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Transient and Permanent Fusion of Vesicles in *Zea mays* Coleoptile Protoplasts Measured in the Cell-attached Configuration

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Abstract. Exocytosis in protoplasts from *Zea mays* L. coleoptiles was studied using patch-clamp techniques. Fusion of individual vesicles with the plasma membrane was monitored as a step increase of the membrane capacitance (C_m). Vesicle fusion was observed as (i) An irreversible step increase in C_m. (ii) Occasionally, irreversible C_m steps were preceded by transient changes in C_m, suggesting that the electrical connection between the vesicle with the plasma membrane opens and closes reversibly before full connection is achieved. (iii) Most frequently, however, stepwise transient changes in C_m did not lead to an irreversible C_m step. Within one patch of membrane capacitance steps due to transient and irreversible fusions were of similar amplitude. This suggests that the exocytosis events do not result from the fusion of vesicles with different sizes but are due to kinetically different states in a fusion process of the same vesicle type. The dwell time histogram of the transient fusion events peaked at about 100 msec. Fusion can be described with a circular three-state model for the fusion process of two fused states and one nonfused state. It predicts that energy input is required to drive the system into a prevailing direction.

Key words: Capacitance — Exocytosis — Endocytosis — Transient and permanent membrane fusion — Maize coleoptile

Introduction

Electron micrographs of grass coleoptile cells show in the cytoplasm a prominent population of vesicles with a diameter of 80–100 nm (Pope et al., 1979; Quaite, Parker & Steer, 1983; Phillips, Preshaw & Steer, 1988). Quantitative analysis of these micrographs revealed that the number of these vesicles increases under the influence of the phytohormone auxin in growing cells (Phillips et al., 1988). The authors hypothesized that these vesicles transport compounds for secretion and cell wall synthesis, and that the vesicles are required for enlargement of the cell membrane of the growing coleoptile. Estimates of the rate of vesicle production revealed that the membrane area supplied by exocytotic vesicles greatly exceeds the membrane area needed for cell elongation (Phillips et al., 1988). The authors proposed that a fraction of secretory vesicles on the plasma membrane (pm) is recycled after a mean resident time of about 17 min.

In a recent investigation we employed capacitance measurements in order to investigate with a higher time resolution the elementary processes underlying fusion and fission of these vesicles. These measurements showed capacitance steps of 60 to 2000 aF with a median value of about 200 aF for exo- and endocytotic events (Thiel et al., 1998). Steps in membrane capacitance (C_m) reflect changes in surface due to the fusion or fission respectively of single vesicles (Neher & Marty, 1982). The size of the C_m changes in coleoptile protoplasts corresponds to those vesicles visible in electron micrographs and confirms that C_m measurements are indeed reporting fusion and fission of these vesicles with the pm (Thiel et al., 1998). Here we consider kinetic aspects of fusion and fission suggesting the existence of two modes of fusion leading to either permanent integration of vesicle membrane into the pm or transient fusion causing only a short lasting reversible fusion of the vesicle with the pm.

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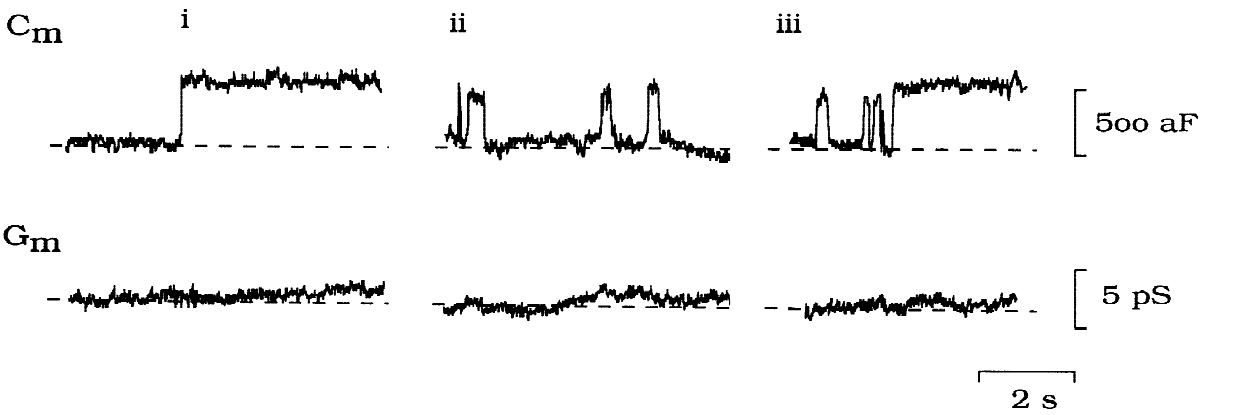

