Volume 333, number 3, 283–286 FEBS 13181 November 1993
© 1993 Federation of European Biochemical Societies 00145793/93/$6.00

Rapid pressure driven exocytosis–endocytosis cycle in a single plant cell

Capacitance measurements in aleurone protoplasts

Robert Zorec[a,*], Mark Tester[b]

[a]*Laboratory of Neuroendocrinology, Institute of Pathophysiology, School of Medicine, 61105 Ljubljana, Slovenia*
[b]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK

Received 15 July 1993; revised version received 3 September 1993

The whole-cell patch-clamp technique has been used to increase intracellular pressure via the pipette while monitoring changes in membrane capacitance (related to membrane area) in single aleurone protoplasts. Increased pressure increased membrane capacitance and diameter, upon the release of pressure both parameters returned to resting levels. Pressure also reversibly increased membrane conductance. Comparison between diameter and capacitance shows that the capacitance changes are due to changes in cell surface area. These results show that pressure can rapidly alter the balance between exo- and endocytosis. Pressure-induced changes in conductance are suggested to be due to insertion/withdrawal of channels with the exo-/endocytosed membrane.

Aleurone; Barley; *Hordeum vulgare*; Capacitance measurement; Pressure; Exocytosis; Endocytosis; Membrane turnover

1. INTRODUCTION

Membrane turnover in eukaryotic cells involves exo- and endocytosis. This has been documented in single animal cells by measurements of membrane capacitance, a parameter linearly proportional to plasma membrane area [1–3]. Although it seems likely that exo- and endocytosis occur in plant cells, we know of only one direct study of these processes in plant cells [4]. Here we have used the whole-cell patch-clamp technique [5] to monitor membrane surface area (membrane capacitance, C_m) in single aleurone protoplasts.

The aleurone surrounds the storage tissue of seeds, and secretes hydrolases, such as α-amylase upon germination [6]. The hydrolytic enzymes then mobilize energy and nutrient reserves to enable the growth of the developing embryo. In intact seeds, the overall process of germination occurs upon imbibition of water, which results in an increase in the turgor of the cells, i.e. an increase in the pressure experienced by the plasma membrane.

2. MATERIALS AND METHODS

Protoplasts from barley aleurone were prepared according to the methods described previously [4,7], with the following modifications. Before the 48 h incubation with cellulase (Onozuka R10, 5%), aleurone layers were predigested for 4 h, then transferred to fresh enzyme-containing solution. All operations were performed in air, not in an N_2 atmosphere. Protoplasts were purified on a Nycodenz step gradient (70%/50%/0%, weight to volume). The bathing solution consisted of (in mM): KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 MES (2[*N*-morpholino]ethanesulfonic acid)/NaOH, pH 5.6. The pipette-filling solution contained: 120 K glutamate, 2 $MgCl_2$, 10 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid)/KOH, pH 7.2, 1 to 2 ATP, 1 to 11 EGTA (ethylene glycol bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), giving a calcium activity of around 30 nM [3]. In some experiments cAMP was added to the pipette solution. Varying the concentration of EGTA and the addition of cAMP had no apparent effect on electrophysiological parameters recorded. All salts were obtained from Sigma.

Standard patch-clamp whole-cell recording techniques were used to measure membrane capacitance [8–10]. Patch pipettes of 3 to 7 MΩ were prepared [19], which resulted in an average access conductance in whole-cell recordings of 114 ± 39 nS (mean ± S.D., $n = 43$). Membrane capacitance (C_m), parallel combination between membrane leak and membrane conductance (G_m), and access conductance (G_a) were measured using a lock-in amplifier (1 mV p-p, 1600 Hz) with a computer performing on-line calculations [9,10]. The computer program was written by Dr. J. Dempster from the University of Strathclyde, Glasgow, UK. Cells were held at −50 to −80 mV, and G_m was estimated from d.c. membrane current and driving potential (holding potential − reversal potential). The average reversal potential at resting conditions was −57 ± 25 mV (mean ± S.D., $n = 22$), which is close to the Nernst potential for K^+ ions in our recording situation when junction potential is taken into account. Osmotic pressures of solutions were measured by a Wescor (5500) vapor-pressure osmometer and adjusted with sorbitol (Calbiochem) to within 10% of each other (1100 mOsm/kg). Experiments were performed at 22°C. Protoplast diameter was measured with an eye-piece micrometer, pipette pressure was measured with a water manometer. Unless stated otherwise statistics are in the format mean ± S.E.M.

