Unitary exocytotic and endocytotic events in guard-cell protoplasts during osmotically driven volume changes

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Abstract Osmotically driven swelling and shrinking of guard-cell protoplasts (GCPs) requires adjustment of surface area which is achieved by addition and removal of plasma membrane material. To investigate the mechanism for adaptation of surface area we have used patch-clamp capacitance measurements. The recorded membrane capacitance (C_m) trace of swelling and shrinking GCPs occasionally revealed discrete upward and downward deflecting capacitance steps, respectively, with a median value of about 2 fF. The observed capacitance steps resulted from the fusion and fission of single vesicles with a diameter of around 300 nm. We conclude that exo- and endocytosis of these vesicles accommodate for osmotically driven surface area changes in GCPs.

Key words: Cell swelling-shrinking; Exocytosis; Endocytosis; Guard-cell; Membrane capacitance; Membrane tension

1. Introduction

During stomatal movement, guard-cells undergo large and reversible changes in cell volume as a result of changes in ion accumulation and net water fluxes. These osmotically driven alterations in volume are accompanied by large changes in plasma membrane surface area of up to 40% [1]. Patch-clamp capacitance measurements of guard-cell protoplasts (GCPs) revealed that expansion and reduction of surface area are the result of addition and removal, respectively, of equivalent portions of membrane material [2]. In that study it was shown that the addition and retrieval of membrane material is Ca^{2+}-independent, but that the rate of changes in surface area is proportional to the osmotic potential difference across the plasma membrane. This suggests that fusion and fission of membrane material is under the control of membrane tension [2,3]. Similarly, osmotically driven changes in surface area have also been reported for animal cells and in particular for neurons [4–6]. Like in GCPs, these surface area changes were found to be independent of Ca^{2+} and interpreted as tension-driven fusion and fission of membrane material [7]. This suggests a common mechanism within eukaryotes for accomplishment of surface area adaptation under unbalanced osmotic conditions.

A remarkable feature of the osmotically driven surface area excursions is the high rate at which changes occur. In opening or closing stomata the surface area of guard-cells can vary by about 14% within 10 min [8]. This raises the question about origin and destination of the large amount of membrane material required for these changes. In the past, these osmotically driven changes in surface area in guard-cells and their protoplasts have been investigated using electron microscopy and uptake of fluid phase markers [9]. Using the latter technique large vesicles with a diameter of about 1 to 3 μm were found to be incorporated during shrinkage of GCPs [9]. However, these giant vesicles were neither observed to re-fuse with the plasma membrane upon re-swelling, nor were giant vesicles found in hyper-osmotically treated intact guard-cells [9]. This questions the physiological relevance of the formation of these giant vesicles in surface area changes. In addition, it still leaves the question of the origin of the membrane required for surface expansion unanswered. A scrutiny of electronmicrographs of osmotically treated guard-cells has so far not identified a reservoir of membrane material which could accommodate for these surface area variations [9]. Thus, currently neither the origin of the large area of membrane incorporated during surface area expansion nor the destination of the membrane retrieved during shrinkage is known.

Recently, the fluorescent membrane marker FM1-43 has been used to monitor changes in surface area in shrinking GCPs [10]. It was found that osmotic contraction of the protoplasts caused an internalization and subsequent diffuse distribution of FM1-43 label throughout the cytosol [10]. This is best explained by the retrieval of FM1-43 label from the plasma membrane and its internalization via vesicles which are too small to be resolved in laser scanning micrographs. On this background we have used patch-clamp capacitance measurements in the whole-cell configuration to further investigate the hypothesis that osmotically induced surface area changes are accomplished by fusion and fission of small vesicles. When compensating the electrical parameters of the cell this technique allows high resolution measurements of surface area changes in single cells [11]. Using this approach we have been able to resolve the fusion and fission of single vesicles as small as 150 nm in swelling and shrinking GCPs. This confirms the view that changes in plasma membrane surface area are accomplished by fusion and fission of small vesicles.