Fig. 1. Spontaneous capacitance (C_m) steps recorded in cell-attached patch on maize coleoptile protoplast. Parts of a recording showing an imaginary part and a real part of admittance, corresponding to patch capacitance (upper trace) and patch "conductance" (lower trace). Illustrated are three different categories of fusion events comprising (i) irreversible increase steps, (ii) rapid stepwise changes in C_m not leading to irreversible steps and (iii) irreversible increase steps preceded by rapid transient stepwise changes in C_m.

Materials & Methods

Protoplasts were prepared from the apex of etiolated grown coleoptiles of maize (cv, mutin, KWS Saatzucht, Einbeck, Germany) as described previously (Thiel et al., 1994). Exocytotic activity of protoplasts kept in 250 mM KCl, 10 mM $CaCl_2$, 20 mM Hepes/KOH, pH 7 was monitored with pach-clamp techniques in the cell-attached mode (Kreft & Zorec, 1997). Patch pipettes were filled with bath solution and had a resistance of 1 to 4 MΩ. The high KCl concentration was chosen for two reasons. Firstly, it assured a free running voltage of the protoplasts at about 0 mV and secondly it enabled an improved seal resistance in pure ionic solution compared to solutions with manitol or sorbitol as osmoticum. Membrane patches were clamped at 0 mV, on which a sine wave voltage (111 mV rms, 6.4 kHz) was superimposed using a SWAM Cell (Henigman, Piran, Slovenia) patch clamp amplifier. Signals were filtered at 10 Hz, −3 dB, low pass, Bessel 6-pole and sampled at 100 Hz by an A/D converter (CED 1401, Cambridge, UK). C_m step amplitudes were measured after correcting for drift in baseline.

Results

Figure 1 illustrates representative segments of a recording of capacitance (C_m) and conductance (G_m) from the plasma membrane of a maize coleoptile protoplast in the cell-attached configuration. In this configuration spontaneous unitary step increases and decreases in C_m could be recorded. Step increases and decreases in C_m report changes in plasma membrane surface due to unitary exo- and endocytotic events, respectively (Neher & Marty, 1982). In general increases and decreases of C_m steps of 50 to 1000 aF were recorded in coleoptile protoplasts; these reflect fusion and fission events respectively of vesicles from this tissue (Thiel et al., 1998).

The exocytotic steps were not stereotypic but could be grouped according to their time course into three categories (Fig. 1): (i) events characterized by a single step increase in C_m, over a period >2 sec (ii) transient C_m events were observed, each step increase followed by a decrease in C_m with an amplitude of approximately the same size and within approx. 100 msec; and (iii) events of irreversible increase steps as in (i) preceded over periods of a few sec by transient C_m steps of the category (ii).

To compare irreversible and transient stepwise capacitance increases, we compared in individual patches the amplitudes of irreversible and transient exocytotic events. To assure resolution of all transient C_m-steps only patches with a signal-to-noise ratio >3 were considered for analysis. Figure 2 illustrates a histogram of step amplitudes from both types of exocytosis. The plot summarizes data from seven patches. The distribution is normalized to the total number of transient and irreversible events. A difference between the distributions lies in the number of observations for transient steps <100 aF. These small steps wer absent from the population of irreversible steps. Further scrutiny of these small steps shows that they are due to very short-lasting events (≤30 msec) without clear plateaus (*data not shown*), and because of their short duration may not have been fully resolved by the recording equipment. At the low-pass filtering frequency of 10 Hz an event needs to exceed a duration of 30 msec to approach within 10% the true amplitude of the C_m step (for details *see* Colquhoun & Sigworth, 1995). With the exception of these small events the relative distributions of irreversible and transient events were very similar. They have a median value at about 400 aF. When considering only capacitance steps >100 aF, a Student's *t*-test reveals that the two populations were not significantly different. Hence, it appears that the vesicles, which undergo irreversible or transient fusion can not be distinguished by their size.