3. RESULTS

Immediately after establishing a whole-cell recording, resting C_m and protoplast diameter were measured. Assuming these cells are spherical [12], we expect C_m to

*Corresponding author. Fax: (386) (1) 302 272.

equal $c \cdot 4 \cdot \pi \cdot r^2$, where c is the specific capacitance and r is the protoplast radius. Linear regression between measured resting C_m (in pF) and calculated membrane area (in μm^2) is shown in Fig. 1A. The slope of the line corresponds to specific capacitance of 0.75 $\mu F/cm^2$, which is similar to that reported in animal cells measured with the same technique [4,13]. This shows that the membrane area changes can be monitored by measurements of C_m, which is also in agreement with results obtained in animal cells [1].

The use of whole-cell patch clamp techniques not only allows the control of electrical parameters of plasma membrane and the composition of the cytosol [14], but also the application of pressure to the pipette interior and therefore to the inside of the cell [15]. Application of positive pressure (1 to 4 mm H_2O; inside relative to the outside of the cells) resulted in an increase in C_m, protoplast diameter and membrane conductance (Fig. 2). The activation of C_m and membrane current appeared to be simultaneous within the time resolution of the recording (25 Hz, see Fig. 3A).

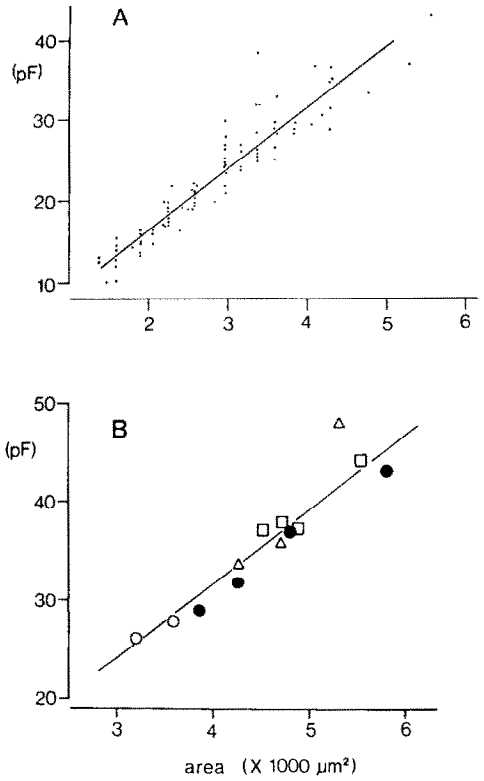

Fig. 1. (A) Correlation between resting membrane capacitance and protoplast membrane area (calculated from diameter, assuming protoplasts are spheres), measured immediately after the establishment of a whole-cell recording configuration. The line is of the form: $y = (0.0075 \pm 0.003)x + (1.6 \pm 0.9)$, with a correlation coefficient of 0.94, $n = 85$. This gives a specific capacitance of 0.75 $\mu F/cm^2$. The constant term is not significantly different from zero. (B) Correlation between pressure-induced changes in the protoplast surface area (calculated as in panel A) and membrane capacitance. The line was drawn according to the equation in panel A. Different symbols denote different cells.