2. Materials and methods

GCPs were prepared from *Vicia faba* L. c.v. Hangdown as described previously [2].

Standard patch-clamp whole-cell recordings were used to measure the membrane capacitance (C_m) [12]. Patch pipettes were prepared as described previously [2]. Protoplasts were clamped at −50 mV to −70 mV, on which a sine-wave voltage (55 mV or 111 mV rms) was superimposed at 1600 Hz. After attaining the whole-cell configuration the bulk of the C_m of the cell was compensated using the cancellation circuitry of the dual-phase lock-in amplifier (SWAM IIC, Henigman,

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Abbreviations: GCP, guard-cell protoplast; C_m, membrane capacitance; G, conductance

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Piror, Slovenia). The phase angle was set such that one of the outputs of the dual-phase lock-in amplifier signal was directly proportional to changes in C_m and the other to changes in the sum of membrane conductance (G) and access G [12]. Signals were filtered at 3 Hz or 10 Hz and acquired at 100 Hz by an A/D converter (DigiData 1200, Axon Instruments, Foster City, CA, USA) and the WCP software (Dempster, University of Strathclyde, UK). Upward deflecting steps in C_m were interpreted as unitary exocytotic events and downward deflecting steps as single endocytotic events [11]. To correct for potential variations in the access G during a measurement we frequently applied calibration pulses with a defined amplitude of 100 fF (e.g. Fig. 1A). The absolute value of step changes in C_m were obtained after scaling to the measured amplitude of the nearby calibration pulse. Recordings were made at room temperature. Data are given as the mean ± S.E.M.

Protoplasts were bathed in a solution consisting of (in mM): 10 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 5 MES-KOH, pH 5.6 adjusted to 520 mosmol/kg with sorbitol. Patch-pipettes were filled with a solution containing (in mM): 170 K-gluconate, 10 KCl, 2 $MgCl_2$, 2EGTA, 2 K_2ATP, 10 HEPES-KOH, pH 7.8, osmolarity was adjusted with sorbitol to 520 mosmol/kg (isotonic pipette solution), 620 mosmol/kg (hyper-osmotic pipette solution) or 420 mosmol/kg and 479 mosmol/kg (hypo-osmotic pipette solution).

3. Results and discussion

For the investigation of the mechanism of surface area changes we have carried out patch-clamp capacitance measurements in GCPs in the whole-cell configuration. Immediately after establishment of the measuring configuration the bulk of the capacitance was compensated which allows monitoring of C_m at a high gain [11]. Quantification of the rms noise level of C_m recordings in GCP in this compensated mode revealed a mean value of 0.5 fF ± 0.1 ($n = 9$). The value resembles the noise level reported from similar recordings in other cell systems where the detection of fusion and fission of vesicles with a diameter as low as about 150 of nm has been achieved [11,13].

In measurements of GCPs under isotonic conditions no change in C_m or G has been observed (Fig. 1A). A blow up of the C_m-trace in Fig. 1A reveals no distinct capacitance steps (Fig. 1B). Thus, under isotonic conditions fusion and fission of a single vesicle is either not taking place or is such a rare event that it evades detection.

