Capacitance Flickers and Pseudoflickers of Small Granules, Measured in the Cell-Attached Configuration

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ABSTRACT We have studied exocytosis of single small granules from human neutrophils by capacitance recordings in the cell-attached configuration. We found that 2.2% of the exocytotic events were flickers. The flickers always ended with a downward step. This indicates closing of the fusion pore. During flickering, the fusion pore conductance remained below 1 nS, and no net membrane transfer was detectable. After fusion pore expansion beyond 1 nS the pore expanded irreversibly, leading to rapid full incorporation of the granule/vesicle into the plasma membrane. Following exocytosis of single granules, a capacitance decrease directly related to the preceding increase was observed in 7% of the exocytotic events. This decrease followed immediately after irreversible pore expansion, and is presumably triggered by full incorporation of the vesicle into the patch membrane. The capacitance decrease could be interpreted as endocytosis triggered by exocytosis. However, the gradual decrease could also reflect a decrease in the "free" patch area following incorporation of an exocytosed vesicle. We conclude that non-stepwise capacitance changes must be interpreted with caution, since a number of factors go into determining cell or patch admittance.

INTRODUCTION

During exocytosis, preformed vesicles fuse with the plasma membrane leading to the release of their content. Exocytosis of single vesicles or granules has been measured as a stepwise increase in membrane capacitance (on-step) in a variety of cell types (Neher and Marty, 1982; Fernandez et al., 1984; Nüsse and Lindau, 1988; Lindau et al., 1993; Scepek and Lindau, 1993; Lollike et al., 1995). The on-step is sometimes followed by a downward off-step of similar size, which suggests that fusion of a vesicle with the plasma membrane can be reversible and transient. This means that a fusion pore can open—connecting the vesicular lumen to the extracellular space—and subsequently close again. This transient opening of a fusion pore has been termed "flicker," analogous to the brief opening of ion channels (Fernandez et al., 1984). These capacitance flickers have been mainly characterized for the very large granules from mast cells (Spruce et al., 1990; Monck et al., 1990; de Toledo et al., 1993). Flickers have also been observed in fusion of cells expressing viral fusion proteins [e.g., hemagglutinin (HA)] with either target cells or artificial bilayers (Spruce et al., 1989, 1991; Tse et al., 1993; Melikyan et al., 1993, 1995; Zimmerberg et al., 1994). Although the initial fusion pore conductance is small, similar to that of an ion channel (Breckenridge and Almers, 1987; Spruce et al., 1990; Melikyan et al., 1995), the pore can expand to a conductance of at least 8 nS and still close (Spruce et al., 1990; Melikyan et al., 1995). During flickering, a net membrane transfer from the plasma membrane to the granule membrane has been observed in mast cells (Monck et al., 1990; Oberhauser and Fernandez, 1996).

In the whole-cell patch-clamp configuration the properties of transient fusion events can only be resolved for very large granules. We recently developed an improved method to measure small capacitance changes in cell-attached patches with a resolution below 0.1 fF (Lollike et al., 1995). This method allows for determination of fusion pore dynamics of vesicles with capacitance as small as 1 fF (Lollike et al., 1995). Recently, this method was combined with amperometric detection of transmitter release by inserting a carbon fiber electrode into the patch pipette. This technique of patch amperometry revealed capacitance flickers in chromaffin cells, which allow for transmitter release without full fusion (Albillos et al., 1997). Here we demonstrate capacitance flickers of small granules in neutrophils. Flickers are thus a general feature of fusion and not confined to exocytosis of giant granules or viral-induced fusion of cells. Due to the improved sensitivity in patch recordings, the properties of such flickers can be resolved in much more detail than in whole-cell recordings. We found that the properties of flickers in neutrophils differ from those reported in mast cells. During flickering of neutrophil granules the fusion pores retained a conductance below 1 nS and the flickering granules never underwent full fusion. We also observed a phenomenon that we termed "pseudoflickering": a gradual capacitance decrease that follows immediately after pore expansion of a preceding exocytotic event. In contrast to regular capacitance flickers, these pseudoflickers cannot be explained by re-closing of the fusion pore. We discuss uncertainties associated with interpreting non-stepwise ad-

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mittance changes that may be affected by factors other than exo- and endocytosis. In view of the increasing applications of patch capacitance measurements (Lollike et al., 1995; Kreft and Zorec, 1997; Albillos et al., 1997) we consider it important to point out the possibility of specific artefacts which could be introduced with this method.

