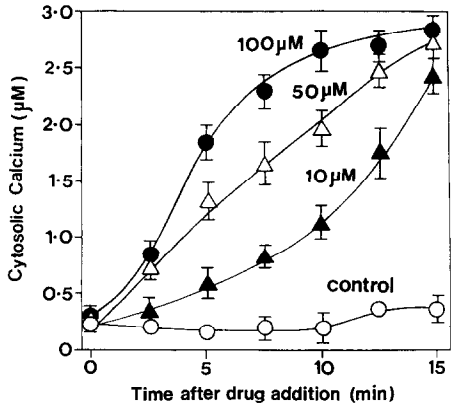

Fig.1. Effect of W7 on cytosolic calcium levels in carrot protoplasts, with 500 μM extracellular Ca^{2+}. Each point represents mean ± SE, $n \geq 3$.

3.3. *Effects of calmodulin antagonists*

On addition of 50–100 μM W7 and W5 $[Ca^{2+}]_i$ was seen to rise, over 15 min, to levels above those reliably detectable with quin 2, $>10^{-6}$ M [12] (with 500 μM extracellular calcium) (figs 1,2). W5 is an analogue of the calmodulin antagonist W7 but with much reduced activity [1]. Therefore the equivalent effects of W7 and W5 suggest that at these concentrations their action was due to a non-specific disruption of membrane functions rather than a specific calmodulin antagonism. Such non-specific effects on membranes at relatively high drug levels have been noted by others [9,13,14]. However, at 10 μM, W7 causes a faster increase in $[Ca^{2+}]_i$ than W5 (figs 1,2) in accordance with the lower affinity of W5 for calmodulin [1].

TFP and the local anaesthetic, tetracaine, are chemically unrelated to W7 and W5 but nevertheless inhibit calmodulin activity [1]. TFP and tetracaine caused a similar increase in cytosolic calcium levels to W7 (with 500 μM extracellular calcium) though requiring approx. 5-fold higher concentrations. This is in agreement with their reduced binding affinity for calmodulin in vitro compared with W7 [1].

3.4. *Effect of EGTA on calmodulin antagonism*

On replacement of 500 μM extracellular Ca^{2+} with 1 mM EGTA the increase in $[Ca^{2+}]_i$ induced by 10 μM W7 is reduced 6-fold over the 15 min of observation (fig.5). This reduction but not total inhibition of the $[Ca^{2+}]$ increase may indicate that

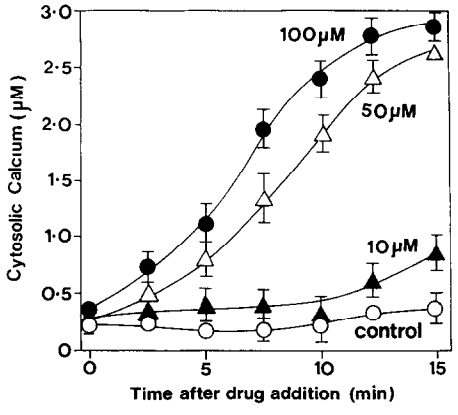

Fig.2. Effect of W5 on cytosolic calcium levels in carrot protoplasts, with 500 μM extracellular Ca^{2+}. Each point represents mean ± SE, $n \geqslant 3$.

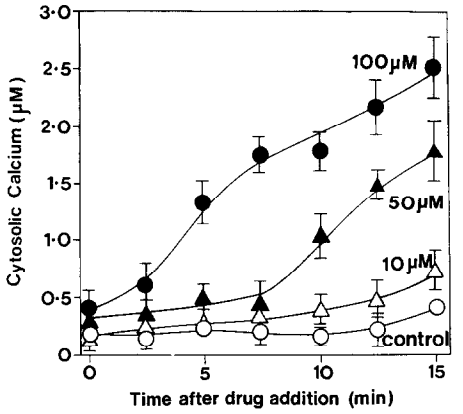

Fig.3. Effect of trifluoperazine on cytosolic calcium levels in carrot protoplasts, with 500 μM extracellular Ca^{2+}. Each point represents mean ± SE, $n = 3$.

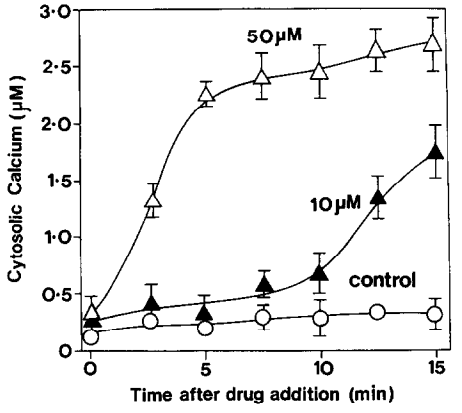

Fig.4. Effect of tetracaine on cytosolic calcium levels in carrot protoplasts, with 500 μM extracellular Ca^{2+}. Each point represents mean ± SE, $n = 3$.