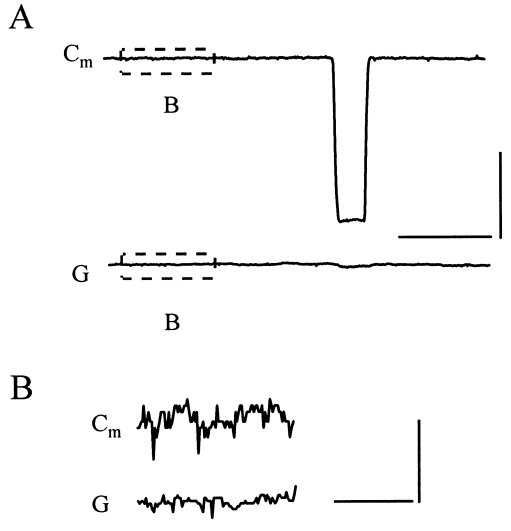

Fig. 1. High resolution measurement of C_m of GCP under steady state conditions. The GCP was dialyzed with isotonic pipette solution. A: Time dependent changes in C_m and G. Note that the C_m calibration pulse is not projected in the G trace. B: Magnified sections of the C_m and G trace marked in (A). Horizontal calibration bars: A: 1 s, B: 500 ms. Vertical calibration bars, A: 50 fF and 1 ns for the C_m trace and G trace, respectively, B: 5 fF and 100 ps for the C_m trace and G trace, respectively.

3.1. Surface area contraction is achieved by vesicular fission of plasma membrane material

For the initiation of shrinking GCPs were dialyzed with a hypo-tonic pipette solution. Within about 3 min of breaking into the whole-cell configuration perfusion of the cytosol by the pipette solution resulted in a constant osmotic potential difference across the plasma membrane [5] and corresponding steady net water efflux and protoplast shrinkage. Fig. 2A illustrates a part of a measurement of C_m and G from a GCP recorded about 4 min after onset of internal perfusion. The C_m trace shows an overall decrease at a rate of 30 fF s^{-1}. The average rate of ΔC_m from the four recordings analyzed was 19.6 fF s^{-1}, ± 12.3 fF s^{-1} [range 6–32 fF s^{-1}]. This is about six times faster than the mean rate of ΔC_m measured under the same osmotic conditions in GCPs in the non-compensated mode [2], but within the large scattering of the measured values. At high gain C_m measurements now allow a microscopic view on the elementary processes of surface area decrease. A blow up of the steadily decreasing C_m record in Fig. 2A reveals infrequently distinct downward deflecting capacitance steps followed by a plateau (Fig. 2B). These steps were not reflected in the G trace confirming the appropriate setting of the phase angle [12]. Therefore each of these steps in C_m can be interpreted as distinct decrease in the surface area of the plasma membrane due to the fission of single endocytotic vesicles. Fig. 2D shows similar endocytotic steps in C_m recorded from three other GCPs under conditions of shrinkage. For general analysis of the data we defined all downward deflecting steps which reached a plateau lasting > 50 ms (five times the sampling rate) as the result of the fission of a single vesicle from the plasma membrane. We further defined the resolution limit as twice the rms noise level of the C_m record. Thus, considering a rms noise level of 0.6 fF for the recording shown in Fig. 2A and taking into account a specific C_m of 8.1 mF min^{-2} for GCPs [2] vesicles as small as 200 nm could be reliably detected. A histogram of the endocytotic capacitance steps obtained with this criteria under conditions of protoplasts shrinking is shown in Fig. 4A. The median value for endocytotic steps was 2.3 fF. This corresponds to the fission of spherical vesicles with a diameter of 300 nm and implies that osmotically induced decrease in surface area is mainly due to the fission of vesicles with a diameter about 300 nm.

However, the bulk of the C_m recording decreased without resolvable discrete steps (e.g. Fig. 2C). Notably, the plateaus that generally followed discrete downward deflecting (endocytotic) capacitance steps led to the assumption that there was no unspecific drift in the recorded C_m trace. This means that the decrease in C_m without resolvable discrete steps (as in Fig. 2C) is most likely due to the fission of vesicles too small be resolved as steps in C_m. Hence, the median value for endocytotic steps deduced from Fig. 4A is probably overestimated. Smaller, unresolved steps resulting from the fission of vesicles below the resolution limit (< 150 nm) most likely also contribute significantly to the reduction of surface area.