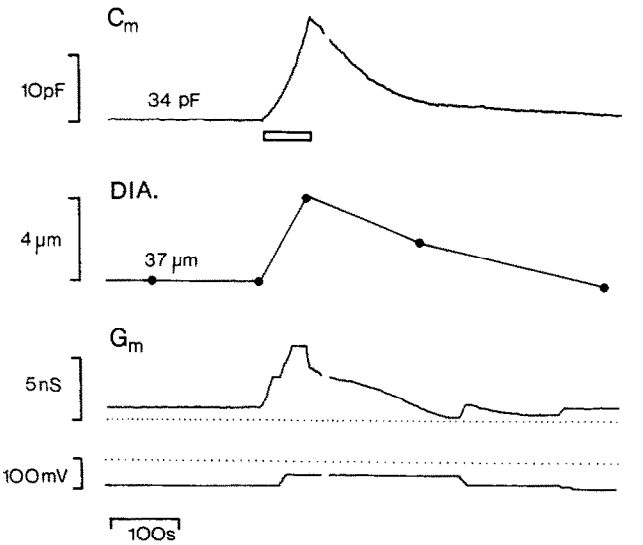

Fig. 2. Pressure-induced changes with time of membrane capacitance (C_m), protoplast diameter (DIA.), and parallel combination of leak and membrane conductance (G_m) in a cell dialysed with pipette filling solution containing 1 mM EGTA, 100 μM cAMP, and 2 mM ATP. Bottom trace indicates changes in holding potential across the cell membrane (V_m). Access conductance of 170 nS was constant during the recording. During the application of pressure (around 4 mm H_2O, indicated by the bar), the holding potential had to be changed due to saturation of the A/D converter. Note that at the beginning and at the end of the experiment, the levels of G_m, and thus levels of the membrane current, are similar. Reversal potential measured before the application of pressure was −67 mV. Initial capacitance was 34 pF and the initial protoplast diameter was 37 μm. Dotted line denotes zero levels on conductance and voltage traces.

After the release of the pipette pressure, C_m returned towards the values before the pressure application. The decline was slower than the rise (Fig. 2). The decay time, estimated as the time taken for half the change in C_m to occur, varied between 50 to 1000 s. Assuming the decay was linear, the rates were from 2 to 89 fF/s (average 21 ± 2 fF/s, $n = 7$). These are at least fivefold slower than the rise time, estimated as the maximal increase in C_m, which was 133 ± 30 fF/s (range 26 to 225 fF/s, $n = 7$). The increase in C_m, protoplast diameter, and membrane conductance could be evoked repeatedly in a single protoplast (not shown).

Application of pressure caused an increase in protoplast diameter and this returned to resting values upon the release of pressure (Fig. 2). When pressure-driven changes in C_m were plotted against the calculated membrane area, the relationship remained constant and not significantly different to the relationship between resting C_m and calculated membrane area (Fig. 1B). The same relationship was found for changes induced by cytosolic Ca^{2+} (see [4]). Such results indicate that the changes in C_m are due to insertion and retrieval of membrane to and from the plasma membrane. This is likely to be due to the processes of exocytosis and endocytosis, respectively.

