

The Exocytotic Fusion Pore of Small Granules Has a Conductance Similar to an Ion Channel

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Abstract. We measured capacitance changes in cell attached patches of human neutrophils using a high frequency lock-in method. With this technique the noise level is reduced to 0.025 fF such that capacitance steps of 0.1 fF are clearly detected corresponding to exo- and endocytosis of single 60 nm vesicles. It is thus possible to detect almost all known exocytotic and endocytotic processes including exocytosis of small neurotransmitter containing vesicles in most cell types as well as endocytosis of coated and uncoated pits. In neutrophils we demonstrate a stepwise capacitance decrease generated by 60–165 nm vesicles

as expected for endocytosis of coated and non-coated pits. Following ionomycin stimulation a stepwise capacitance increase is observed consisting of 0.1–5 fF steps corresponding to the different granule types of human neutrophils from secretory vesicles to azurophil granules. The opening of individual fusion pores is resolved during exocytosis of 200 nm vesicles. The initial conductance has a mean value of 150 pS and can be as low as 35 pS which is similar to the conductance of many ion channels suggesting that the initial fusion pore is formed by a protein complex.

EXOCYTOSIS and endocytosis is mediated by vesicles and granules of variable size. Fusion and fission of single vesicles can be measured as stepwise changes in plasma membrane capacitance (24) and the initial opening of the fusion pore between the granular lumen and the extracellular space has been characterized by electrophysiological measurements (7, 9, 20, 33). However, these studies were limited to giant granules with a diameter $>1 \mu\text{m}$ and the interpretations of the results remain controversial (1, 21). It has been proposed that the initial fusion pore is formed by a protein channel, similar to a gap junction which connects the cytoplasmic space of two adjacent cells (1, 2). Alternatively it was proposed that the fusion pore is completely lipidic with pore opening induced by tension generated by a protein scaffold (21–23). These two models lead to different expectations for the initial electrical pore conductance. Whereas ion channels formed by *trans*-membrane proteins usually have conductances below 400 pS (13), the conductances of lipidic pores are generally larger than 1 nS (26, 34). Measurements of pore conductance thus provide a tool to distinguish between these two possibilities. Previous experiments on pore conductances led to mean values of 250–350 pS (7, 33) which are closer to ion channels than lipid pores,

but these states were very short-lived and could possibly reflect an unstable state formed when the lipid pore opens. The demonstration of metastable fusion pores with conductances below 200 pS would be strong evidence for a fusion pore similar to a *trans*-membrane ion channel.

Most exocytotic and endocytotic granules and vesicles have a diameter between 60 and 300 nm corresponding to capacitance steps of 0.1–2.3 fF. These cannot be resolved in whole cell recordings and nothing is known about the properties of the fusion pore which forms during exocytosis of such vesicles. The low noise in recordings from small membrane patches provides increased resolution for the measurement of capacitance steps (24). We measured capacitance changes in cell attached patches of human neutrophils which contain four classes of granules or vesicles with different size (4, 5, 15). We improved the technique by using a much higher sine wave frequency and demonstrate that capacitance steps as small as 0.1 fF can be detected corresponding to exo- and endocytosis of 60 nm vesicles. Furthermore we were able to study the dynamics of single fusion pore openings in vesicles as small as 200 nm, which exhibit metastable fusion pores with conductances lower than previously observed.

Materials and Methods

Cell Preparation

Human neutrophils were isolated as previously described (6) from volunteer donors by venipuncture of 8 ml blood into 2 ml anticoagulant (25 mM so-

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dium citrate, 126 mM glucose). The anticoagulated blood was mixed with an equal volume of 2% dextran in saline (0.9% NaCl), and after 15 min the leukocyte rich supernatant was removed and centrifuged at 200 *g* for 10 min at 4°C (all following steps were performed at 4°C). The resulting pellet was resuspended in 30 ml of saline and underlaid with 15 ml of Lymphoprep (Nycomed Pharma A.S., Oslo, Norway) and centrifuged for 22 min at 400 *g*. To lyse contaminating erythrocytes the pellet was resuspended in water for 30 s after which an equal volume of 1.8% NaCl was added to restore tonicity. After a final centrifugation of 6 min at 200 *g* the pellet was resuspended in external saline (ES)¹ containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes (pH 7.2–7.3 adjusted with NaOH) and 10 mM glucose. The resulting neutrophil suspension was >95% pure and was kept at room temperature or on ice for up to 4 h until patched. No cytochalasin treatment was used in the experiments described here.