METHODS

Isolation of human neutrophils

Human neutrophils were isolated from peripheral blood from volunteer donors as previously described (Boyum, 1968; Lollike et al., 1995), and resuspended in ES buffer [140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.2–7.3)] at 4°C and used for patching within 3 h.

Cell-attached patch-clamp capacitance measurements

Patch-clamp capacitance measurements were performed as previously described (Lollike et al., 1995). In short, a sine wave voltage with a frequency of 8 kHz and 50 mV rms amplitude centered at 0 mV was applied to the pipette in the cell-attached configuration. At this frequency fusion pore conductances are well-resolved in neutrophils (Lollike et al., 1995). The resulting sine wave current output from the patch-clamp amplifier (EPC7, Heka-electronics, Lambrecht, Germany) was fed into a lock-in amplifier (SR830 DSP, Stanford Research Systems, Stanford, CA) for phase-sensitive detection. The built-in low pass filter of the lock-in amplifier was set to 3 ms, 24 dB. The in-phase [real component (*Re*) corresponding to conductance] and 90° out-of-phase [imaginary component (*Im*) corresponding to capacitance] currents were sampled every 12.5 ms by a computer (Falcon 030, Atari, Sunnyvale, CA) equipped with a 16 bit A/D converter (DAS1159, Analog Devices, Norwood, MA). The slow capacitance compensation was set to 0.2 pF, 0.2 μS, and the sine wave current was minimized using C-fast compensation. The approximate phase of the lock-in amplifier was set before recordings by changing the slow capacitance compensation of the EPC7 and adjusting the phase of the lock-in amplifier as described (Neher and Marty, 1982).

Patched cells were stimulated by ejecting ionomycin (200 μM) (Sigma, St. Louis, MO) from a pipette positioned ~100 μm from the cell. All recordings were performed at room temperature.

Data analysis

The correct phase setting was inspected by eye in the recorded traces. If C steps in the imaginary part (Im) were consistently accompanied by a projection into the real part (Re), or if conductance changes were accompanied by a projection into the *Im* trace, then the phase was rotated until the phase setting was correct. This phase shift was usually within ±5°. When the fusion pore conductance is small, the signal in the Im trace is reduced compared to the size that is obtained when the vesicle is completely fused. Simultaneously, a signal appears in the Re trace as long as the fusion pore conductance G_p is comparable to ωC_v, with C_v being the capacitance of the fusing vesicle and ω being $2\pi f$ (f = sine wave frequency). For convenience of notation we use *Re* and *Im* for the changes in the real and imaginary trace after baseline subtraction. These changes are given by $Re = [(\omega C_v)^2/G_p]/(1 + (\omega C_v/G_p)^2)$ and $Im = \omega C_v/(1 + (\omega C_v/G_p)^2)$ and can be thus be used to calculate the true values of C_v and G_p (Lindau, 1991):

$$C_v = [(Re^2 + Im^2)/Im]/\omega \quad (1)$$

$$G_p = (Re^2 + Im^2)/Re \quad (2)$$

To avoid division by zero, C_v was set to zero for $Im = 0$. G_p was set to zero for $Re = 0$, and $Im < C_v/2$. Determination of fusion pore conductance was discontinued when $Re \leq = 0$ while $Im > C_v/2$.

