

A novel Ca^{2+} -dependent step in exocytosis subsequent to vesicle fusion

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Abstract Exocytosis begins with formation of a small fusion pore which then expands allowing rapid release of granular contents. We studied the influence of cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) on the conductance of the initial pore and on the dynamics of subsequent expansion in horse eosinophils using the patch clamp technique. The mean initial conductance is ~ 200 pS independent of $[\text{Ca}^{2+}]_i$. This value is close to that previously found in beige mouse mast cells. The pore subsequently expands by 18 nS/s at $[\text{Ca}^{2+}]_i < 10$ nM, by 40 nS/s at $[\text{Ca}^{2+}]_i = 1.5$ μM and by 90 nS/s at $[\text{Ca}^{2+}]_i = 10$ μM . These results show that the structure of the initial fusion pore is independent of cytoplasmic Ca^{2+} . However, the pore expansion is a Ca^{2+} -dependent process modulating secretion at a step later than vesicle–plasma membrane fusion.

Key words: Exocytosis; Calcium; Eosinophil; Fusion pore

1. Introduction

Exocytosis is an ubiquitous cellular function including constitutive incorporation of vesicles into the plasma membrane as well as regulated secretion performed by nerve terminals, endocrine and exocrine cells, granulocytes, mast cells and many other cell types. The common feature is that cytoplasmic vesicles containing preformed material fuse with the plasma membrane releasing their contents through the thus formed fusion pore. The formation and expansion of the fusion pore can be recorded electrically in single fusion events using the whole cell patch clamp technique providing the fusion pore conductance as a measure of the size of the pore [1–3]. In beige mouse mast cells the initially formed fusion pore is small having a mean conductance of 230–330 pS [1,2]. Within milliseconds the pore then expands allowing rapid release of granular contents [2,4]. In endocrine cells and nerve terminals various steps preceding fusion and the initiation of fusion itself are controlled by $[\text{Ca}^{2+}]_i$ [5–7]. However, the Ca^{2+} dependence of neither the initial fusion pore conductance, nor of the pore expansion have yet been investigated. We studied the influence of $[\text{Ca}^{2+}]_i$ on the initial pore conductance and on the rate of the subsequent conductance increase in horse eosinophils during degranulation stimulated by intracellular application of GTP γ S [8]. These cells have large exocytotic granules with a correspondingly large capacitance of the granule membrane [8] which makes it possible to measure the current transient charging the membrane of the fusing vesicle to the holding potential.

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Abbreviations: EGTA, ethylene glycol-bis(β -amino-ethyl ether)- N,N,N',N' -tetraacetic acid; HEDTA, N -hydroxyethylethylenediaminetriacetic acid; GTP γ S, guanosine-5'- O -(3-thiotriphosphate).

2. Materials and methods

2.1. Cell preparation

Eosinophils were isolated from fresh blood from the jugular vein of two 2–3-year-old horses and purified by centrifugation over discontinuous Percoll gradients [8]. The purified eosinophils were stored in Medium 199 (Biochrom) in the presence of 4 mM glutamine, 4.2 mM NaHCO_3 and penicillin/streptomycin at room temperature and used within 2–4 days after preparation.

2.2. Patch clamp capacitance measurements

Patch clamp experiments were done in whole cell voltage clamp at room temperature (22–24°C). The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES/NaOH, 15–20 mM glucose at pH 7.2. The pipette solution contained: 125 mM K-glutamate, 10 mM NaCl, 7 mM MgCl_2 , 10 mM HEPES/NaOH pH 7.2, 1 mM $\text{Na}_2\text{-ATP}$, 20 μM GTP γ S. $[\text{Ca}^{2+}]_i$ was adjusted using the following buffers: 7 mM EGTA, no added CaCl_2 (< 10 nM); 5 mM EGTA, 4.5 mM CaCl_2 (1.5 μM) or 6.5 mM HEDTA, 1 mM CaCl_2 (10 μM) added to the pipette solution. The change of cell membrane capacitance was measured as the imaginary component of the admittance change of the cell using a lock-in-amplifier [9,10] and a EPC-9 patch-clamp amplifier (HEKA, Darmstadt). Command voltage was 800 Hz, 20 mV (rms) sine wave. The lock-in outputs were sampled by a computer every 22 ms.