Ionomycin Stimulation

Fura-2 loading was performed by a 30-min incubation in the presence of 100 nM fura-2 AM (Molecular Probes, Eugene, OR) added from a stock of 1 mM DMSO containing 20% pluronic. For stimulation 10 μ l of 0.1 mM ionomycin (Sigma Chem. Co., St. Louis, MO) was added to the recording chamber giving a final concentration of 2 μ M, or a solution containing 200 μ M ionomycin (from 1 mM stock in ethanol) was ejected for 25 s at a pressure of 10 mm water from a pipette positioned about 100 μ m from the patched cell. Both methods led to a three- to sixfold change of fura-2 fluorescence ratio measured with excitation filters of 358 and 390 nm.

Patch Clamp Capacitance Measurements

For patch recordings cells were added to a recording chamber containing ES. Recordings were made using an EPC-7 patch clamp amplifier (HEKA-elektronik, Lambrecht, Germany). Pipettes contained ES and had a typical resistance of 5 M Ω . After seal formation capacitance and conductance changes in the membrane patch under the pipette were recorded with a lock-in amplifier (model SR830 DSP lock-in; Stanford Research Systems, Stanford, CA) using a 50 mV (rms) sine wave with a frequency of 800 Hz or 8 kHz. In the cell attached configuration the higher frequency provides larger current changes and allows measurement of fusion pore conductance in smaller vesicles. The current output of the patch-clamp amplifier was directly connected to the lock-in input with the filters of the EPC-7 switched off. A more detailed description of the method can be found in (17, 19, 24). The two outputs of the lock-in amplifier were low pass filtered using the built-in filters. At 800 Hz the time constant was set to 10 ms, 24 dB and at 8 kHz to 3 ms, 24 dB. These settings correspond to 3dB corner frequencies of 7 and 23 Hz, respectively, and an equivalent noise bandwidth of 8 and 26 Hz. The rise time (from 10 to 90%) following a step change generated by these filters is 15 ms at the 3 ms setting and 50 ms at the 10 ms setting. The filtered outputs were sampled by a computer every 25 or 12.5 ms. For conversion of capacitance changes into changes in membrane area, a specific capacitance of 8 fF/ μ m² was used.

When the conductance of the fusion pore is low, the capacitance channel Y2 provides a reduced signal $Im = \omega C_V/N$ (imaginary part) and an apparent conductance signal $Re = (\omega C_V)^2/G_P/N$ (real part) appears at the Y1 output (7). In these formulas C_V denotes capacitance of fusing vesicle and G_P the conductance of the fusion pore, with $N = 1 + (\omega C_V/G_P)^2$. From the two measured quantities Re and Im , the time course of C_V and G_P can be calculated: $C_V = ([Re^2 + Im^2]/Im)/\omega$ and $G_P = (Re^2 + Im^2)/Re$ (see also reference 17).

