The measurement of intracellular calcium levels in protoplasts from higher plant cells

S. Gilroy, W.A. Hughes* and A.J. Trewavas

Department of Botany, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, Scotland and **Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, England*

Received 24 February 1986

The measurement of cytosolic calcium concentration, $[Ca^{2+}]_i$, in the higher plant cells has proved difficult due to the negligible uptake of $[Ca^{2+}]_i$ indicator. The uptake of the fluorescent $[Ca^{2+}]_i$ indicator, quin 2, as its permeant ester, quin 2/AM, proved unsuccessful when used with plant cells and cell protoplasts. However, electrically induced membrane permeabilisation, electroporation, has allowed quin 2 uptake into mung bean root protoplasts to 10^{-4} M. Resting $[Ca^{2+}]$ was measured as 171 ± 41 nM. The loaded quin 2 was responsive to changes in the Ca^{2+} environment of the protoplasts, indicating a reversible fall in $[Ca^{2+}]$ to 17% of the initial value, over 10 min, on incubation with EGTA.

Calcium estimation *Electroporation* *Quin 2* *(Mung bean root)* *Protoplast*

1. INTRODUCTION

In vivo variations in $[Ca^{2+}]_i$ in plants have only been directly measured and correlated with physiological processes in a few cases. These have been limited to giant algal cells, suited to calcium indicator microinjection [1], or a few specialised systems where indicator uptake as a permeant ester [2] has proved successful, i.e. *Haemanthus* endosperm [3] and barley aleurone protoplasts [4]. However, there is a lack of a suitable, generally applicable method for the intracellular uptake of the impermeant $[Ca^{2+}]_i$ indicators to small, vacuolated cells of higher plants.

We report here an investigation of the possible methods of uptake of the $[Ca^{2+}]_i$-indicating fluorescent dye, quin 2, by higher plant cell protoplasts. A simple reversible membrane permeabilisation procedure, 'electroporation' [5], has proved effective and represents the first general method of application of this indicator suitable for use with populations of plant protoplasts.

Abbreviations: DMSO, dimethyl sulphoxide; FDA, fluorescein diacetate; quin 2, methoxyquinolinebis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; quin 2/AM, tetraacetoxymethyl ester of quin 2; DTPA, diethylenetriaminepentaacetic acid

2. MATERIALS AND METHODS

2.1. *Protoplast isolation*

Protoplasts from the apical 2–4 mm of root tips of mung beans (*Phaseolus mungo*) were prepared as in [6]. Protoplast suspensions were stored at 4°C and used within 30 min of production.

2.2. *Electroporation*

Immediately prior to electroporation protoplasts were sedimented ($100 \times g$, 10 min) and resuspended at 4°C to 10^6/ml in electroporation buffer containing (mM): 385 mannitol, 100 KCl, 100 sucrose, 20 NaCl, 0.1 $MgSO_4$, 0.1 KH_2PO_4, 10 Hepes, 6 quin 2 (Sigma). Two controls were carried out: (i) omission of quin 2 and (ii) replacement of quin 2 with 6 mM EGTA to simulate the Ca^{2+}-chelating action of quin 2. Ca^{2+} was not included as it reduced the efficiency of, and survival of protoplasts after, permeabilisation if present while membranes

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

were permeabilised (unpublished). 10^7 protoplasts were subjected to 2 permeabilising pulses of 50 μs duration (time constant), maximum d.c. field strength 3.8 $kV \cdot cm^{-1}$, 30 s between pulses, using equipment essentially as in [7]. After incubation for 10 min at 4°C and 150 min at 25°C, to reseal membranes, protoplasts were washed 3 times (sedimented at 100 × *g*, 10 min, with resuspension to fresh buffer supplemented with 500 μM $CaCl_2$, to stabilise resealed membranes). After this procedure no quin 2 could be detected in the supernatant from the final wash. Permeabilisation and resealing were assessed by the loss and reattainment of the ability to exclude ethidium bromide (0.01%) for 1–2 min [7]. In unelectroporated or resealed protoplasts ethidium bromide uptake took 10 min; on permeabilisation uptake was instantaneous.

2.3. *Fluorimetric assay*

Assays were carried out in a Baird Nova spectrofluorimeter with excitation at λ 339 nm (slit 10 nm) and emission at λ 490 nm (slit 10 nm). Prior to each assay 7% (v/v) percoll was added to slow protoplast settling.