2.3. Measurement of current transients

When the first electrically conductive connection between the granular lumen and the extracellular space is formed, the capacitance of the granule membrane is charged to the holding potential present across the plasma membrane by a measurable transient current [1]. We applied holding potentials between -80 and $+80$ mV. 170 ms long current records were measured in the absence of the sine wave with a sampling rate of 25 μs (PDP 11/73 with Indec interface). Then the sinusoidal voltage (800 Hz, 20 mV rms) was added to the holding potential for 30 ms to measure the cell membrane capacitance with the lock-in-technique. Due to a dead time of another 20 ms from the data acquisition program the sequence was repeated every 220 ms. If a capacitance step > 30 fF was detected on-line, 3 capacitance values preceding the step and 3 values after the step were saved on disk together with the current records measured between these points.

The current transients in the segments measured during the period where the capacitance step occurred were analysed off-line as previously described [2]. The running integral of the current transient yields the charge which has been moved during the opening of the fusion pore and thus provides the time course of granule potential. The initial potential present across the granule membrane before fusion was calculated by dividing the total charge (from integration of the current transient) by the capacitance step size and subtracting the holding potential. Dividing the current trace by the potential trace yields the time course of the fusion pore conductance.

3. Results

3.1. Initial fusion pore opening

To determine the initial fusion pore conductance 170 ms long current records were measured between periods where the cell membrane capacitance was monitored with the lock-in amplifier technique adding a 800 Hz, 20 mV (rms) sine wave to the command voltage. When a capacitance step > 30 fF was detected on-line, the current segments were stored and analyzed

off-line. When the holding potential was -80 or $+80$ mV, 70% of the current segments measured during the period where the capacitance step occurred contained a detectable current transient. This transient charges the fused granule to the holding potential (Fig. 1A,B) and can be used to estimate the pore conductance approximately 100 μ s after fusion. The relative frequency of 70% for detectable transients at these potentials is close to the fraction of time (77%) during which current was recorded. The missing 7% are probably due to transients which were too small to be resolved. At a holding potential of 0 mV current transients were only rarely observed (8%).

Integration of the current transient provides the total charge. The potential present across the granule membrane before fusion was calculated by dividing the total charge by the capacitance step size and subtracting the holding potential. Granule potentials were distributed around a peak close to 0 mV (-11 ± 81 mV, mean \pm S.D., $n = 579$) explaining why current transients were rare at 0 mV holding potential. The time course of the granule potential can be calculated from the charge which has been moved (running integral of the current transient) and the time course of fusion pore conductance is then calculated by dividing the current trace by the potential trace and the initial pore conductance was taken as the first observable quasistable level in this trace (Fig. 1C).

The initial pore conductance values observed during degranulation stimulated by intracellular perfusion with 20 μ M GTP γ S in the presence of 1.5 μ M free Ca^{2+} is variable (Fig. 1C). Correspondingly we obtained a broad frequency distribution of the measured initial pore conductance (Fig. 1D). The distribution is asymmetric and is very similar to that previously obtained for the initial fusion pore during fusion of giant granules in beige mouse mast cells [2]. Plots of initial pore conductance vs. vesicle size showed random scatter (not shown) indicating that the initial pore conductance was not correlated with vesicle size. When the experiments were performed at low $[\text{Ca}^{2+}]_i$, the frequency distribution (Fig. 1E) was not significantly different (Wilcoxon test). The mean values are 224 ± 10 pS (S.E.M., $n = 278$ pore openings measured in 105 cells) for high $[\text{Ca}^{2+}]_i$ and 186 ± 10 pS (S.E.M., $n = 206$ openings in 89 cells) for low $[\text{Ca}^{2+}]_i$. The average initial pore conductance is thus about 200 pS independent of $[\text{Ca}^{2+}]_i$. This value is remarkably close to the initial fusion pore conductance of 230 – 330 pS observed in beige mouse mast cells [1,2].

3.2. Fusion pore expansion

The current transients can provide the fusion pore conductance only for a very short period until the membrane potential is charged to the holding potential. However, when a sinusoidal voltage is applied, the induced charging and discharging currents can be used to estimate the fusion pore conductance for much longer times, but with lower time resolution. During the fusion of a single granule with the plasma membrane a low pore conductance G_p (Fig. 1A) reduces the capacitance signal by the factor $1/[1 + (\omega C_V/G_p)^2]$ and provides an apparent conductance signal proportional to $[(\omega C_V)^2/G_p]/[1 + (\omega C_V/G_p)^2]$ [11]. Knowing the granule capacitance from the final step size, we calculated the time course of G_p from the capacitance trace and confirmed it by comparison of the predicted and measured conductance trace [3].