Results

Capacitance Steps Reflecting Endocytosis

Fig. 1 *A* shows a cell attached recording from a human neutrophil of patch capacitance (top) and conductance (bottom) recorded within the first two minutes after obtaining a giga seal using a 50 mV (rms), 8 kHz sine wave command voltage. The trace shows a decrease by 10 fF in steps of variable size. Such downward capacitance steps were usually observed

during the first minutes after formation of the giga seal, indicating endocytotic events associated with a stepwise decrease in membrane area when vesicles are internalized. Part of the recording is shown on an expanded scale in Fig. 1 *B*. The noise level of the capacitance measurement (top) was quantified in sections between steps. A second order polynomial fit was used as the mean capacitance in sections between steps (smooth line between the first and second downward step) giving an rms noise level of 0.025 fF. Using a specific capacitance of 8 fF/ μ m², this value can be converted to rms fluctuations of membrane area by 0.003 μ m². Under these conditions capacitance steps of 0.1 fF corresponding to a vesicle diameter of 60 nm can thus be reliably detected. The trace shows a number of downward steps with a size of 0.1–0.4 fF corresponding to endocytosis of 60–130 nm vesicles. The step size distribution of these downward steps (Fig. 1 *C*) shows that most of the steps are smaller than 0.8 fF, with a maximum at the smallest step size between 0.1 and 0.2 fF, corresponding to 60–165 nm vesicles.

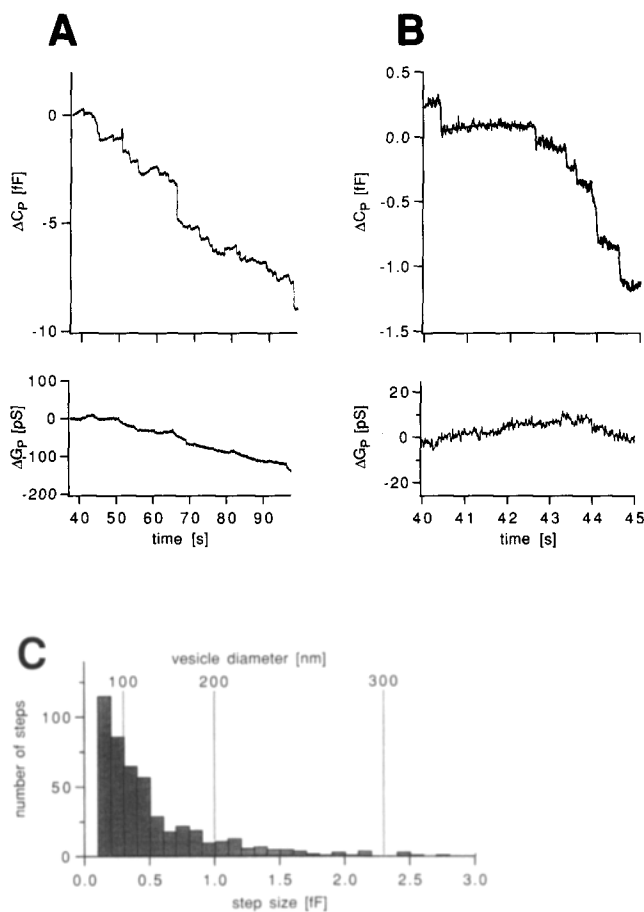


Figure 1. Endocytosis in a membrane patch during the first minutes after obtaining a giga seal on a human neutrophil. (*A*) In this patch a capacitance decrease by 10 fF is measured during 1 min (*top trace*) indicating a decrease of patch membrane area by about 1.25 μ m². Only minor changes are seen in the conductance (*bottom trace*), which is drawn to scale with the capacitance traces. (*B*) An expanded section of *A* showing small downward steps. (*C*) The frequency distribution of downward capacitance steps (493 steps from 25 cells) shows that 80% of the steps have a size between 0.1 and 0.7 fF.