2.4. *Quin 2 content and [Ca^{2+}]*

Protoplast quin 2 content was estimated by comparison of Ca^{2+}-saturated quin 2 fluorescence from loaded protoplasts after lysis (0.5% Triton X-100) or addition of 5 μM A23187 (Sigma), to that of a known [quin 2] under equivalent conditions. 250 μM Ca-DTPA was added to remove any quin 2 quenching ions released on lysis [9].

[Ca^{2+}] was estimated according to either [2] or [10], assuming cytosolic [Mg^{2+}] of 1 mM and pH of 7.1. In the first 4 experiments both methods gave identical [Ca^{2+}] and therefore only the former method was used in subsequent experiments.

3. RESULTS

3.1. *Quin 2 uptake*

Table 1 indicates a range of methods that we exhaustively investigated to allow the uptake of quin 2 by higher plant cells and protoplasts. We have previously found that uptake as the permeant ester of quin 2, quin 2/AM [2], was unsuccessful due to an esterase activity associated with both plant cells and protoplasts [11]. This esterase hydrolysed the quin 2/AM to the extracellular medium with no

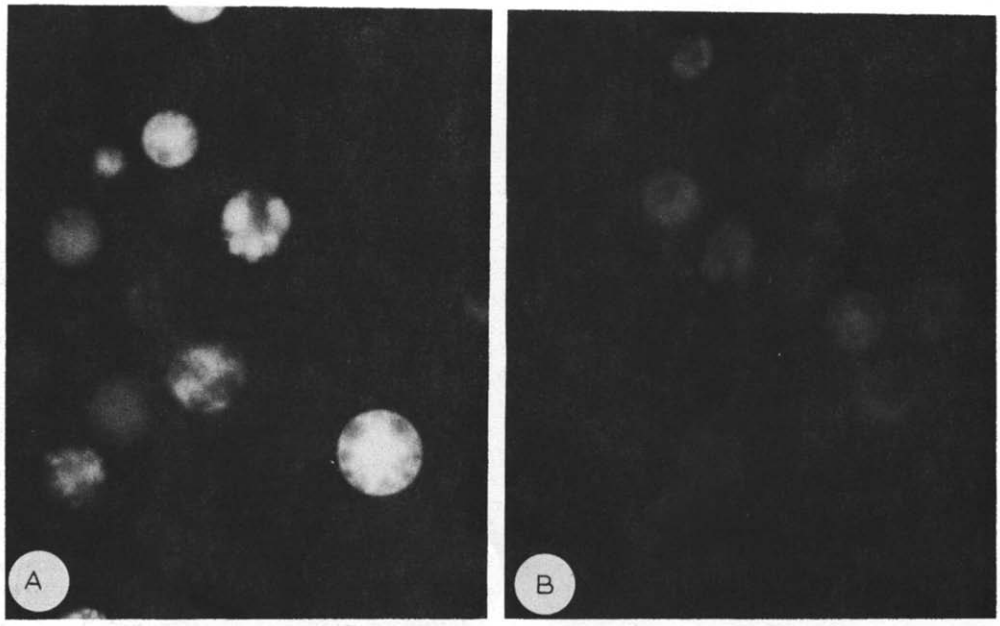

Fig.1. Mung bean root protoplasts, washed and + 500 μM Mn^{2+}: (A) loaded to 10^{-3} M quin 2 by electroporation and resealing in 60 mM quin 2; (B) as above but unelectroporated. × 100 Micrographs were taken using a Leitz Ortholux II microscope with Ploemopak fluorescence illuminator, and Ilford HP5 400ASA black and white print film.