RESULTS

Fusion pore flickering

Fig. 1 *A* depicts part of a capacitance recording from a human neutrophil in the cell-attached configuration showing spontaneous exocytotic activity. In the capacitance trace (Im) initially two large capacitance steps occur in rapid succession. The associated transients in the conductance trace (Re) are as expected from the opening of the fusion pores. Besides the transient increase, the Re trace shows no persistent change associated with exocytosis of the two large granules, indicating that the phase setting is correct. A few seconds later, the capacitance on-step (marked I) is followed by an off-step of the same size. Subsequently, several more on/off-steps are seen (II–VI) until the capacitance remains at the lower level. The multiple upward and downward capacitance steps seen in the Im trace are all of the same size (1.15 fF ± 0.05 fF), indicating that a single granule opens and closes its fusion pore repeatedly. In 670 individual exocytotic events (on-steps), only 57 steps were followed by another on-step of similar size (±0.05 fF). The probability that two subsequent capacitance steps have the same size at our experimental resolution is thus 8.5%. The probability that the trace in Fig. 1 *A* represents single exocytotic events of six different granules, all of the same size, is thus vanishingly small (4×10^{-7}). The close agreement between the size of on- and off-steps indicates that no detectable membrane transfer occurred during flickering.

The capacitance flickers are associated with clear deflections in the Re trace (Fig. 1 *A*), indicating a low fusion pore conductance during flickering. Part of the recording is shown on an expanded scale in Fig. 1 *B* together with the capacitance of the vesicle (C_v) and the fusion pore conductance (G_p) calculated from *Im* and *Re* using Eqs. 1 and 2, respectively. The C_v trace shows clear transitions between two levels. Sometimes the fusion pore opening is very brief (*asterisk* in Fig. 1 *B*) such that the full capacitance level is not attained due to the filtering of the lock-in output. This effect is analogous to the reduced amplitude of short events in single channel recordings (Colquhoun and Sigworth, 1995). The G_p trace shows that the fusion pore conductance is either unmeasurably small (pore closed), or fluctuates around 500–600 pS (pore open). The histogram of Fig. 2 *A*, indicating the time this pore spent at different conductance levels, shows a clear peak around 500–600 pS. During the total flicker, as measured from the first on-step (Fig. 1 *A*, I) to the last off-step (Fig. 1 *A*, VI), the pore was open 76% of the time. For the last, long flicker (VI) the fusion pore conductance could not be determined due to changes in the slope of the baseline in Im during the flicker.

We observed a total of 25 flickers in 1158 exocytotic events, giving a frequency of 2.2%, similar to the value