1. Abbreviation used in this paper: ES, external saline.

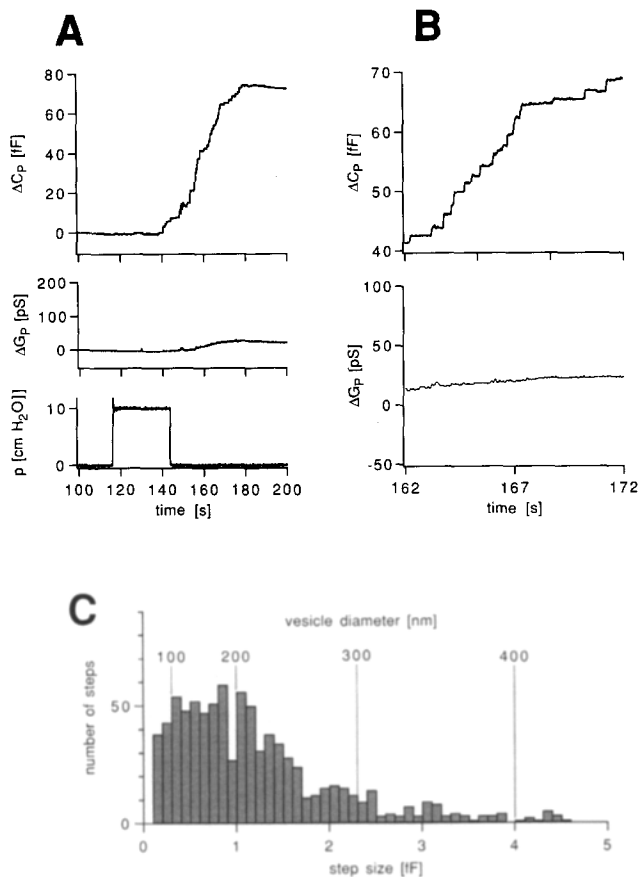


Figure 2. (A) Stimulation with ionomycin induces a marked increase of patch capacitance (ΔC) with only minor changes in the conductance trace (ΔG). Ionomycin was applied by pressure ejection from a second pipette (p trace). (B) The capacitance increase occurs in steps of variable size as expected for the wide range of granule sizes. (C) The frequency distribution of positive capacitance steps covers the range for all neutrophil granule types. In this distribution data from recordings with a detection limit of 0.4 fF (800 Hz measurements) and 0.1 fF resolution (8 kHz measurements) were pooled. The number of steps in the bins between 0.1 and 0.4 fF were thus multiplied with a normalization factor $F = (\text{total number of steps } >0.4 \text{ fF}) / (\text{number of steps } >0.4 \text{ fF measured at 8 kHz})$.

Exocytosis Stimulated by Ionomycin

To stimulate exocytosis, the calcium ionophore ionomycin was added either to the bath solution or directly applied onto the cell using a second pipette. This stimulation led to a variable capacitance increase of up to 100 fF in the patch. The

increase appeared to be correlated to patch area since a large increase was only obtained when the patch could be seen under the microscope inside the pipette. Fig. 2 A shows a 80 fF capacitance increase which consists of discrete steps indicating exocytotic fusion events (Fig. 2 B). These steps ranged between 0.1 and 5 fF (Fig. 2 C) which corresponds to fusion of vesicles with diameters between 70 and 450 nm. This is the range expected for the different granule types (see Table I).

Fusion Pores in Neutrophil Granules

The fusion process of a single vesicle starts with the opening of an initial pore of molecular dimensions (pore diameter $\sim 2\text{--}3$ nm) followed by a subsequent dilation as previously shown for the giant granules of mast cells (7, 33) and eosinophils (20). In the patch recordings on neutrophils we were now able to resolve the time course of fusion pore conductance in vesicles which are much smaller. Fig. 3 A shows the analysis of such an event revealing formation of an initial pore conductance of 70 pS in a 350 nm vesicle (step size 3.14 fF) which is stable for >200 ms, before the pore starts to expand rapidly. Another fusion event of a 220 nm vesicle (step size 1.23 fF) is shown in Fig. 3 B. The initial pore conductance is 130 pS and the conductance increases very little during the next 70 ms. In Fig. 3 C an event is shown where the initial pore conductance was 35 pS followed by a slow increase by 260 pS/s during the next 500 ms (2.4 fF, 310 nm diam). In most cases rapid expansion occurred when the pore conductance exceeded 150 pS.