Table 1

Potential methods for Ca^{2+} indicator uptake to the cytosol of plant cells and protoplasts

General approach	Treatment
Ester-mediated quin 2 uptake	(i) incubation with 10–300 μM quin 2/AM [2] (ii) repeated incubation with 50 μM quin 2/AM to maintain [quin 2/AM] gradient (iii) incubation with 10–250 μM quin 2/AM with treatments to inhibit extracellular esterase activity: trypsin, 0.1–0.5%; pH_o 2–10; eserine[a], 1–50 μM; incubation at 4°C
Uptake of free quin 2	(i) incubation with 50 μM–50 mM quin 2 (ii) incubation with 1–50 mM quin 2 under conditions to modulate its charge: pH_o 2–10; $CaCl_2$, 0–50 mM (iii) fusion of quin 2-containing liposomes
Reversible disruption of the plasmalemma	(i) cycles of osmotic swelling and shrinkage (ii) centrifugation (iii) mild sonication (iv) chelation of membrane Ca^{2+} with EGTA (v) high-voltage electric discharge, 'electroporation'

[a] Eserine is a potent esterase inhibitor [15]

Methods were investigated using mesophyll protoplasts of tobacco and petunia, suspension cultures of petunia and oil palm and root tip protoplasts of Mung bean

evidence of intracellular hydrolysis and release of free quin 2 to the cytosol [11]. Methods to saturate or inhibit this external esterase activity proved unsuccessful (table 1). Of the alternative methods of quin 2 uptake investigated (table 1), only electroporation has proved effective (83% successful, $n = 18$) and the following results relate to this procedure as applied to mung bean root tip protoplasts.

3.2. *Reversible membrane permeabilisation*

Directly after electroporation ethidium bromide exclusion (over 1–2 min) decreased from 88 ± 11 to 23 ± 15% ($n = 8$) indicating a loss of plasmalemma integrity. This recovered to 68 ± 8% ($n = 8$) after the membrane resealing incubation, at which point protoplasts showed >70% FDA staining (retention of intracellular fluorescein) for >2 h. This indicated that the plasmalemma had resealed.

3.3. *Quin 2 uptake after electroporation*

Electroporation allowed the intracellular trapping of quin 2 (fig.1). Electroporation in 6 mM

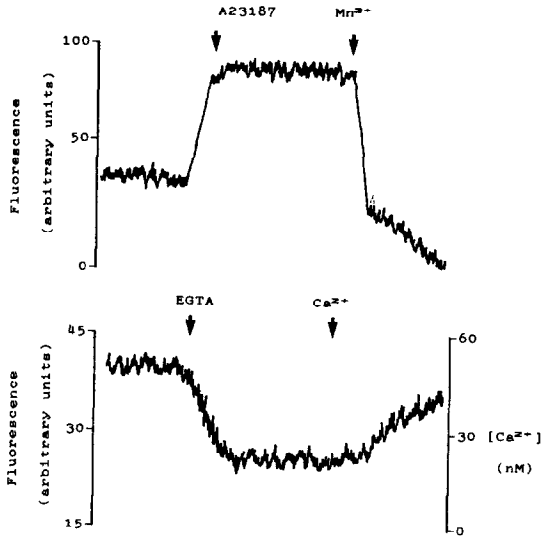

Fig.2. Fluorescence, above autofluorescence, 10^6 protoplasts/ml loaded to 10^{-4} M quin 2, after sequential addition of 2 μM A23187 and 500 μM Mn^{2+} (upper), or 5 mM EGTA and 6 mM $CaCl_2$ (lower). A23187 was added from a 1 mM stock in DMSO.

quin 2 trapped, on average, 800 ± 230 pmol quin 2 per 10^6 protoplasts (n = 8). This represents a [quin 2] of 1.9×10^{-4} M assuming a mean protoplast volume of 4 pl (average protoplast diameter 20 μm) and a uniform intracellular quin 2 distribution. At these levels of quin 2 uptake protoplast autofluorescence represented 15–50% of the total fluorescence signal at 490 nm.

3.4. *Intracellular location of quin 2*

The fluorescence of the trapped quin 2 was unaffected by the addition of 500 μM Mn^{2+}, a potent quin 2 fluorescence-quenching ion [10] (fig.1). On addition of 2 μM A23187, a divalent cationophore, quin 2 became accessible to either the high [Ca^{2+}] of the medium (500 μM), which increased the Ca^{2+}-dependent fluorescence of quin 2 3–4-fold, or to quenching by 500 μM extracellular Mn^{2+} (fig.2). These results are consistent with an intracellular location of the indicator. It is therefore protected from rapid changes in the medium by the protoplast plasmalemma unless an ionophore, which translocates cations across membranes, is present.