In eight patches with prominent transient fusion events (total of 410 increase steps) we estimated the relative occurrence of transient fusion events (Fig. 1(ii))

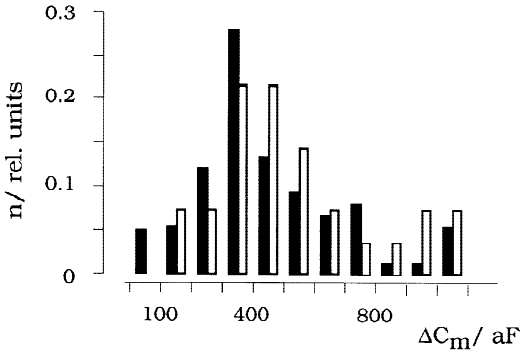

Fig. 2. Comparison between amplitudes of transient and irreversible increase steps. Data from 3 patches with 98 transient events (solid columns) and 34 irreversible (open columns) events.

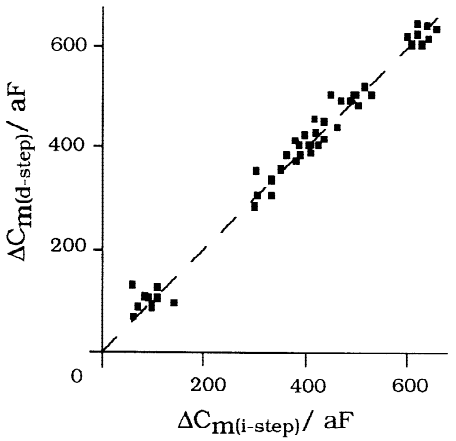

Fig. 3. Plot of increase step (i-step) amplitudes *vs.* subsequent decrease step (d-step) amplitudes. Data fall into three distinct clusters suggesting that at least three different vesicles fuse repeatedly with the examined patch of plasma membrane.

and irreversible events (Fig. 1, category (i) and final increase step in (iii)). This analysis showed that the majority (71%) of events were in these patches of transient nature. This estimation is based on the assumption that each transient event is due to the fusion of a different individual vesicle. In the case of steps with similar amplitudes (e.g., Fig. 1(ii)) it was impossible to determine whether the transient C_m events were caused by an individual vesicle which fused repetitively with the membrane or whether these events were due to the fusion of different vesicles with approximately the same size.

To determine the relative size of transient increase steps within one patch we further examined a patch in which the amplitude of unitary events revealed a large range from 55 aF to 650 aF. Figure 3 shows a plot of increase step amplitudes *vs.* subsequent decrease step amplitudes from this patch. From this plot it becomes evident, that data group into three distinct clouds along a line with a slope of one. Hence, from the three distinct clusters it can be assumed that at least three different vesicles repeatedly fuse within the patch of membrane studied. The short time gaps between fusion events of the same amplitude (e.g., Fig. 1(ii)) suggest that the respective vesicles remained docked to the pm between transient fusion events. Recycling of the same vesicle in such a short period of time appears unlikely.

To examine the life time of transient capacitance steps we measured the time between increase and subsequent decrease steps. To ensure that recordings were related to the fusion/fission of the same vesicle only steps were considered in which a decrease step occurred with an amplitude ±5% of the increase step. Figure 4*A* illustrates two transient fusion events on an expanded time scale showing that events shorter than 50 msec are still detectable. The dwell-time histogram from one patch is illustrated in Fig. 4*B*, and shows a maximum at about 100 msec. To judge the effect of data filtering on the estimation of dwell times we calculated (Corquhoun & Sigworth, 1995) the deviation of the measured life-time of the fused-vesicle state from the true life time of simulated square steps with durations ≥10 msec. This procedure corrected the measured data for filtering artefacts. The corrected dwell time histogram is illustrated as solid bars in Fig. 4*B*. This corrected distribution reveals that the decreasing number of observations of <100 msec is not due to unresolved short C_m steps and the dwell time histogram has a real maximum. Histograms with maxima were observed in four further patches. Figure 4*C* illustrates a cumulative histogram with a maximum at about 100 msec for the dwell times of transient capacitive steps (after correction for filter artefacts) collected from five patches.