The time course of fusion pore expansion ~ 100 ms after fusion is also variable (Fig. 2A), but the increase can be approximated

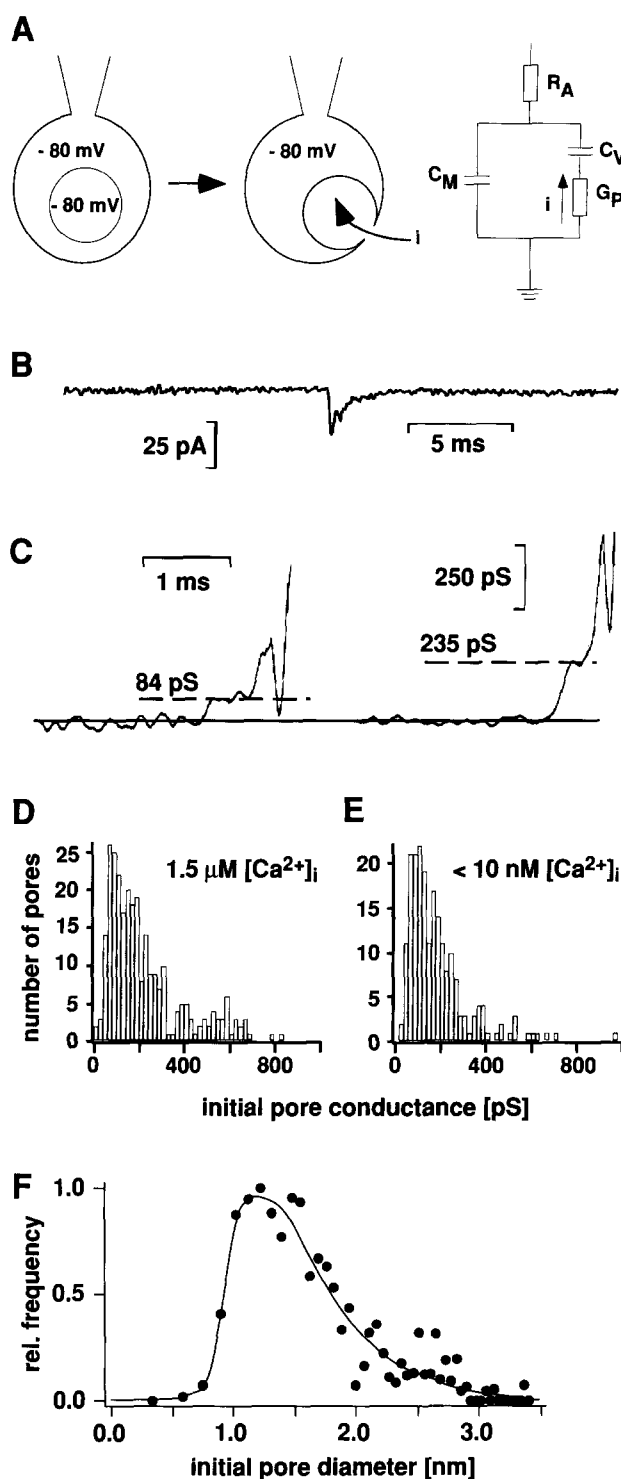


Fig. 1. Initial fusion pore conductance from analysis of current transients. (A) Upon fusion the capacitance C_V of the vesicle is added to the plasma membrane (C_M) and is rapidly charged via the fusion pore conductance G_p to the holding potential set by the pipette voltage. (B) A typical current transient measured at -80 mV holding potential. (C) Two representative fusion events showing different initial fusion pore conductances. (D,E) Frequency distribution of initial pore conductance values obtained with this technique at low (7 mM EGTA/no added Ca^{2+}) and high $[\text{Ca}^{2+}]_i$ (5 mM EGTA/ 4.5 mM CaCl_2). (F) Frequency distribution of pore diameters obtained by converting the pooled data from (D) and (E) assuming a pore length of 15 nm. The continuous line is drawn by eye.