3.5. *[Ca^{2+}] and indicator responsiveness*

In the presence of 500 μM external quin 2, intracellular [Ca^{2+}] was estimated at 171 ± 41 nM (10^{-4} M intracellular quin 2, n = 15). On removal of extracellular Ca^{2+} (+ 5 mM EGTA) the [Ca^{2+}] reported by quin 2 fell, over 5 min, to 17% of the original level (n = 2). This effect was reversed with 6 mM $CaCl_2$ (fig.2).

4. DISCUSSION

Plasmalemma permeabilisation by electroporation allowed intracellular uptake of the [Ca^{2+}]-indicating, fluorescent dye, quin 2, to 10^{-4} M, by protoplasts of higher plant cells. After 1–2 h at 25°C these permeabilised protoplasts recovered their membrane integrity, trapping the quin 2 intracellularly.

It is likely that intracellular quin 2 is partitioned between various cell compartments. Therefore, the [Ca^{2+}] reported here cannot be directly related to [Ca^{2+}]$_i$ until the extent of such partitioning, which is currently under investigation, has been determined. However, there are reasons to believe the intracellular quin 2 is unbound and has access to the cytosol (which represents some 20–30% of the root tip protoplast volume) as: (i) electroporation is thought to permeabilise preferentially the plasmalemma [5], therefore initially quin 2 uptake must be to the cytosol; (ii) the quin 2 family of [Ca^{2+}] indicators, in the cytosol of animal cells, is thought not to cross organelle membranes [8,12]; (iii) intracellular binding of quin 2 should change its fluorescence spectrum [8] but no such change was observed (not shown); (iv) intracellular quin 2 did respond to changes in extracellular calcium levels. Thus the indicator is in a functional, Ca^{2+}-sensitive and Ca^{2+}-responsive state and site.

The apparent dependency shown by intracellular [Ca^{2+}] on extracellular [Ca^{2+}] may truly reflect the state in intact protoplasts. Alternatively, it may result from a short-term loss of membrane function/semi-permeability on electroporation from, e.g. the leaching of low-M_r cellular constituents whilst permeabilised. However, membrane integrity was seen to recover prior to the [Ca^{2+}] measurements and other investigators have found the technique non-disruptive of both membrane and cellular function [13,14].

Thus electroporation represents a general purpose method for ion indicator uptake which should prove applicable to a wide range of both indicators and previously inaccessible higher plant systems.

ACKNOWLEDGEMENTS

The authors would like to thank Dr P.B. Garland for use of the Baird fluorimeter and Mr R. Hart for valuable technical assistance.

REFERENCES

[1] Williamson, R.E. and Ashley, C.C. (1982) Nature 296, 647–651.
[2] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68–71.
[3] Keith, C.H., Raten, R., Maxfield, F.R., Bajer, A. and Selanski, M.L. (1985) Nature 316, 848–850.
[4] Bush, D. and Jones, R. (1985) in: Molecular and Cellular Aspects of Calcium in Plant Development (Trewavas, A.J. ed.) Plenum, New York, in press.
[5] Mehrle, W., Zimmerman, U. and Hampp, R. (1985) FEBS Lett. 185, 89–94.
[6] Xu, Z.H., Davey, M.R. and Cocking, E.C. (1981) Z. Pflanzenphysiol. 104, 289–298.

[7] Watts, J.W. and King, J.M. (1984) Biosci. Rep. 4, 335–342.
[8] Rink, T.J. and Pozzan, T. (1985) Cell Calcium 6, 133–144.
[9] Rink, T.J., Sanchez, A., Grinstein, S. and Rothstein, A. (1983) Biochim. Biophys. Acta 762, 593–596.
[10] Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. (1983) J. Biol. Chem. 288, 4876–4882.
[11] Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1985) in: Molecular and Cellular Aspects of Calcium in Plant Development (Trewavas, A.J. ed.) Plenum, New York, in press.
[12] Almers, W. and Neher, E. (1985) FEBS Lett. 192, 13–17.
[13] Zimmerman, U., Vienken, J. and Pilwat, G. (1983) in: Investigative Microtechniques in Medicine and Biology, vol.1 (Chayen, J. and Bitensky, L. eds) pp.89–167, Marcel Dekker, New York.
[14] Langridge, W.H.R., Li, B.J. and Szalay, A.A. (1985) Plant Cell Rep. 4, 355–359.
[15] Gambhir, S.S. and Das, P.K. (1980) J. Med. Res. 71, 654–660.