To sum up, the present results demonstrate that patch-clamp capacitance measurements in the compensated mode

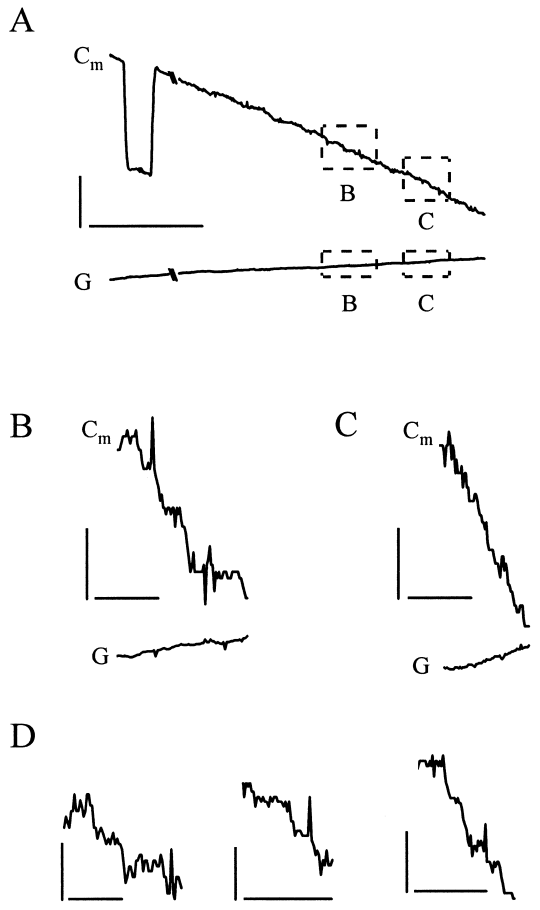

Fig. 2. High resolution measurement of C_m of GCP under the conditions of shrinking. The GCP was dialyzed with hypo-osmotic pipette solution. A: Time dependent changes in C_m and G. Note that the C_m calibration pulse is not projected in the G trace. B,C: Magnified sections of the C_m and G trace marked in (A) showing discrete downward deflecting (endocytotic) capacitance steps (B) and continuous change in C_m without resolvable steps (C). D: Sections of high resolution measurements of C_m showing endocytotic steps recorded from three different GCPs under conditions of shrinking. Horizontal calibration bars, A: 1 s B–D: 250 ms. Vertical calibration bars, A: 50 fF and 1 nS for the C_m trace and G trace, respectively, B–D: 5 fF and 100 ps for the C_m trace and G trace, respectively.

allow the detection of steps in C_m resulting from the interaction of single vesicles with the plasma membrane in GCPs. In shrinking GCPs the resolved steps in C_m were all reflecting a decrease in surface area. Thus shrinkage of GCPs is indeed associated with retrieval of vesicles well below 0.5 μm from the plasma membrane.

Following fluid-phase uptake of fluorescence markers in the course of osmotically driven shrinking led to the detection of a few endocytotic vesicles of 0.5 μm to 3 μm in diameter in onion epidermal cells [14], rye leave protoplasts [15], *Chenopodium album* protoplasts [16] and *Pisum sativum* guard-cell protoplasts [9]. Endocytosis of vesicles of this size would result in capacitance steps in the range of 6–230 fF. In the four recordings analyzed we never observed steps >9 fF e.g. vesicle diameter >0.6 μm. This furthermore supports the view that formation of the giant vesicles is not obligatory in the process of surface area contraction in GCPs. In addition, the interpretation that in a diffuse internalization of fluorescent label observed in shrinking GCPs is due to fission and internalization of small vesicles from the plasma membrane [10], is also in accordance with this view. It can therefore be concluded that internalization of small vesicles is the default mechanism by which protoplasts reduce their surface area.

