brief communication

Cytoplasmic calcium stimulates exocytosis in a plant secretory cell

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ABSTRACT Although exocytosis is likely to occur in plant cells, the control of this process is the subject of speculation, as no direct measurements of vesicle fusion to the plasma membrane have been made. We used the patch clamp technique to monitor the secretory activity of single aleurone protoplasts by measuring membrane capacitance (C_m), while dialyzing the cytosol with different Ca^{2+} containing solutions. Secretory activity increased with $[Ca^{2+}]_i \sim 1$ μM. This demonstrates directly the existence of exocytosis in plant cells, and suggests that both plant and animal cells share common mechanisms (cytosolic Ca^{2+}) for the control of exocytotic secretion.

INTRODUCTION

Animal cells secrete macromolecules stored in membrane bound vesicles by the fusion of these vesicles to the plasma membrane (Almers, 1990), which also seems to be the case for plant cells (Jones and Robinson, 1989). This process (exocytosis) is generally controlled, at least in animal cells, by changes in cytosolic Ca^{2+} activity $[Ca^{2+}]_i$ (Douglas, 1968; Penner and Neher, 1989). However, the role of $[Ca^{2+}]_i$ in controlling secretion from plant cells is controversial (compare Steer, 1988; and Jones and Robinson, 1989). We therefore investigated the secretory activity of single aleurone protoplasts, a classical plant secretory cell, with patch clamp technique. Membrane capacitance was measured (C_m), a parameter proportional to plasma membrane area, which is fluctuating due to processes of exocytosis and endocytosis (Neher and Marty, 1982). Aleurone cells from the seeds of barley (*Hordeum vulgare* L. cv Himalaya) secrete hydrolyzing enzymes such as α-amylase when stimulated by the hormone gibberellic acid (GA_3, [Chrispels and Varner, 1967]); this hormone is produced by the embryo upon germination (Radley, 1967), and the hydrolases enable mobilization of energy reserves stored in the endosperm to be used by the growing plant (Jones, 1985; Fincher, 1989). GA_3-stimulated secretion of α-amylase is dependent upon external $[Ca^{2+}]$ (Chrispels and Varner, 1967), suggesting that Ca^{2+} directly regulates the exocytotic secretion of hydrolases (Jones and Jacobsen, 1983), a suggestion consistent with the observation that prolonged incubation of aleurone protoplasts in GA_3 almost doubles $[Ca^{2+}]_i$ (Bush and Jones, 1988). However, until now, there has been no direct evidence for exocytosis and for Ca^{2+} role in exocytosis in any plant cell.

The use of the patch clamp technique to measure changes in capacitance of the plasma membrane of a wide range of animal cells has shown that, at least for excitable cells, cytosolic Ca^{2+} is a central control of exocytosis; however, in nonexcitable cells, Ca^{2+} may only modulate secretion (Penner and Neher, 1988). In this work, we have monitored secretory activity of single aleurone cells by measuring membrane capacitance while dialyzing the cytosol with various Ca^{2+}-containing media.

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MATERIALS AND METHODS

Aleurone protoplasts were prepared according to the methods described previously (Bush et al., 1988) 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). Protoplasts were bathed in (mM): 10 MES (2[*N*-Morpholino]ethanesulfonic acid), 10 KCl, 1 $CaCl_2$, 2 $MgCl_2$, pH 5.6/NaOH. The pipette-filling solution contained: 120 K glutamate, 2 $MgCl_2$, 10 Hepes (*N*-2-Hydroxyethylpiperazine-*N*ethanesulfonic acid), pH 7.2/KOH, to which various amounts of EGTA and Ca-saturated EGTA (Neher, 1988) was added (all salts from Sigma Chemical Co., St. Louis, MO).