With a measuring sine wave frequency of 8 kHz the opening of the fusion pore could be resolved in 31 out of 49 steps with a size between 1.2 and 3.8 fF. In 21 of these an initial well defined state was detectable preceding the subsequent expansion. For steps between 0.8 and 1.2 fF the initial pore conductance could be determined in 12 out of 38 steps. Fig. 3 D shows that the initial fusion pore conductance is independent of vesicle size at least between 0.8 and 4 fF. The frequency distribution of the initial pore conductance values is shown in Fig. 3 E. It has a peak around 100 pS and the mean value is 149 ± 13 pS (SEM, $n = 43$). With an 8 kHz sine wave and vesicle capacitance around 2 fF fusion pore conductances around 100 pS can be well resolved but values less than 30 pS are difficult to detect. To determine if a smaller pore conductance precedes these fusion pore states, we used a measuring frequency of 800 Hz which should allow for detection of 10 times smaller conductance states. At 800 Hz the noise level is larger and the lock-in output was thus filtered at a lower frequency. Under these conditions the fusion pore opening was generally too fast to be resolved. In 3 out of 271 steps >1.2 fF we found an indication for conduc-

Table I. Types and Size of Human Neutrophil Granules and Vesicles

Granule type	Granule size (nm)	Reference	Expected step size (fF)	
			Peak	Range ($\pm \sigma$)
Azurophil granule	320 ± 80	(28)	2.4	1.4–3.8
Specific granule	236 ± 52	(16)	1.3	0.8–2.0
Gelatinase granule	187 ± 43	(16)	0.8	0.5–1.2
Secretory vesicle	~ 100	(8)	0.25	