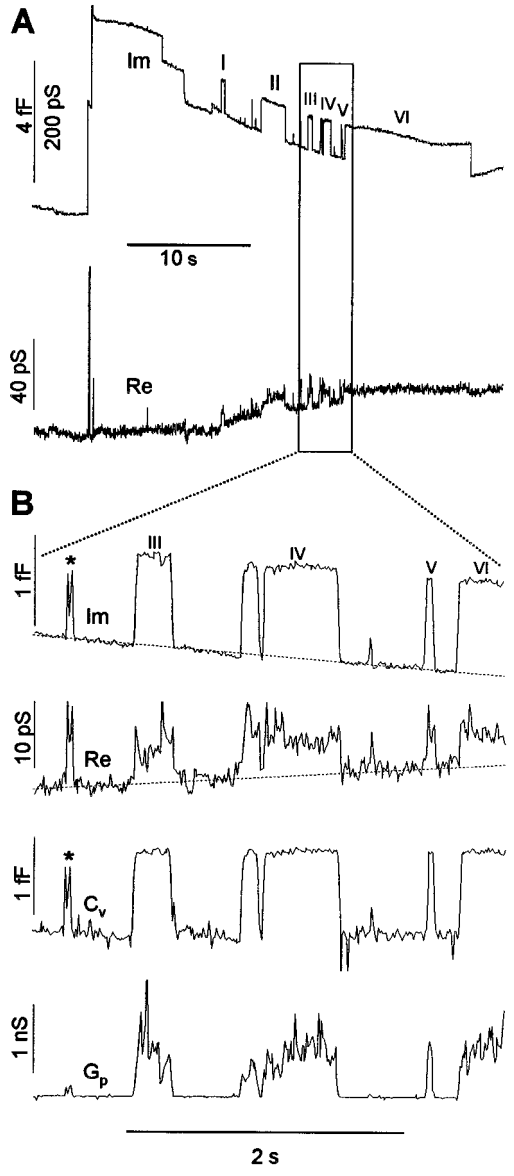

FIGURE 1 Capacitance flicker. (*A*) Part of a recording showing the imaginary part (Im, *upper trace*) and real part (Re, *lower trace*), corresponding to patch capacitance and patch conductance, respectively. Note the increased scaling in the Re trace. At (I) a flicker starts, with several distinct openings and closings of the fusion pore in a single granule. In addition to several very short flickers, six flickers are long enough to be fully resolved. The total duration of the flicker is 20.7 s, and the longest time the pore stays open is 10.5 s (VI). (*B*) The part within the rectangle of (*A*) shown on an expanded scale. The top and second trace are Im and Re, respectively. The third and fourth trace show C_V (vesicle capacitance) and G_p (fusion pore conductance) as calculated using Eqs. 1 and 2. The baselines used to calculate the C_V and G_p traces are indicated by dotted lines in the Im and Re trace.

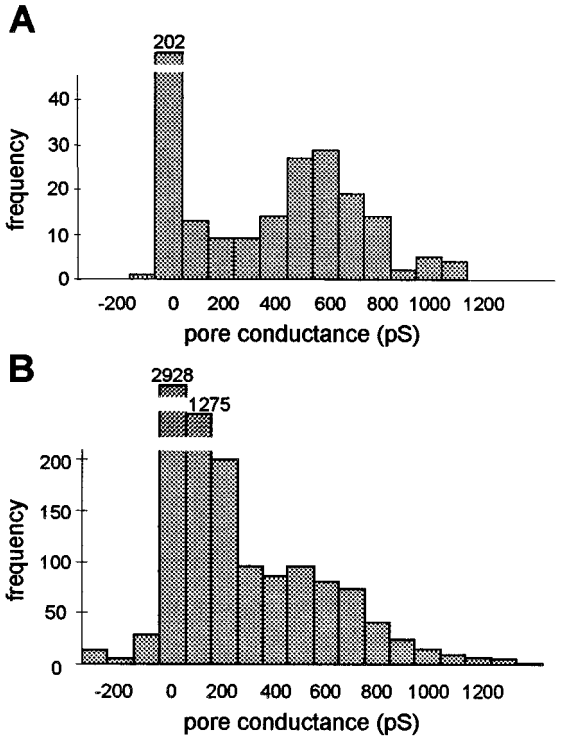

FIGURE 2 Histograms showing the distribution of fusion pore conductances from the data of Fig. 1 *B* (*A*) and from all our analyzable flickers ($n = 19$) (*B*). Bins size 100 pS.

reported from recordings of flickers in beige mice mast cells (Spruce et al., 1990). Seven flickers occurred spontaneously before ionomycin stimulation, whereas the other flickers occurred at variable time following stimulation. Seventy-two percent (18 of 25) of the flickers were single flickers with an on-step followed by a single off-step; 28% were multiple flickers, as the event of Fig. 1 *A*. All flickers ended with an off-step, indicating that full fusion was unsuccessful. The range of flicker on-steps was 0.38–5.48 fF, corresponding to granules with a diameter from 120 to 680 nm. This indicates that all four vesicle/granule types of human neutrophils (Lollike et al., 1995) are able to flicker. Furthermore, all off-steps had the same size (within ± 0.05 fF) as their preceding on-step.

The fusion pore conductance could be estimated in 19 flickers. While the pore was open, its apparent conductance was <1 nS at least 98% of the time. Fig. 2 *B* shows the conductance histogram from all flickers. The bar at 0 pS indicates the closed state. There is also a significant amount of time where the conductance ranged between 50 and 250 pS, reflecting smaller fusion pore stages as previously described (Lollike et al., 1995).