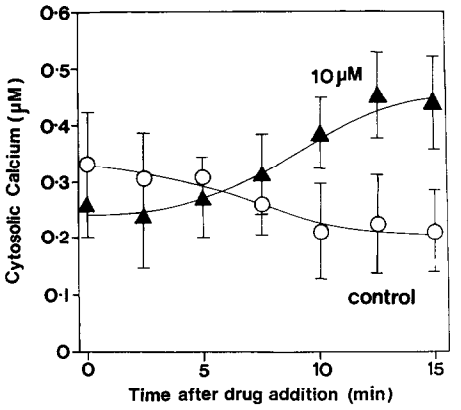

Fig.5. Effect of W7 on cytosolic calcium levels in carrot protoplasts, with 1 mM extracellular EGTA. Each point represents mean ± SE, $n \geqslant 3$. Note change in scale.

the effect of W7 on Ca^{2+} influx is not solely at the plasmalemma. There will also be an effect on the discharge of calcium from intracellular stores, such as the endoplasmic reticulum or vacuole [9].

4. DISCUSSION

The technique of intracellular loading of the $[Ca^{2+}]_i$-indicating dye, quin 2, by electroporation has allowed the measurement of intracellular calcium levels in mung bean root protoplasts [11], carrot suspension culture protoplasts and barley mesophyll protoplasts (unpublished). Circumstantial evidence for the uptake of the dye to the cytosol of loaded protoplasts has been presented elsewhere [11] but further to this the carrot protoplasts used in this study are highly cytoplasmic (approx. 10% vacuolated) and isolated vacuoles and the particulate matter from protoplasts accumulate only 5–6% of the available quin 2 (not shown). This would suggest that the quin 2 is mostly in the cytosol at the time of $[Ca^{2+}]$ measurements and so will monitor cytosolic calcium levels. The resting $[Ca_2{}^{+}]$ of 361 nM indeed is comparable with that found in giant algal cells using alternative methods to measure free cytosolic calcium levels [15,16]. However, the subcellular localisation of quin 2 is currently under fuller investigation.

The rise in $[Ca^{2+}]$ from 361 nM to $>10^{-6}$ M observed on incubation with calmodulin antagonists (at concentrations specific for anticalmodulin activity) may arise from the reported calmodulin dependency of the Ca^{2+}-ATPase Ca^{2+} efflux pump at the plasmalemma and endoplasmic reticulum Ca^{2+}-sequestering activity [8,9]. Both are involved in pumping Ca^{2+} from the cytosol to maintain the low resting Ca^{2+} levels and so if inhibited would be expected to cause an increase in $[Ca^{2+}]_i$. The decrease in antagonist-induced $[Ca^{2+}]_i$ rise on reduction of extracellular calcium suggests that a major fraction of cytosolic Ca^{2+} influx is normally across the plasmalemma but that even when this flux is reduced the discharge of, unidentified, intracellular Ca^{2+} stores can cause $[Ca^{2+}]_i$ to rise as expected if the cytosolic Ca^{2+} efflux pumps were inhibited. However, many other plant enzymes are known to be calmodulin-dependent [7] and could equally well affect Ca^{2+} levels through a general disruption of cellular metabolism.

The efficiency with which the calmodulin antagonists induce the rise in $[Ca^{2+}]$, W7 > tetracaine > W5 and TFP is in general agreement with their ability to bind calmodulin in vitro [1] reinforcing the view that, in this case, their action may be specific for calmodulin antagonism. However, non-specific effects of these drugs have been noted [9,13,14] and often attributed to detergent-like effects. However, 0.05% Triton X-100 caused intracellular $[Ca^{2+}]$ to approach instantaneously that of the medium (fig.6). This ef-

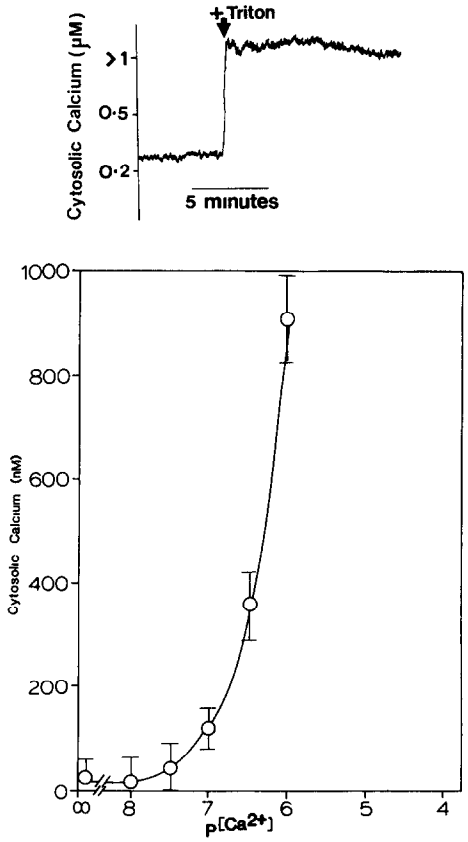

Fig.6. (a) Effect of 0.05% Triton X-100 on cytosolic calcium levels, with 500 μM extracellular Ca^{2+}. The fluorescence trace is representative of that obtained in 3 separate experiments. (b) Effect of 0.05% Triton X-100 on cytosolic calcium levels, with extracellular Ca^{2+} set by 1 mM EGTA + appropriate amounts of $CaCl_2$ (calculated by a reiterative computer program). Each point represents mean ± SE, $n = 3$.

fect is unlike the time-dependent action of the calmodulin antagonists (figs 1–4), which are therefore less likely to be acting through any non-specific detergent-like activity they may possess.

In summary, the large changes in $[Ca^{2+}]_i$ observed on addition of apparently specific levels of calmodulin antagonists may provide an alternative explanation of the effects of these drugs on a wide range of physiological processes in plants to a direct involvement of calmodulin in these processes.

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