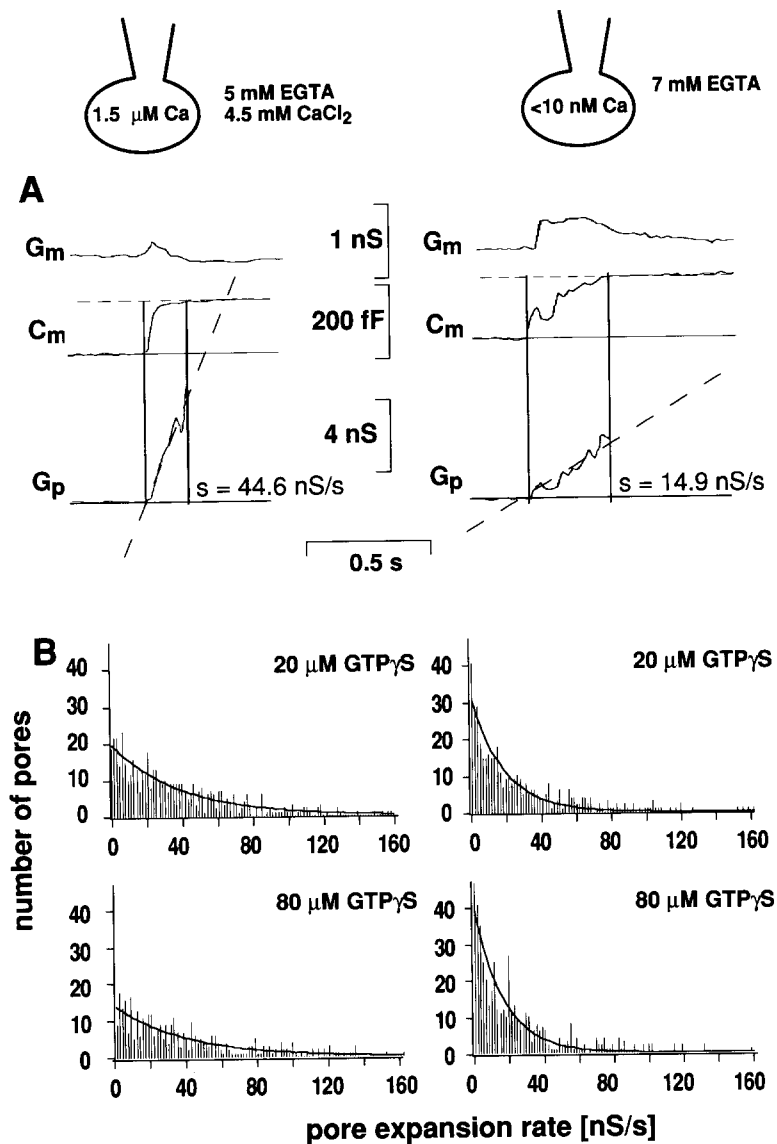


Fig. 2. Fusion pore expansion recorded with the lock-in technique at high (left) and low (right) $[Ca^{2+}]_i$. (A) During pore expansion the low value of G_p leads to a reduced change in the capacitance trace (C_m) and a correlated transient increase in the conductance trace (G_m). G_p was calculated from the C_m trace and the expansions were approximated by straight lines providing the expansion rate in nS/s. (B) The frequency distributions of the measured expansion rates can be approximated by single exponentials (solid lines), with slow expansions being most frequent. The expansion rates are not affected by the GTP γ S concentration, but are altered by $[Ca^{2+}]_i$.

by a straight line providing the slope of the conductance increase. As for the initial pore conductance, a dependence of the expansion rate on vesicle size was not observed. The expansion rates are exponentially distributed both at low and high $[Ca^{2+}]_i$ (Fig. 2B, top panels). The mean fusion pore expansion rates obtained from single exponential fits to the data shown in Fig. 2B, are strongly affected by intracellular Ca^{2+} . The mean values are 40 ± 2 nS/s at high $[Ca^{2+}]_i$ ($n = 493$ fusion pores from 26 cells) and 19 ± 1 nS/s at low $[Ca^{2+}]_i$ ($n = 423$, 39 cells). When the GTP γ S concentration was increased to 80 μM , the distributions (Fig. 2B, bottom panels) and the mean slopes were not affected (40 ± 3 nS/s at high $[Ca^{2+}]_i$, $n = 361$, 18 cells and 17 ± 1 nS/s at low $[Ca^{2+}]_i$, $n = 474$, 36 cells). To test if an additional population of very slow openings occurs at low $[Ca^{2+}]_i$, the fits to the low $[Ca^{2+}]_i$ data were repeated ignoring the first three or

five bins in the histogram. The fits then gave values of $25\text{--}27 \pm 3$ nS/s which are still significantly lower than those obtained at high $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ was further elevated to 10 μM , the distribution could also be fitted with a single exponential (data not shown) giving a mean slope of 89 ± 11 nS/s ($n = 374$, 26 cells, 20 μM GTP γ S).