The distribution of dwell times due to a reaction scheme with a preferred direction as in Fig. 5 (*see* Discussion) can be described according to Colquhoun and Hawkes (1995), with the sum of two exponentials

$$n(t) = a \cdot ((\exp(-t/\tau_1) - \exp(-t/\tau_2)) \quad (1)$$

where τ_1 and τ_2 are the time constants and a is the number of observations multipled by a normalizing factor. Fitting in the present case the individual and cumulative dwell time histograms with Eq. (1) yielded two time constants of $\tau_1 = 117/106$ msec and $\tau_2 = 55/40$ msec for the individual and cumulative histogram, respectively.

Discussion

In a previous study it had been shown that measurements of cell membrane capacitance are suitable to monitor in maize coleoptile protoplasts the fusion and fission of single vesicles with and from the plasma membrane, respectively (Thiel et al., 1998). In the present investiga-

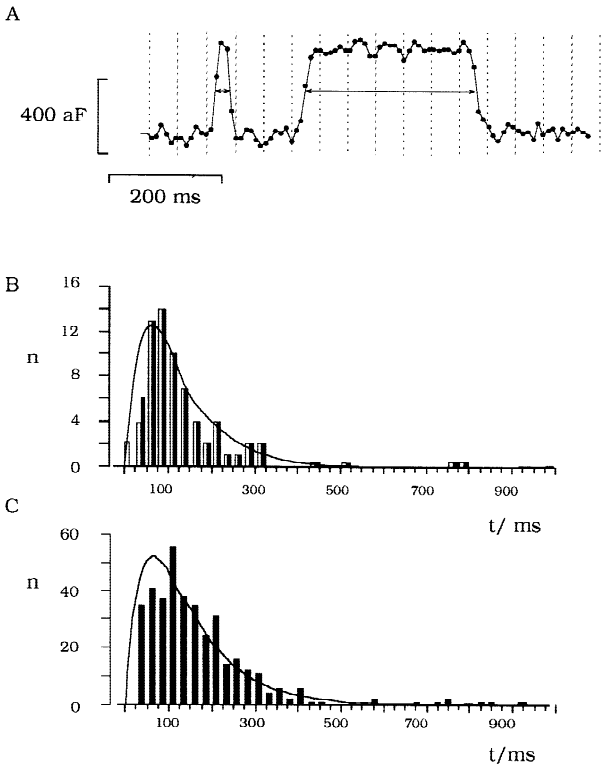

Fig. 4. Estimation of lifetime for transient fusion events. (*A*) C_m trace on expanded time scale with two transient fusion events. Dwell time was estimated from the time between crossing 50% of the C_m step amplitude in both directions (indicated by arrows). (*B*) Histogram of the duration of transient fusion events from one patch. Open bars report measured data. Solid bars show data after correction for filter artefact. (*C*) Cumulative histogram of data collected from five patients. Data fitted (line) with sum of two exponentials $n(t) = a \cdot ((\exp(-t/\tau_1) - \exp(-t/\tau_2))$, where a comprises the amplitudes of the exponentials. Fit yields two time constants of $\tau_1 = 117/106$ msec and $\tau_2 = 55/40$ msec in B/C, respectively.