Capacitance pseudoflickers

In 7% (81 of 1158) of the total exocytotic events we observed pseudoflickers, i.e., an exocytic event followed by a capacitance decrease and with properties distinct from those of regular flickers. Four typical examples are shown in Fig. 3, *A–D*. All events are characterized by a gradual (non-stepwise) increase in the Im trace followed by a gradual decrease to a level higher than the baseline before the event. The gradual increase is accompanied by a transient increase in the Re trace, as expected for the opening and

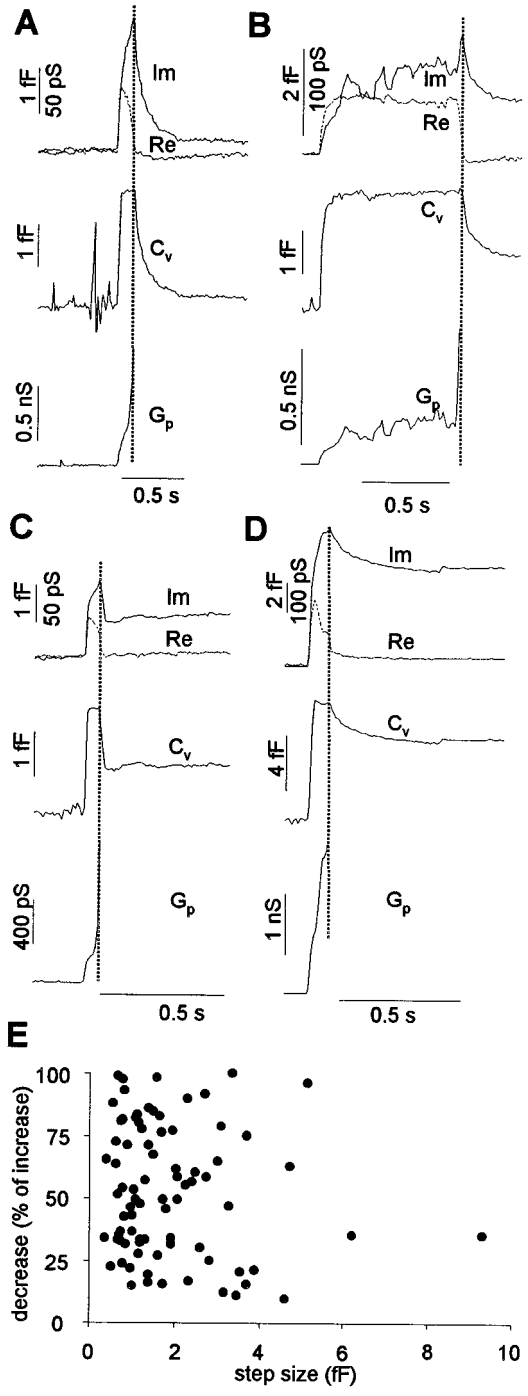

FIGURE 3 (*A–D*) Four examples of CDFE with different features. The point at which the capacitance decrease starts is indicated by vertical dotted lines, and in all cases occurs at the same time as the steep rise in fusion pore conductance (G_p). (*E*) The relative capacitance decrease as related to the size of the preceding capacitance step.

expansion of a fusion pore. When C_v is calculated using equation 1, then the increase in capacitance becomes, once again, a step. This demonstrates, that exocytosis is not associated with conductance changes. Thus, the added vesicle membrane does not contribute additional conductance, e.g., ion channels. Also, the decay in the Im trace is not accompanied by a transient change in the Re trace. The capacitance decrease following exocytosis (CDFE) thus does not reflect the re-closing of the fusion pore and cannot be explained by endocytosis of a single vesicle. If the CDFE reflects endocytosis, then it must be rapid endocytosis of many small vesicles. At least for the slow decays of Fig. 3, *A, B,* and *D*, the decay phases show no steps >0.1 fF, indicating that individual endocytosed vesicles must be <60 nm.

The C_v trace is always characterized by a plateau, which persists while the fusion pore conductance is small. The mean plateau time (time between C_v step and start of capacitance decrease) was 0.45 ± 0.08 s (SEM, $n = 81$), ranging from the limit imposed by our time resolution of 12.5 ms to 3.3 s. The decrease commences at the time where rapid pore expansion occurs. In those events where we could resolve the fusion pore, the last reliable value of the fusion pore conductance had a mean value of 1050 ± 100 pS (SEM, $n = 30$).