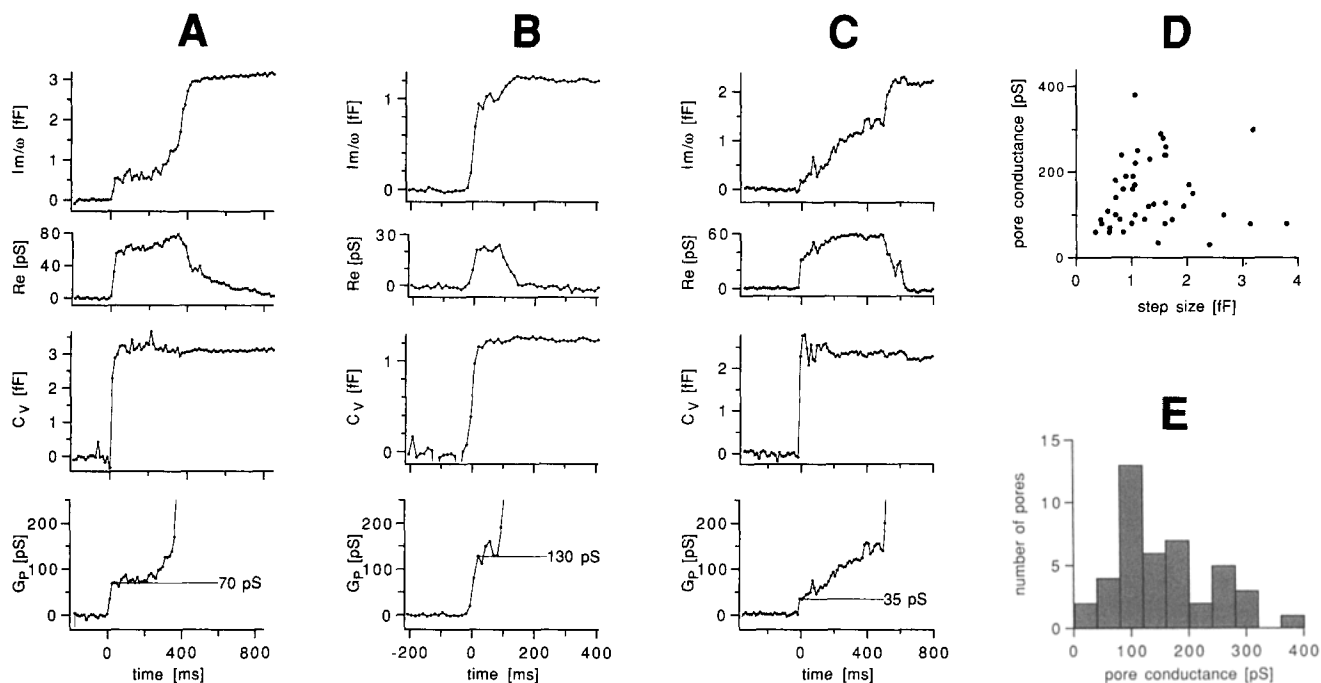


Figure 3. (A–C) Fusion pore openings in single fusion events measured with 8 kHz, 50 mV (rms) sine wave. When the fusion pore conductance G_p is comparable to the ωC_v (C_v = vesicle capacitance), then the Y2 output of the lock-in amplifier (Im , top trace) is smaller than ωC_v and an increase in Y1 (Re , second trace) occurs. As the pore conductance expands, Im/ω approaches C_v and Re returns to the baseline level. From the two quantities Re and Im , the time course of C_v and G_p can be calculated (see Methods) according to $C_v = [(Re^2 + Im^2)/Im]/\omega$ (third trace) and $G_p = (Re^2 + Im^2)/Re$ (bottom trace). The rise time of the C_v trace indicates the high time resolution of the measurement. (D) The measured G_p values are independent of C_v . (E) Frequency distribution of initial pore conductance levels.

tances between 10 and 30 pS, but due to the low time resolution these values were not as accurate as those measured at 8 kHz frequency.

Discussion

Cell Attached Recordings Can Resolve Exo- and Endocytosis of Single 60 nm Vesicles

In the whole cell configuration a cell is internally perfused with the pipette solution leading to wash-out of cytosolic factors which may be important for certain cellular functions. In this configuration mast cells do not degranulate in response to receptor-directed stimulation (18) and in hair cells endocytosis following exocytosis is inhibited (27), but these functions can be observed in permeabilized patch recordings where the cytosolic composition is not disturbed. The cell attached configuration also makes it possible to record from intact cells. In addition, the lower noise level greatly improves the resolution of current measurements.

Exocytosis and endocytosis of single vesicles has been demonstrated as stepwise capacitance changes in a variety of cell types in whole cell patch clamp recordings using a lock-in amplifier (17). However, due to the unavoidable noise of the whole cell recording configuration only steps larger than 2 fF could be detected (19). The low noise in recordings from small membrane patches was instrumental in recording the opening and closing of single ion channels (11) and it was shown in the pioneering work of Neher and Marty that this method also provides increased resolution for the measurement of capacitance steps (24). We improved this technique

by using an 8 kHz sine wave frequency and a lock-in amplifier with improved performance. The resolution is increased by more than an order of magnitude compared to whole cell recordings and allows the detection of 0.1 fF capacitance steps associated with exocytosis and endocytosis of 60 nm vesicles. Most exo- and endocytotic vesicles are larger than this and even single fusion events of small synaptic vesicles should be detectable in many preparations.

The cell attached configuration is illustrated in Fig. 4 together with a highly simplified equivalent circuit consisting only of patch capacitance C_p and capacitance of the remaining plasma membrane C_m . The conductances due to ion channels are omitted for clarity. For 5 M Ω pipettes the patch area is typically 1–10 μm^2 (29) corresponding to $C_p = 8$ –80 fF. In our experiments we applied strong suction to increase the patch area which will thus be close to 10 μm^2 . The total plasma membrane area of a neutrophil is about 300–400 μm^2 (25). This corresponds to $C_p < 0.1$ pF and $C_m \sim 3$ pF. During degranulation fusion of granules changes C_p as well as C_m . However, C_m is at least 30 times larger than C_p and the measured capacitance $C = (C_p \cdot C_m)/(C_p + C_m)$ is thus approximately equal to C_p . Even a large increase of C_m has only a negligible effect on C . In contrast, an increase of C_p from 100 to 200 fF leads to a measured change in C of 91 fF. The measured capacitance changes thus report the membrane area of the fusing vesicles accurately within 10%.