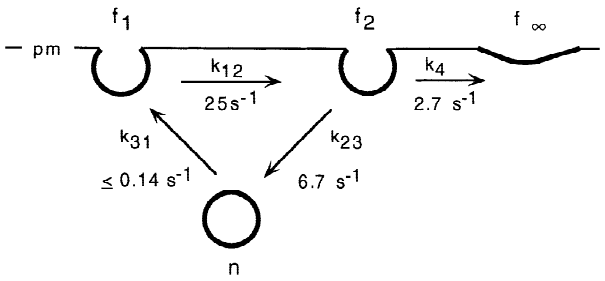

Fig. 5. Kinetic minimal model for transient fusion of a vesicle with the plasma membrane (pm). Data are consistent with a circular reaction mechanism with two kinetically distinct fused states (f_1, f_2) and a nonfused state (*n*). Reaction occurs in a preferred direction in the sense that a fusion process always starts in one fused state and then proceed via the second fused state before reentering the nonfused state. In the general model such a preferred direction of the fusion process can be obtained if $k_{12} \cdot k_{23} \cdot k_{31} \neq k_{21} \cdot k_{32} \cdot k_{13}$ where k_{21}, k_{32}, k_{13} are the back reactions of k_{12}, k_{23}, k_{31}, respectively.

tion, kinetics and amplitudes of unitary C_m increases and decreases were employed to elucidate elementary processes of exocytotic fusion in this plant cell. A key observation here is that unitary exocytotic events can either be irreversible or transient. Characteristic for transient C_m changes was that increase steps were followed within some 100 msec by a decrease step of about the same amplitude. In principle, the latter observation could mean that each increase step and each decrease step is due to independent fusion and fission of different vesicles. However, in any one patch we always observed increase steps to be followed within $\leq$2 sec by a decrease step. In a 5 min period 25 such increase steps were observed. The likelihood, that this strict sequence is due to a superposition of exactly the same number of independent fission events following by chance a fusion event is only 1.6×10^{-14} (*see* Krengel, 1998 for statistical treatments of "runs"). So, while it is very unlikely that the step changes in C_m are due to independent fusion/fission events, the present data can be understood along the lines of interpretations of similar events recorded in animal cells (e.g., Albillos et al., 1997; Breckenridge & Almers, 1987; Zimmerberg et al., 1987; Alvarez de Toledo & Fernadez, 1988; Lollike, Borregaard & Lindau, 1998; Monk, Alvarez de Toledo & Fernandez, 1990). Accordingly, single increase events as in Fig. 1(i) reveal that an exocytotic vesicle can in a single step form a fusion pore with the pm leading to irreversible incorporation of the vesicle membrane into the pm. The observation that the extra membrane that is added to the pm often appears and disappears several times before the fusion process is finalized (Fig. 1(ii)) shows that after formation of a fusion pore between vesicle and pm the pore opening can reversibly close several times before a fusion process is finalized. We also found that exocytotic events frequently did not run to completion i.e., transient stepwise changes in C_m were not followed by an irreversible fusion step (Fig. 1(ii)). The best explanation for this is that coleoptile cells possess a so called "kiss and run" mechanism, just as in animal cells. In this case a vesicle forms only transiently a fusion pore with the pm. It may be speculated that a vesicle buds off from the membrane upon delivery of its soluble cargo for recycling (Albillos et al., 1997).

For a mathematical description of the elementary fusion process we propose a markovian model with discrete states such as fused (*f*) and nonfused (*n*). A spontaneous change in C_m is therefore based on a change of the underlying state with constant transition probabilities or rate constants as defined in Fig. 5. Such a markovian model is justified because dwell times of fused and nonfused states are long compared to the times over which the transition takes place. Previous kinetic analysis of transient fusion events in degranulating mouse mast cells have revealed fused dwell times which could be de-

scribed with a single exponential (Oberhauser, Monk & Fernandez, 1992; Fernandez-Chacon & Alvarez de Toledo, 1995). Guided by the statistical analysis of single channel activity (Colquhoun & Hawkes, 1995) such a monotonically decreasing distribution of dwell times suggests that a simple reversible reaction between one fused state and one nonfused state is sufficient to describe the transient fusion mechanism. However, the bulk dwell time distributions in the present work pass a maximum. This requires a more complex model to describe the dominant process in maize protoplasts appropriately. The present data can be described with a model comprising a circular reaction mechanism with two fused states (f_1, f_2) and a nonfused state (n).