The extent and the time course of the CDFE were variable. Fig. 3 *A* shows an event with a large capacitance decrease. The retarded pore expansion in Fig. 3 *B* is associated with a correspondingly long plateau in the C_v trace. The event in Fig. 3 *C* has a rapid decrease, and Fig. 3 *D* depicts an event with a small decrease compared to the preceding increase.

In Fig. 3 *E* the relative CDFE is plotted versus the preceding step size. Although the relative decrease scatters widely, it does not depend on the size of the triggering granule. The CDFE never exceeded the preceding capacitance increase, although in a few cases the size of the CDFE was similar to that of the increase. In these cases, the events were clearly distinguished from flickers by the non-stepwise decrease in the C_V trace. Also, the off-steps of flickers were never preceded by expansion of the fusion pore. The mean relative decrease was 52%.

DISCUSSION

Fusion pore flickering

When a fusion pore forms, connecting the lumen of a secretory vesicle to the extracellular space, the size of the fusion pore can fluctuate and the pore can close again. Both the fluctuation and the closure of the fusion pore have ambiguously been termed flicker in the literature. We reserve the term flicker for a fusion pore that alternates between the open and the closed state, whereas we define variations in the conductance (size) of an open fusion pore as fluctuations (see also Melikyan et al., 1995).

In our cell-attached recordings from human neutrophils, 2.2% of the exocytotic events were flickers. It was demonstrated for the first time that also granules as small as 120 nm could flicker. The frequency of 2.2% is similar to that reported for mast cells (Spruce et al., 1990) which have very large granules and in fusion of cells expressing HA with erythrocytes (Spruce et al., 1991). This therefore suggests

that the mechanism responsible for the flicker phenomenon is independent of size and is not restricted to large granules. As in chromaffin cells (Albillos et al., 1997), flickers can be observed in neutrophils in the cell-attached configuration, leaving the cytoplasm unperturbed. Flickering is thus not artificially introduced by the recording configuration. Neither leakage of cytosolic components into the patch pipette in the whole-cell configuration, nor the stress imposed on the membrane patch in the cell-attached configuration, seems to induce or inhibit flickering. Flickering appears to be an inherent property of both exocytotic and viral fusion that occurs occasionally regardless of cell type, granule/cell size, or recording configuration. However, the frequency of observing flickers in fusion varies widely (Spruce et al., 1991; Melikyan et al., 1993; Oberhauser and Fernandez, 1996), and in fusion of cells expressing baculovirus GP64 no flickers are detected (Plonsky and Zimmerberg, 1996).

There are, however, some important differences between the well-characterized flickers in mast cells and the flickers in neutrophils. In mast cells, fusion pores can expand to a conductance of at least 8 nS and still close again (Spruce et al., 1990). Also, in mast cells flickers are eventually followed by irreversible fusion. In contrast, during flickers of small granules in neutrophils the fusion pore conductance remained below 1 nS and the flickers in neutrophils always ended with an off-step, indicating that irreversible fusion did not occur. Combined capacitance measurements and amerometry (Albillos et al., 1997) have recently shown that flickering chromaffin granules (similar in size to neutrophil granules) also have a pore conductance around 500 pS, and do not later undergo full fusion. Fusion pores formed between HA-expressing cells and lipid bilayers showed flickering states with 1–2 nS conductance (Melikyan et al., 1993). However, these pores could also close from conductance states up to 20 nS (Melikyan et al., 1995).