Standard patch clamp whole-cell recording techniques were used to measure membrane capacitance (Neher and Marty, 1982), by the automated "noncompensated" method (Lindau and Neher, 1988; Zorec et al., 1991a). Patch pipettes of 3–7 MΩ were prepared as reported (Corey and Stevens, 1983), which resulted in an average access conductance (G_a) in whole cell recordings of 114 ± 39 nS (mean ± SD, n = 43). Membrane capacitance (C_m), parallel combination between membrane conductance and membrane leak (G_m), and G_a were measured using a lock-in amplifier with a computer performing on-line calculations (Lindau and Neher, 1988; Zorec et al., 1991*a*, *b*). The computer program was written by Dr. J. Dempster from the University of Strachclyde (Glasgow, UK). Cells were held at −50 to −90 mV, and G_m was estimated from d.c. membrane current and driving potential (holding potential − reversal potential). Varying holding potential between −50 to −80 mV had no effect on the timecourse of C_m. The average reversal potential was −57 ± 25 mV (mean ± SD, n = 22), which is close to the Nernst potential for K^+ ions in our recording situation when junction potential of around −10 to −20 mV (negative inside pipette) 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 (1,100 mOsm/kg), usually slightly lower in the pipette-filling solution. Variation between changes in osmotic pressure did not affect changes in C_m. Experiments were performed at 22°C. Protoplast diameter was measured with an eyepiece micrometer. Protoplast surface area was calculated from diameters, assuming a perfect sphere. Unless stated otherwise statistics are in the format, mean ± SEM.

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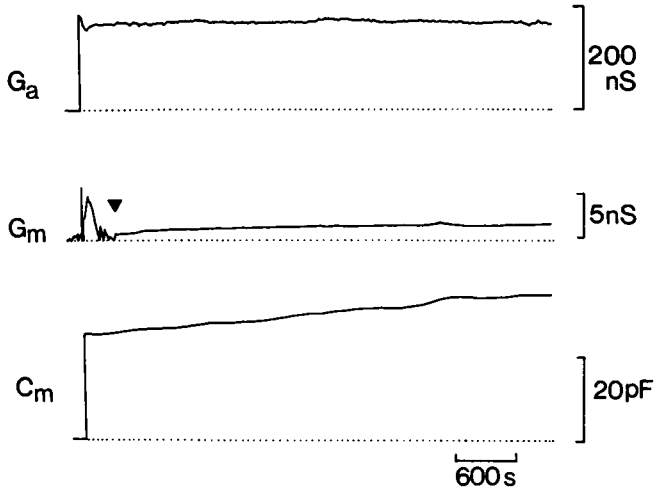

FIGURE 1 Changes with time in access conductance (G_a), parallel combination of leak and membrane conductance (G_m), and membrane capacitance (C_m) in a cell dialyzed with 950 nM Ca^{2+} (1 mM EGTA, 6 mM Ca-EGTA). Initial surface area 2,968 μm^2, which increased to 4,275 μm^2, at the end of the recording (see Fig. 2, *filled diamonds*). Filled triangle indicates time when holding potential was set to −55 mV.

RESULTS AND DISCUSSION

Membrane capacitance measured immediately after the formation of whole-cell recordings was related to surface area (A) for 85 protoplasts in which such measurements were possible, using the following relationship:

$$C_m = c \cdot A + b, \quad (1)$$

where specific capacitance, c, equalled 7.5 ± 0.3 mF/m² (correlation coefficient = 0.94, $P > 0.0001$), and b was 1.6 ± 0.9 pF, which is not significantly different from zero ($P < 0.05$, Student's t test). The origin of the constant term b is probably due to statistical error introduced by electrical and morphological measurements, although in larger protoplasts, surface area could have been overestimated because these may be slightly flattened against glass coverslips. Specific capacitance is similar to measurements obtained in animal cells using similar techniques (e.g., Pusch and Neher, 1988; Zorec et al., 1991b).