3.2. Surface area increase is due to fusion of vesicles with the plasma membrane

On this background we examined whether the reverse mechanism namely fusion of vesicular membrane with the plasma membrane is accomplishing surface area expansion. We have therefore carried out whole-cell patch-clamp capacitance measurements in GCPs which were dialyzed with hyper-osmotic pipette solution, a procedure resulting in continuous swelling of the protoplast. Fig. 3A shows part of a measurement of C_m and G in a GCP after capacitance compensation

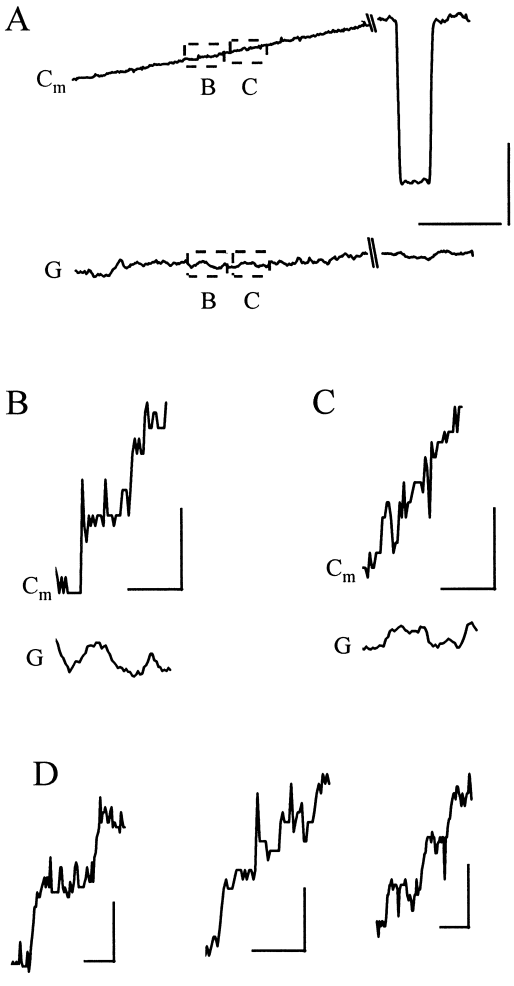

Fig. 3. High resolution measurement of C_m of GCP under the conditions of swelling. The GCP was dialyzed with hyper-osmotic pipette solution. A: Time dependent changes in C_m and G. Note that the C_m calibration pulse is not projected in the G trace. B,C: Magnified sections of the C_m and G trace marked in (A) showing discrete upward deflecting (exocytotic) capacitance steps (B) and continuous change in C_m without resolvable steps (C). D: Sections of high resolution measurements of C_m showing exocytotic steps recorded from three different GCPs under conditions of swelling. Horizontal calibration bars, A: 1 s B–D: 250 ms. Vertical calibration bars, A: 50 fF and 1 ns for the C_m trace and G trace, respectively, B–D 2 fF and 100 ps for the C_m trace and G trace, respectively.

recorded about 15 min after establishment of the whole-cell configuration. In the present example the overall increase in C_m was 7.5 fF s^{-1}. The average rate of increase in C_m was 4.7 fF s^{-1}, ±2.3 fF s^{-1} (range 2–9 fF s^{-1}) for the five recordings analyzed. This is similar to what has been obtained from measurements in the non-compensated mode in GCPs with the same osmotic potential difference [2]. The blow-up of the steadily increasing capacitance trace reveals infrequently discrete upward deflecting steps in C_m followed by a plateau (Fig. 3B). These C_m steps were not correlated with changes in the G trace and can therefore be interpreted as the result of an increase in surface area due to the fusion of single vesicles with the plasma membrane. Fig. 3D represents similar upward deflecting (exocytotic) steps from measurements of C_m in three other GCPs under conditions of swelling. As for shrinking GCPs, the plateau which followed the discrete upwards deflecting steps (Fig. 3B and D) suggests that there is no unspecific drift in the C_m trace. Again, it is therefore most likely that the rise in C_m without discrete capacitance steps (Fig. 3C) resulted from the fusion of vesicles too small to be resolved with the recording equipment. Considering a resolution limit twice the rms noise level, the detection of exocytotic steps in the measurement presented in Fig. 3A is limited to events >0.8 fF. Hence, fusion of a spherical vesicle with a diameter >180 nm can be reliably detected. The distribution of all resolved exocytotic steps from five recordings analyzed is presented in Fig. 4B. The histogram exhibits a median value for exocytotic steps of 2.1 fF. This corresponds to the fusion of vesicles with a diameter of 290 nm.