Monck and co-workers have reported a difference in size between the on- and off-step of flickers in mast cells (Monck et al., 1990; Oberhauser and Fernandez, 1996) suggesting that a net membrane transfer from the plasma membrane to the granule membrane occurs during flickering. In contrast, in human neutrophils the size of the off-steps was not different from that of the on-steps, even though the high resolution of our recordings allows detection of membrane transfer 300 times slower than that observed in mast cells. This indicates that net membrane transfer did not occur. In our experiments we cannot observe lipid transfer per se. We can only detect net membrane transfer leading to a measurable change in patch admittance. The absence of net membrane transfer in neutrophil flickers can be explained if the net driving force is zero. Either the granule membrane is not under tension, as suggested in mast cells (Monck et al., 1990), or the membrane tension in the granule membrane and in the plasma membrane are exactly balanced. In previous experiments on HA-mediated fusion, where fusion pore conductance and transfer of fluorescent lipids were measured simultaneously, it was found that lipid transfer may only occur when the fusion pore has expanded beyond a critical conductance of ~500 pS (Tse et al., 1993; Zimmerberg et al., 1994). It is thus possible that the small fusion pore during neutrophil flickers with conductance below 1 nS may not allow for membrane transfer.

Capacitance decrease following exocytosis

Seven percent of the exocytotic events were followed by a capacitance decrease producing pseudoflickers. In contrast to regular flickers the decrease is gradual and does not reflect re-closing of a fusion pore. The frequency of CDFE events varied substantially among patches. Two patches with 100 and 57 exocytotic events showed no detectable CDFE. Since the average CDFE frequency is 7%, the likelihood that these two patches show no CDFE is $7 \cdot 10^{-4}$ and $1.6 \cdot 10^{-2}$, respectively, if CDFE would occur randomly.

Endocytotic mechanisms

If this CDFE is interpreted as endocytosis, it must be a novel type of endocytosis strictly correlated with exocytosis of a single granule. It is triggered by irreversible fusion pore expansion and could be explained by rapid endocytosis of several very small (<60 nm) vesicles. Decreases in plasma membrane tension upon exocytosis can cause endocytosis (Dai et al., 1997). The endocytosed membrane might originate either from the plasma membrane, as usually envisioned (Fig. 4 *A*), or from the granule membrane. Once the fusion pore expands and the granule is incorporated into the plasma membrane, focal points at which the granule membrane is attached to the cytoskeleton (Rothwell et al., 1989) could be ripped off and "left behind" (Fig. 4 *B*). Such a mechanism would be strictly correlated with incorporation of the granule membrane into the plasma membrane, and the relative capacitance decrease would vary depending on the degree of attachment to the cytoskeleton.

Other mechanisms

Besides endocytotic processes, other mechanisms generating the CDFE under our experimental conditions must be considered. In the cell-attached configuration the fraction of "free" versus total area of the patch membrane is ~30% (Sakmann and Neher, 1995). Thus, a significant part of the patch membrane is attached to the pipette glass, contributing to the giga seal. The exocytotic events we monitor in the cell-attached configuration occur in the free area of the patch membrane. The full incorporation of a vesicle into the plasma membrane leads to a change in the overall geometry of the patch. In some cases, this could lead to increased attachment of membrane to the glass and a corresponding decrease of the free area contributing to the patch capacitance (Fig. 4 *C*). This would electrically be detected as a decrease in capacitance, and could thus also explain the CDFE.