To study the role of $[Ca^{2+}]_i$ in the control of secretory activity, we dialyzed cells with various Ca^{2+}-containing media. A representative increase in membrane capacitance in a cell dialyzed with 950 nM free Ca^{2+} is shown in Fig. 1 (*bottom trace*). Increases were observed in 17 of 22 cells (in a range of activities from 750–1,200 nM); these tended to be larger with higher $[Ca^{2+}]_i$. Calcium-induced changes in C_m were measured as the percentage of the initial resting value to the maximum recorded (Table 1). Due to variability in lengths of recordings, rates of C_m changes were measured. In cells dialyzed with 950 nM Ca^{2+} the average rate of increase in C_m was 3.3 ± 1.3 fF/s

TABLE 1 **Dependence of the change in C_m on $[Ca^{2+}]_i$**

$[Ca^{2+}]_i$	Maximal change in C_m	Number of cells	Average time
nM	%		*s*
≈30	−0.6 ± 2.0	8	1,280 ± 674
750	6.7 ± 4.4	4	1,293 ± 595
950	10.5 ± 3.9	15	1,563 ± 465
1,200	14.0 ± 8.2	3	614 ± 128

Change in C_m was measured as the percentage of the initial (resting) value to the maximum recorded. Experiments were performed as in Figs. 1 and 3. Solution with calcium activity of around 30 nM was prepared by including 3.5 to 11 mM EGTA in the pipette solution, whereas other activities were prepared by mixing CaEGTA and EGTA in the following way: for 750 nM $[Ca^{2+}]_i$ 5 mM CaEGTA, and 1 mM EGTA; for 950 nM $[Ca^{2+}]_i$ 9.5 mM CaEGTA, and 1.5 mM EGTA; and for 1,200 nM $[Ca^{2+}]_i$ 8 mM CaEGTA, and 1 mM EGTA. Statistics are in the form mean ± SEM. Average time denotes the average length of recordings.

(n = 15), assuming the change was linear. Given that an average vesicle diameter is 120 nm (Fernandez and Staechelin, 1985), ~10 vesicles fuse per second to account for the rise in the membrane area at 950 nM $[Ca^{2+}]_i$. An increase in diameter was observed with an increase in C_m (Fig. 2), thus maintaining a constant specific capacitance. This suggests that membrane has been inserted into the plasma membrane.

No large increases in C_m were observed in cells dialyzed with low $[Ca^{2+}]$ (~30 nM; Fig. 3); in fact, a small

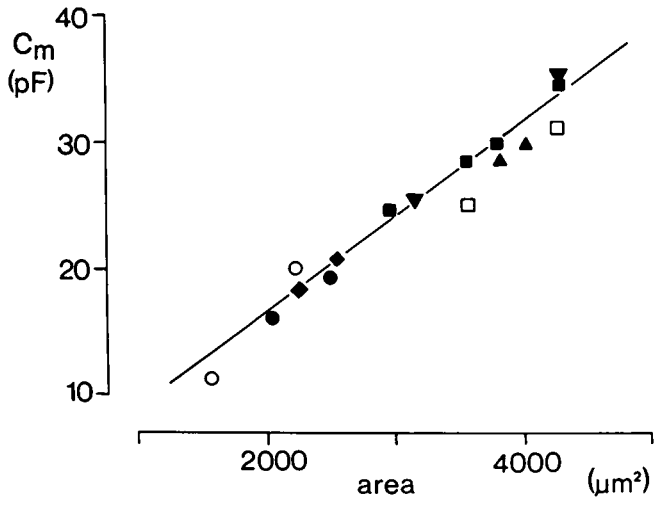

FIGURE 2 Correlation between cell surface area and membrane capacitance (C_m). Aleurone protoplasts appear to be spherical with diameters of 20–40 μm. Therefore, we would expect C_m to equal $c \cdot \pi \cdot d^2$, where c is specific capacitance and d, diameter (see text). Line was drawn according to the Eq. 1 of slope 7.5 mF/m². Filled symbols of the same shape represent measurements taken on the same cell as they increased in diameter and capacitance upon dialysis with 750 or 950 nM Ca^{2+} (1 mM EGTA and 5 or 6 mM Ca-EGTA). Open symbols show representative examples where cells were dialyzed with solutions containing EGTA only (1 or 5 mM) and a decrease in diameter was observed. See Fig. 1 for methods.