Endocytosis of Vesicles with the Size of Coated and Uncoated Pits

The typical size of coated pits and non-coated endocytic vesi-

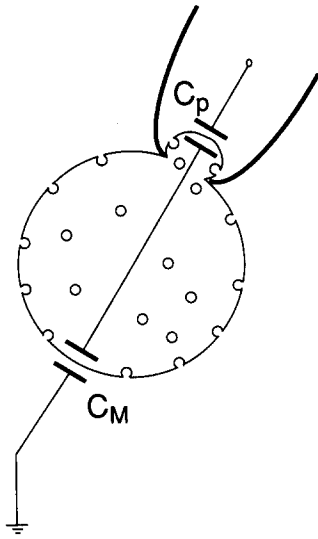


Figure 4. Schematic representation of the cell attached configuration with a highly simplified equivalent circuit consisting only of patch capacitance (C_P) and capacitance of the remaining plasma membrane (C_M). The conductances due to ion channels are omitted for clarity. Exocytosis and endocytosis lead to changes of C_P as well as C_M . However, the two capacitors C_P and C_M are in series and the resulting measured capacitance C is given by the equation $1/C = 1/C_P + 1/C_M$. C_P of patches obtained with pipettes of the size used here is typically <0.1 pF whereas C_M of a human neutrophil is ~ 3 pF, 30

times larger than the patch capacitance. Complete degranulation leads to an increase of plasma membrane capacitance by 6 pF (25). However, for a 0.1 pF patch, a 6 pF increase of C_M would result in a change of the measured capacitance from 97 fF to 99 fF, a change which is negligible. In contrast, fusion of vesicles in the patch contributing a total capacitance of 100 fF would increase the measured capacitance by 91 fF. The measured capacitance changes in the cell attached configuration thus report the membrane area of fusing vesicles with an accuracy of 10%.

cles is 70–160 nm (12, 30), corresponding to capacitance steps of 0.1–0.7 fF. The improved resolution of our recordings allowed for the first time to detect endocytosis of these small vesicles as capacitance steps. 392 out of 493 endocytic steps (i.e., 80%) were in the expected range. The endocytotic step size distribution is thus in excellent agreement with the size of endocytic vesicles.

The endocytic activity did not require a particular stimulus and could thus be constitutive. However, in experiments where cells were loaded with fura-2, small calcium increases were observed when the seal was made (data not shown), indicating that the interaction with the glass of the patch pipette activates the cell. It was previously shown that contact with surfaces provides a stimulus for neutrophils inducing changes in shape and intracellular calcium (14). We thus suggest that the initial phase of endocytosis observed here may have been induced by the sealing process. Although the stepwise nature of the endocytic events was clearly detectable, the associated capacitance steps were too small to resolve the time course of individual fission pore closures during internalization of a single vesicle.

Inomycin Stimulates Exocytotic Fusion of All Granule Types

Degranulation of human neutrophils can be stimulated via receptor activation, calcium ionophores, or intracellular application of GTP γ S (3, 25, 32). In the patch clamp whole cell configuration a capacitance increase from 3 to 9 pF is induced by GTP γ S, corresponding to a threefold increase of plasma membrane area due to incorporation of granule membranes (25). Neutrophils contain four distinct classes of exocytotic vesicles and granules with different contents and

different size (Table I). Among these, only fusion of azurophil granules has previously been detected as capacitance steps in the whole cell configuration (25).

The present experiments show that in intact cells ionomycin induces exocytotic fusion events. The total capacitance increase in the experiment of Fig. 2 is about 80 fF corresponding to about 1.5% of the capacitance increase measured during degranulation in the whole cell configuration (25). As mentioned above, the area of cell attached patches is usually in the range of 1–10 μm^2 (29) corresponding to 0.3–3% of the total plasma membrane area. The fraction of the capacitance increase in the patch relative to that measured in a whole cell is thus comparable to the fraction of membrane measured in the cell attached configuration. This indicates that exocytosis occurs normally in the patch.