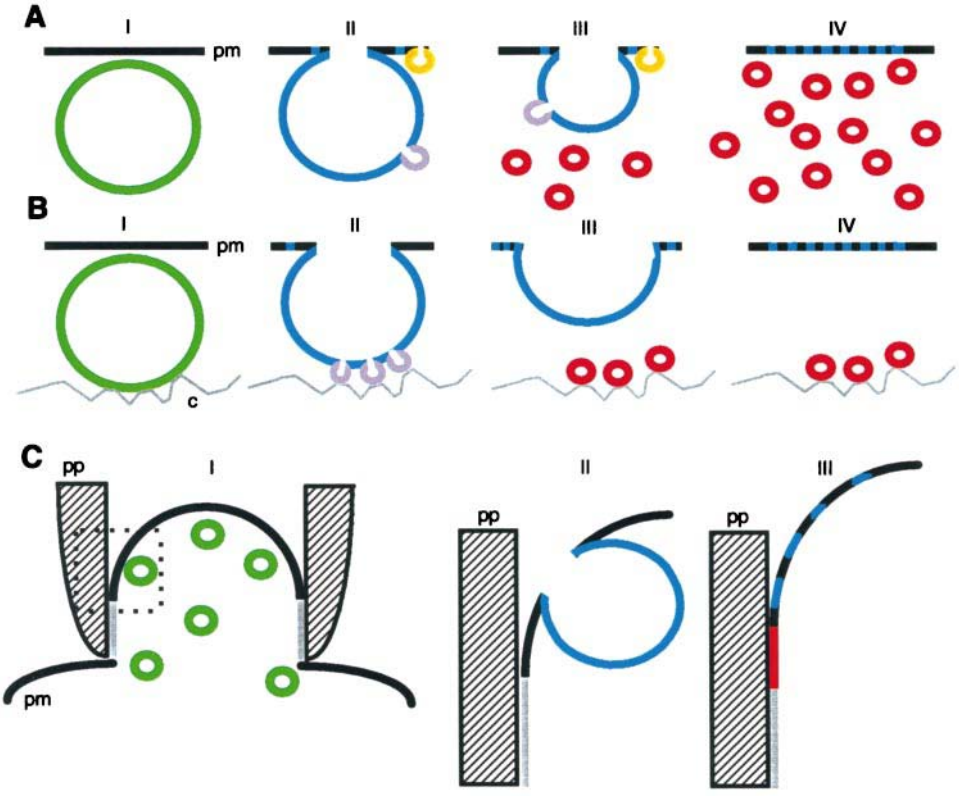

FIGURE 4 Illustration of three alternative explanations for the CDFE. In (*A*) a conventional endocytotic mechanism retrieves membrane from the exocytosed granule (purple) or the plasma membrane (yellow). The size of endocytosed vesicles is <50 nm. In (*B*) the loss of membrane occurs when the exocytosed granule is rapidly detached from the cytoskeleton. In (*C*) the CDFE is attributed to increased attachment of membrane to the patch pipette tip (pp). II and III show the inset in I on an expanded scale. The other colors indicated: black, plasma membrane (pm) or patch pipette (pp); green, cytoplasmic granule; blue, exocytosed granule or granule membrane; red, "lost" membrane; gray, cytoskeleton (c) or patch membrane contributing to seal. Only black, blue, purple, and yellow membranes contribute to the measured capacitance.

Since the CDFE is non-stepwise we cannot distinguish between an endocytotic mechanism and a geometrical change in the patch following exocytosis of a single granule. The high resolution of our recordings indicates that if the CDFE are due to endocytosis, then it must be mediated by unusually small vesicles. It should thus be emphasized that gradual changes in capacitance must be interpreted with caution, since changes in patch admittance may reflect other effects than exo- or endocytosis introduced in the cell-attached configuration.

"Safe size" of fusion pore of small granules

The properties determining the fate of the fusion pore must reside in the fusion protein(s), the membrane lipid composition, or the mechanical forces driving fusion. From our data it seems that 1 nS is a critical size of fusion pore conductance of small granules. Thus, at pore conductances <1 nS the pore is fully capable of closure, whereas at pore conductances above 1 nS the pore expands rapidly and irreversibly. Similar conductance levels were observed during flicker in chromaffin cells (Albillos et al., 1997). In contrast, the pore conductance could be as high as 8 nS in mast cells (Spruce et al., 1990), and 20 nS in fusion of HA expressing cells to bilayers (Melikyan et al., 1995), and in both cases the fusion pore could still close. A straightforward explanation of this discrepancy could be the difference in size, the mast cell granules, and HA expressing cells being much larger than neutrophil or chromaffin granules. The high curvature of the small granules, having an at least 10 times smaller diameter, could contribute to the driving force for rapid pore expansion leading to complete fusion. Alternatively, the properties of the granular matrix, which differ among different granule types, might be responsible for the ability of mast cell granules to re-close expanded fusion pores. However, this can hardly explain the ability of large viral fusion pores to re-close. It has recently been suggested that increased tension in the plasma membrane might favor expansion of the fusion pore (Sheetz and Dai, 1996). In the cell-attached configuration, increased tension will be present in the patch membrane, which might promote rapid irreversible pore expansion once a critical pore size has been reached.

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