The two transient fused states reflect the fact, that the dwell time histogram of transient fusion require at least two exponentials i.e., two time constants for description. Futhermore, the maximum in the dwell time histogram demands that the reaction occurs in a preferred direction. This violation of microscopic reversibility can only be explained if the reaction cycle is coupled to an exergonic process; i.e., energy input is required to keep the system away from its thermodynamic equilibrium (for details *see* Colquhoun & Hawkes, 1995). In the general case such a preferred direction comes about if the product of rate constants in the forward direction is unequal to the product of rate constants in the backward direction. With respect to the irreversible fusion events the model requires an additional final fused state (f_∞) with back reaction. Addition of this step is justified by the observation that irreversible and reversible C_m increases are not different in amplitude. This implies that the irreversible fusion is one distinct kinetic state of the same type of vesicles undergoing reversible fusion.

The minimal model with the least number of rate constants is shown in Fig. 5. The key feature of this model is that it has a preferred direction in the sense that a fusion process must always start in one fused state and then proceed via the second fused state before reentering the nonfused state. In the special case of this minimal model the rate constants can be determined as follows: The rate constant k_{31} for leaving the nonfused state n is given by the inverse of the measured mean life-time of the nonfused state. This value is difficult to measure, because in recordings such as in Fig. 1 it is unknown whether the gaps between transient fusion events are due to only one vesicle or several. A crude estimation of the mean nonfused life-time from dwell times of gaps between transient fusion events with the same amplitude, gave a value of about 7 sec. This corresponds to a rate constant k_{31} of 0.14 sec^{-1}, a value that must be taken as an upper limit estimate. Rate constants determining fused-times are k_{12}, k_{23} and $k_{2\infty}$. Because fused dwell times were only measured for those vesicles that had completed a whole cycle $n \rightarrow f_1 \rightarrow f_2 \rightarrow n$, vesicles ending in state f_∞ are not contained in the distribution, albeit this transition shortens the life-time of state f_2. From the fact, that backward reactions are not allowed in this model, the distribution of fused-times is given by the distribution of the sum of two exponentially distributed intervals (Eq. 1) with the mean life-times τ_1 and τ_2 (Colquhoun & Hawkes, 1995). These life-times of the states f_1 and f_2, respectively are given by the corresponding rate constants:

$$\tau_1 = 1/k_{12} \text{ and } \tau_2 = 1/(k_{23} + k_{2\infty}) \quad (2)$$

From the ratio of observations N_∞ in which a fused vesicle remained fused compared to the observations N_n where the vesicle reentered the nonfused state n we obtain an equation for the ratio of the competing rate constants k_{23} and $k_{2\infty}$.

$$N_\infty/N_n = k_{2\infty}/k_{23}. \quad (3)$$

In the present case the ratio of transient (71%) *vs.* permanent fusion (29%) was about 0.41. Combining Eq. (2) and (3) we obtain the rate-constants $k_{23} \approx 6.7$ sec^{-1} and $k_{2\infty} \approx 2.7$ sec^{-1}. Modulation of this step could serve as a regulatory mechanism to switch between transient and permanent fusion (Alés et al., 1999). All rate constants of the reaction scheme are summarized in Fig. 5.

The interpretation of the present data in the context of a reaction scheme with a preferred direction implies that the reaction steps are coupled to a source of energy. Against the background of recent advances in understanding membrane fusion in animal cells it can be assumed that this input of energy is generated by SNARE proteins (Jahn & Hanson, 1998). This hypothesis is fostered by the fact that SNARE-like proteins have now also been found abundantly in the plant genome (rev. Battey et al., 1999; Blatt et al., 1999).

Circumstantial evidence has already suggested in the past that exocytotic vesicles in plant cells are recycled (rev. Low & Chandra, 1994). The present data now uncover that plant cells are apparently also capable of a "kiss and run" type of exocytosis. In physiological terms this could mean that secretory vesicles discharge their soluble diffusable content to the cell exterior by only forming a short-lasting fusion contact with the plasma membrane. It may be speculated that in this way the vesicles retain for economic reasons the vesicular matrix content. This could include for example proteins required for the maturation of secreted cell wall components or proteins (Brummell, Camirand & MacLachlan, 1990). The retained vesicle content might be re-used in subsequent cycles.

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