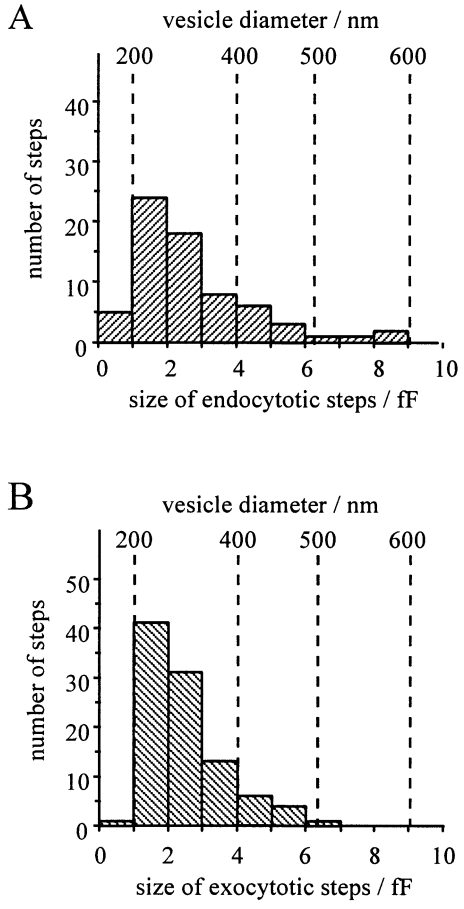

Fig. 4. A: Distribution of step amplitudes of endocytotic capacitance steps ($n=68$, recorded in four GCPs) B: Exocytotic capacitance steps ($n=83$, recorded in five GCPs).

These measurements indicate that exocytosis of small vesicles accounts for osmotically driven surface area expansion. Again, it is most likely that fusion of vesicles with a diameter below the resolution of the recordings also contributed to this process (see above).

3.3. A pool of small vesicles is proposed to participate in surface area changes

The distribution of the exocytotic steps is very similar to that of the endocytotic steps. Thus, based on size there is no difference between vesicles incorporated during surface area expansion and those retrieved during shrinkage. This view is consistent with observations in *Chenopodium album* protoplasts [16]. In that study it was shown that a fluid phase marker which had been taken up by vesicles in the course of shrinking was discharged into the external medium from these vesicles upon re-swelling of the protoplasts. We therefore propose shuttling of vesicles between a cytoplasmic pool and the plasma membrane in the course of osmotically driven changes in surface area. In this context it is reasonable to assume that lysis of swelling protoplasts occurs once the pool of vesicular membrane is depleted [17]. The average maximum increase in surface area of GCPs before bursting was found to be 60%. Individual protoplasts could even increase by up to 90% [2]. Hence, the size of a putative vesicle pool must be envisaged large enough to accommodate for the extent of these changes. Taking into account the average initial size and capacitance of GCPs protoplasts [2] a change in surface area of 60% is equivalent to a change in capacitance of 4.3 pF. Considering furthermore 300 nm (2.3 fF) as a representative value for the diameter of exocytotic vesicles an increase in surface area by 60% corresponds to at least 1900 vesicles which should at minimum be present in the cytosol. The volume of a GCPs of 15 μm in diameter is 1.8×10^{-15} m^3 of which about 10% is cytoplasmic. The 1900 vesicles expected to be found in a GCP would occupy a volume of 2.7×10^{-17} m^3 which corresponds to 15% of the cytoplasmic volume.

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