An increased pressure inside the protoplast also re-

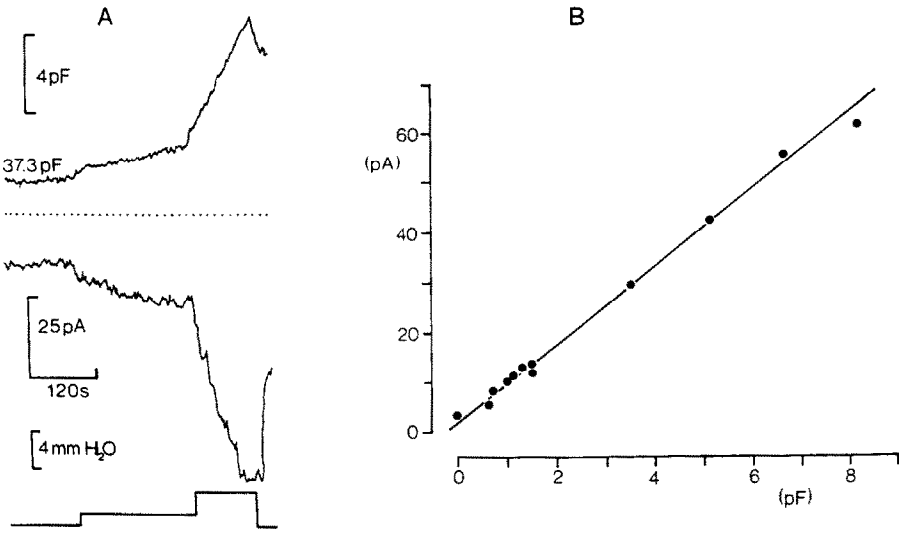

Fig. 3. (A) Representative recording of pressure induced changes in membrane capacitance (top) and membrane current (middle) and pressure application protocol, indicated by the bottom trace. Dotted line denotes zero membrane current. The holding potential was −84 mV, with a reversal potential of −77 mV. Pipette fitting solution as in Fig. 2. The resting membrane capacitance of 37.3 pF was subtracted from the top trace (bandwidth in top and bottom trace is 25 Hz, −3dB, Butterworth). (B) Correlation between pressure-induced change in membrane capacitance (abscissa) and membrane current (ordinate) from recordings in panel A. The signal was sampled every 30 s and amplitudes were measured relative to the stationary levels before the pressure application. The line is of the form $y_1 = 2.2 + 7.9x_1$, correlation coefficient is 0.99, $n = 12$.

sulted in a reversible increase in membrane conductance (d.c. membrane current). The increase in d.c. current is very slow and continues over the entire period of increase in C_m (Fig. 3A). As activation of mechanosensitive channels is much faster than this, with a time constant of less than a second [15], it seems more likely that this increase in current is due to the addition of more channels with the inserted membrane. A linear relationship between pressure induced C_m and current is consistent with this proposal (Fig. 3B).

4. DISCUSSION

In this paper, we describe a rapid increase in exocytosis in response to small increases in intracellular pressure. Pressure-driven exocytosis may contribute to the Ca^{2+}-dependent exocytotic secretion upon imbibition of water by the seed. As water enters the seed, turgor of cells will increase (at least in the early stages of germination), and this may well promote the secretion of hydrolytic enzymes into the endosperm.

There is a wealth of data in animal systems, where a role of osmotic pressure in exocytotic secretion has been suggested [16]; and recent direct measurements of increased capacitance upon osmotic swelling of human epithelial cells supports earlier work [17]. Given the sensitivity of secretion of these cells to cytosolic Ca^{2+} [4], it would also be interesting to know to what extent the pressure-induced increase in C_m is sensitive to changes in cytosolic Ca^{2+} activity.

A notable feature of the measurements presented here on plant cells is the extreme sensitivity of the exocytosis to increased pressure. In the work of Okada et al. [17], large gradients of osmotic potential were applied to cells; and calculations of the active volume by Heinemann et al. [18] also suggest large pressures are required for the perturbation of exocytosis in animal cells. Activation volume, ΔV, is determined by:

$$\ln\theta = P\Delta V/kT$$

where θ is the ratio of the time constants at normal pressure and at the higher pressure (P), k is Boltzmann's constant and T is the absolute temperature (see [18]). Calclations for the barley aleurone cells used in the current work indicate an activation volume of 3×10^{-22} m^3, almost 10^6 times greater than in the mast cells and chromaffin cells used by Heinemann et al. [18]. This suggests that exocytosis in plants is about 10^6 times more sensitive to changes in pressure compared to the similar process in animals. This may be related to the high turgor pressure found in plant cells; however, the molecular basis for such differences definitely merits further attention.

The pressure-driven cycle of exo-endocytosis (Fig. 2) can be best explained by a sequential coupling between the two processes [19]. The coupling mechanism is not known. On the one hand, a biochemical link can be postulated, where changes in cytosolic Ca^{2+} may play an important role. This may be the case at least in triggering exocytosis, but endocytosis appears to be insensitive to changes in Ca^{2+} [19]. On the other hand, coupling could be established simply by an unknown biophysical property of the lipid bilayer itself [20]. Lipowsky [20] has reviewed low energy cost mechanisms which may contribute to shape changes of cell-sized liposomes.