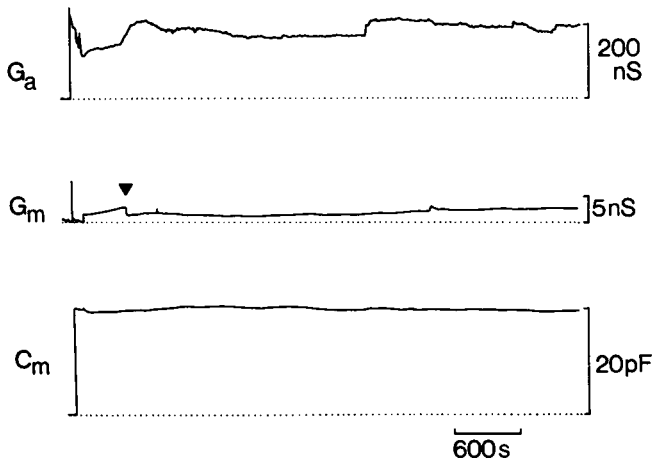

FIGURE 3 Changes with time in G_a, G_m, and C_m (as in Fig. 1), but cell dialyzed with low Ca-pipette filling solution (only 3.5 mM EGTA added, giving $[Ca^{2+}]_i$ of ~30 nM [Zorec et al., 1991b]). Note that relatively large changes in G_a are not projected to the time course of C_m. Filled triangle indicates time when holding potential was set to −85 mV. In this cell there was no significant change in diameter at the end of the experiment.

decrease in C_m was observed in 50% of cells ($n = 8$). The average change in C_m was a decrease of ~1% (see Table 1); the average rate was -0.1 ± 0.4 fF/s, which is significantly lower than for cells dialyzed with high $[Ca^{2+}]$ (950 nM). Cells with a large decrease in C_m decreased in diameter (Fig. 2).

This demonstrates an important role of cytosolic $[Ca^{2+}]$ in controling changes in the area of plasma membrane in aleurone protoplasts. As in animal secretory cells (Penner and Neher, 1989), the increase in C_m of aleurone protoplasts can be related to the increased secretory activity due to exocytosis (more precisely, the rate of exocytosis being greater than that of membrane retrieval). The decrease in C_m with low $[Ca^{2+}]$ (~30 nM) indicates a greater rate of membrane retrieval due to endocytosis, which is apparently insensitive to very low levels of $[Ca^{2+}]$, as in animal cells (von Grafenstein et al., 1986). Decreases in C_m due to low activities of cytosolic $[Ca^{2+}]$ suggests a reduction in the basal rate of exocytosis which normally balances membrane retrieval. This is consistent with reports of basal secretion in unstimulated aleurone protoplasts (see Fig. 3 of Bush et al., 1986).

In summary, using membrane C_m measurements we have shown that increases in membrane area depend on increases in cytosolic $[Ca^{2+}]$, which demonstrates directly the existence of exocytosis in plant cells. This is likely to be physiologically relevant to the stimulation of secretion of hydrolases by GA_3. In the presence of GA_3 it has been shown that cytosolic $[Ca^{2+}]$ increases (Bush and Jones, 1988). Moreover, the abscisic acid (ABA) inhibition of secretion of hydrolases, is correlated with a decrease in cytosolic $[Ca^{2+}]$ in this tissue (Wang et al., 1991).

These results show similarities between control mechanisms regulating exocytotic secretion and membrane turnover in animal and plant cells, although the structure of the latter is very different. This is not unexpected given the similarities that are being discovered in the properties of ion channels in animal and plant cells (Hille, 1984; Tester, 1990).

This work was started when Mark Tester visited Robert Zorec on a Royal Society/Yugoslavian Academies of Sciences Exchange Fellowship, and was completed with support from the Research Councils of Slovenia and of Australia. We particularly thank Drs. S. D. Tyerman, B. R. Terry, and colleagues of The Flinders University of S.A.; we also thank Dr. John Dempster for providing the computer software. Robert Zorec would like to thank Professor M. Kordaš for support.

Received for publication 19 November 1991 and in final form 17 March 1992.

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