The size distribution of capacitance steps shows that all granule and vesicle types contribute to the capacitance increase. We have not detected a significant correlation between step size and time after stimulation. The exact contribution of the different granule types in generation of steps with a certain size cannot be estimated from these data since the size of the different granule types overlap and the morphometric size distributions are not accurate enough. The separation of the different granule classes by capacitance step sizes will be possible when cells are used where the granule sizes are better separated, e.g., in eosinophils.

Occasionally we observed unusually large capacitance steps which could exceed 10 fF. These events presumably represent exocytosis of multigranular compounds indicating that ionomycin may also induce granule–granule fusion leading to compound exocytosis as previously observed in response to GTP γ S stimulation of eosinophils (31).

The Fusion Pore in Neutrophil Granules Is Small

The increased resolution allows the study of single fusion pore openings in vesicles as small as 200 nm in the cell attached configuration. We found that the exocytotic fusion of neutrophil granules starts with a fusion pore of low conductance. In about 40% of the fusion events >1.2 fF, stable fusion pore conductances of 35–380 pS conductance were detected when the appropriate measuring frequency (8 kHz) was used. It is possible that the first electrically conductive connection between the vesicle interior and the extracellular medium is variable and only occasionally as small as 35 pS. However, another interesting possibility is that in most cases the transition to a higher pore conductance is fast such that the first detected state is already somewhat expanded. If the transitions between the intermediates of fusion are rapid compared to the time resolution of the measurement, and are stochastic in nature, then one would expect only very few events where a generally short-lived intermediate exists long enough to be detected. It is thus possible that exocytosis of small granules generally begins with a channel of 35 pS or less.

Previous whole cell measurements on guinea pig eosinophils have revealed stable fusion pore intermediates with conductance values between 70 and 300 pS (20) and the initial fusion pore conductance in the giant granules of beige mouse mast cells (7) as well as horse eosinophils (Hartmann, J., and M. Lindau, manuscript submitted for publication) is in the same range. We have shown here that the initial fusion pore conductance observed during fusion of 200–400 nm

vesicles in intact cells is similar. In contrast to previous measurements on giant granules, we found that in the case of the small neutrophil granules the small fusion pores are frequently stable or expand very slowly, on the 100 ms time scale. Our experiments show that on this time scale fusion pore conductances can be as low as 35 pS revealing a novel early intermediate of fusion. The longer life time of low conductance fusion pores could be a particular property of the neutrophil granules. However, it should be kept in mind that our experiments were performed in the cell attached configuration whereas previous data were obtained in whole cell recordings. It is thus possible that cytoplasmic factors which stabilize the low conductance states are washed out in whole cell recordings.

The molecular nature of the initial fusion pore has been an object of controversial speculation and has been proposed to be a protein structure similar to an ion channel (1, 2), or alternatively a completely lipidic membrane breakdown pore (21, 22). Pores in lipid membranes formed during electrical breakdown have conductances in the μS range (34) and are thus larger than exocytotic fusion pores by many orders of magnitude. The pores formed during mechano-electrical breakdown of mast cell granule membranes had a mean conductance of 1,100 pS (26), which is still an order of magnitude larger than the fusion pore conductances observed here. The fusion pore conductances are indeed much more similar to those of many ion channels formed by single proteins or protein complexes. The nicotinic acetylcholine receptor has a conductance of 44 pS in chromaffin cells (10). The conductance of the big calcium dependent K^+ channel is ~ 100 pS and that of the voltage-dependent anion channel ~ 400 pS (13). The conductance measurements thus strongly suggest that the initial fusion pore is a protein structure similar to an ion channel and not a purely lipidic pore. Since the method allows to resolve in detail the fusion pore opening in vesicles with a diameter of 200 nm it will be suitable to investigate the properties of fusion pores also in neuropeptide containing dense core vesicles.

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