Also of interest from these results is the increase in conductance concomitant with the increase in capacitance. It seems likely that this is due to the insertion of channels with the exocytosed membrane. The ionic selectivity of these channels in our experimental conditions has not been investigated in detail; the pressure-induced current, which was always an inward current (Fig. 3A), could be due to a range of channels, as the plasma membrane was held at potentials negative of the equilibrium potential for several ions (including K^+). It is interesting to note that an increase in membrane conductance was not apparent with Ca^{2+}-stimulated increases in membrane capacitance [4]. This suggests to us that the pressure-stimulated increases in membrane capacitance may not be mediated by Ca^{2+}; it also suggests that two different populations of vesicles are fusing to the plasma membrane, with fusion stimulated by different events. The possibility of the existence of two populations of vesicles means it is also possible that the vesicles stimulated by pressure do not contain hydrolytic enzymes, and thus may not be involved directly in the secretion during germination; this proposal is currently being tested.

To conclude, our experiments have provided evidence for membrane turnover in plant cells, which is pressure-sensitive. This may contribute to controlling exocytotic secretion of hydrolytic enzymes during germination of seeds. In addition, pressure-driven changes in membrane capacitance were paralleled by a reversible change in membrane conductance, which is proposed to be due to insertion and retrieval of channels with the membrane vesicles being inserted into and retrieved from the plasma membrane. Although there are some similarities in the control of exocytosis in plant and animal cells (such as stimulation by cytosolic Ca^{2+}; (see [4]), there also appear to be clear differences, particularly in the sensitivity of the processes to hydrostatic pressure.

Acknowledgements: We particularly thank Drs. S.D. Tyerman and B.R. Terry of the Flinders University of S.A.; also Dr. J. Dempster for providing the computer software, Prof. W. Almers for helpful comments on the manuscript, and Prof. M. Kordaš for general support. Financial support from the Ministry of Science and Technology of Slovenia and from the Research Council of Australia are acknowledged.

REFERENCES

[1] Penner, R. and Neher, E. (1989) Trends Neurosci. 12, 159–163.
[2] Almers, W. (1990) Annu. Rev. Physiol. 52, 607–624.
[3] Zorec, R., Sikdar, S.K. and Mason, W.T. (1991) J. Gen. Physiol. 97, 473–497.
[4] Zorec, R. and Tester, M. (1992) Biophys. J. 63, 864–867.
[5] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Archiv 391, 85–100.
[6] Chrispeels, M.J. and Varner, J.E. (1967) Plant. Physiol. 42, 398–406.
[7] Bush, D.S., Hedrich, R., Schroeder, J.I. and Jones, R.L. (1988) Planta 76, 368–377.
[8] Neher, E. and Marty, A. (1982) Proc. Natl. Acad. Sci. USA 79, 6712–6716.
[9] Lindau, M. and Neher, E. (1988) Pflügers Archiv 411, 137–146.
[10] Zorec, R., Henigman, F., Mason, W.T. and Kordaš, M. (1991) Methods Neurosci. 4, 194–210.
[11] Corey, D.P. and Stevens, C.F. (1983) in: Single-Channel Recording, (Sakmann, B. and Neher, E., Eds.) pp. 53–68, Plenum, New York.
[12] Bush, D.S., Cornejo, M.-J., Huang, C.-N. and Jones, R.L. (1986) Plant Physiol. 82, 566–574.
[13] Pusch, M. and Neher, E. (1988) Pflügers Archiv 411, 204–211.
[14] Marty, A. and Neher, E. (1983) in: Single-Channel Recording, (Sakmann, B. and Neher, E., Eds.) pp. 107–121, Plenum, New York.
[15] Morris, C.E. (1990) J. Membrane Biol. 113, 93–107.
[16] Finkelstein, A., Zimmerberg, J. and Cohen, F.S. (1986) Annu. Rev. Physiol. 48, 163–174.
[17] Okada, Y., Hazama, A., Hashimoto, A., Maruyama, Y. and Kubo, M. (1992) Biochim. Biophys. Acta 1107, 201–205.
[18] Heinemann, S.H., Conti, F., Stuhmar, W. and Neher, E. (1987) J. Gen. Physiol. 90, 765–778.
[19] von Grafenstein, H., Roberts, C.S. and Baker, P.F. (1986) J. Cell. Biol. 103, 2343–2352.
[20] Lipowsky, R. (1991) Nature 349, 475–481.