4. Discussion

4.1. Universal structure of the initial fusion pore

The formation of the fusion pore connecting the lumen of the vesicle to the extracellular space is a key event in exocytosis. This initial fusion pore appears to be a universal structure since very similar distributions around mean values of approximately 200 pS are observed in beige mouse peritoneal mast cells [1,2]

and eosinophils isolated from horse blood used in the present work. Metastable states of the early fusion pore in the same range were observed in guinea-pig eosinophils [12].

The molecular nature of the fusion pore has been an object of controversial speculation [13–16] and the initial pore has been proposed to be lipidic or alternatively a protein structure similar to an ion channel. Pore conductances measured in lipid membranes during electrical breakdown are in the μS range increasing with an initial slope of 0.3 S/s and are thus larger by many orders of magnitude [17]. The pores formed during mechano-electrical breakdown of mast cell granule membranes measured with a technique similar to that used here, had a mean conductance of 1100 pS [18], about five times larger than the conductance of the fusion pore. The properties of the membrane breakdown pores thus appear to differ from those of the fusion pore. If we assume a fusion pore structure traversing two membranes in series, the granule membrane and the plasma membrane, the length of the pore should be about 15 nm. Using the specific resistance of extracellular saline of 60 Ωcm , we converted the distribution of pore conductance into the distribution of diameters (Fig. 1F). The typical size of early fusion pores may be quantified as the range between the half-maximal values of the distribution being 0.9–1.8 nm.

4.2. Expansion of the fusion pore is modulated by intracellular Ca^{2+}

The subsequent pore expansion has previously been investigated systematically only during the first millisecond of its existence where a mean slope of 200 nS/s was obtained at $[\text{Ca}^{2+}]_i \approx 150 \text{ nM}$ [2]. This value is larger than the 17–42 nS/s measured here on the time scale of more than hundred milliseconds. However, individual fusion events observed in mast cells show that after 10 ms the conductance slope decreases [1] to 5–50 nS/s on the 100 ms time scale [1,2,19,20] in agreement with the results shown here. In our experiments the initial pore conductance was usually stable for some time and did not grow linearly during the first millisecond (Fig. 1C). We have thus not made an attempt to assign a conductance slope to the early expansion process.

Once the pore has widened to the nS conductance range, it is lipidic in nature [21]. The expansion of such a pore may be driven by forces of membrane curvature and membrane tension [22]. We found that the pore expansion rate depends on $[\text{Ca}^{2+}]_i$ in the micromolar range. The calcium dissociation constants of phospholipid head groups are larger by many orders of magnitude (80 mM for the negatively charged phosphatidylserine [23]), and even in the presence of a significant surface potential the binding of Ca^{2+} ions to lipid head groups below 10 μM free Ca^{2+} is negligible [24]. It is thus unlikely that the pore expansion is a passive property of the lipid membrane. Our results suggest instead that the forces expanding the pore are generated by proteins. It should be noted that during the reverse process of endocytosis, studied in nerve terminals, the fission pore closes with similar speed (–16 nS/s) [25]. The process of endocytosis is well known to be mediated by a protein machinery [26] indicating that the rates measured here for the expansion of the fusion pore may be achieved by protein forces.

$[\text{Ca}^{2+}]_i$ controls the speed of various steps in exocytosis. We have shown that the initial structure of the fusion pore itself is independent of $[\text{Ca}^{2+}]_i$ and has very similar properties in different cell types. Although it is possible that granular material leaks out via a small pore, an expanded pore is required to allow for rapid release of the bulk material [27]. The Ca^{2+} dependence of the pore expansion thus reveals that in addition to processes preceding fusion, the actual release is also modulated by $[\text{Ca}^{2+}]_i$ at a stage following the vesicle–plasma membrane fusion event.

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