**The Dynamics of Exocytosis in Human Neutrophils**

Oliver Nüße and Manfred Lindau

Biophysics Group, Department of Physics, Freie Universität Berlin, D-1000 Berlin 33, Germany

**Abstract.** We have investigated the dynamics of exocytosis in single human neutrophils. The increase of membrane area associated with granule fusion was followed by time-resolved patch-clamp capacitance measurements. Intracellular application of 20 μM guanosine-5'-O(3-thiotriphosphate) (GTPγS) in the presence of 2.5 mM ATP stimulated exocytosis and led to an increase of membrane capacitance from 3.0 to ~8.4 pF corresponding to a 540 μm² increase of membrane area. This capacitance change is very close to the value expected from morphological data if all primary and secondary granules fuse with the plasma membrane. High resolution measurements revealed stepwise capacitance changes corresponding to the fusion of individual granules. GTPγS-stimulated exocytosis did not require pretreatment with cytochalasin B and the amplitude was independent of the intracellular-free calcium concentration between 10 nM and ~2.5 μM. In the absence of GTPγS elevation of intracellular-free calcium concentration to the micromolar range led to the fusion of only a limited number of granules. Degranulation stimulated with GTPγS started after a lag phase of 2-7 min and was usually complete within 5-20 min. The time course was affected by the intracellular ATP and calcium concentration. Exocytosis was markedly accelerated by pretreatment with cytochalasin B. Our results demonstrate that the final steps leading to primary and secondary granule fusion are controlled by a guanine nucleotide-binding protein and do not require an elevation of intracellular calcium. Calcium and other factors are, however, involved in the regulation having pronounced effects on the dynamics of exocytosis.

**Neutrophils** (polymorphonuclear leukocytes) play a major role in host defence against microbial infections. Phagocytosis and exocytosis are important functions accomplished by these cells. They discharge their granular contents in response to various stimuli such as aggregated immunoglobulin G (16) or the chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (5). The two major granule types, the primary and secondary granules, are believed to have distinct functions (12) and their activation may be under differential control (4). Endo- and exocytosis are associated with significant changes in membrane area. When a secretory granule fuses with the plasma membrane, the area of the plasma membrane increases by the area of the granule membrane. As the membrane area is proportional to the membrane capacitance with a specific capacitance of ~1 μF/cm², such membrane area changes can be measured in single cells by capacitance measurements using the whole-cell patch-clamp technique (20). In the whole-cell configuration the cytoplasmic composition is determined by the pipette solution. Various substances like calcium buffers, nucleotides, or proteins can thus be introduced into the cell at well-defined concentrations. The method is thus similar to the microinjection. In addition, the capacitance measurement provides a direct and quantitative record of the time course of granule fusion. The technique has previously been used to demonstrate (14) and characterize (13) the stimulation of exocytosis in mast cells by guanine nucleotides. We have applied the method to study the dynamics of exocytosis in response to guanosine-5'-O(3-thiotriphosphate) (GTPγS) and calcium in single human neutrophils.

**Materials and Methods**

**Cell Preparation**

Human neutrophils were isolated from heparinized fresh blood by dextran sedimentation, centrifugation through a ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ) layer and hypotonic lysis of remaining red blood cells as described (7). Isolated cells were suspended in a medium containing 117 mM NaCl, 4.2 mM KCl, 0.8 mM MgCl₂, 16 mM CaCl₂, 8.3 mM Hepes/NaOH, 5.6 mM Glucose, 2.3 mM HCl, 0.33 mM NaH₂PO₄, pH 7.3-7.4, stored at 37°C, 7% CO₂ and used within 6 h.

**Patch-Clamp Experiments**

For the experiments ~200 μl of the cell suspension were dropped into a chamber with a glass bottom (coverslip). After settling of the cells the medium was exchanged for our standard external solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes/NaOH, 15-20

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1. **Abbreviations used in this paper:** (Ca²⁺), intracellular-free calcium concentration; G protein, guanine nucleotide-binding protein; GTPγS, guanosine-5'-O(3-thiotriphosphate); fMet-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine.
of the nucleus was estimated to cover 20% of the cell profile in agreement with previous studies.

**Estimation of the Total Granule Membrane Area**

The total number $n$ of granules of each type was estimated assuming four spheres and was thus estimated to occupy ~5% of the cell volume. The volume of the nucleus was approximated as a sphere, giving ~1.9 primary granules/μm² and ~3.7 secondary granules/μm². The measurement of the total granule membrane area was accordingly estimated to be ~360 μm² for primary and ~240 μm² for secondary granules.

**Results**

**Intracellular GTPyS Stimulates Exocytosis**

Fig. 1 shows the time course of membrane capacitance and conductance recorded from a cell that was dialyzed with a pipette solution containing 20 μM GTPyS, 2.5 mM ATP, and 500 μM EGTA. The cells in the recording chamber were perfused with our standard external saline supplemented with cytochalasin B (10 μg/ml) since it is well-established that receptor-mediated exocytosis in neutrophils is markedly enhanced by this pretreatment. The small negative deflection in the beginning of the capacitance trace is an artifact resulting from the strong suction pulses which were applied to break into the cell. The capacitance started to increase after a lag phase of ~3 min. After this delay the capacitance increased from the initial value $C_i = 4.0$ pF to a final value $C_f = 9.2$ pF, presumably reflecting a proportional increase in membrane area as a consequence of granule fusion. The morphological appearance of many cells changed during the course of the experiment. The most obvious effects were changes in shape that were not reproducible and not correlated with the capacitance changes. Since the vast majority of neutrophil granules are too small to be well-resolved by light microscopy, morphological changes associated with degranulation could not clearly be identified. In many experiments, however, several small particles were seen in the vicinity of the cell after degranulation, presumably corresponding to the largest granules of the cell. In some cells we have observed a capacitance decrease after degranulation but usually we did not record long enough from individual cells to investigate this phenomenon in detail.

**High Resolution Capacitance Measurements Reveal Discrete Fusion Events**

The fusion of a secretory granule is a discrete event and...
should result in a stepwise capacitance increase as previously demonstrated in chromafin cells (24), rat mast cells (14), and pancreatic acinar cells (22). These capacitance steps can be measured at high resolution by the phase-sensitive detection method (20, 24). We have applied this technique to neutrophils stimulated by intracellular GTPγS. In Fig. 2 a stepwise capacitance changes of 2–4 fF (dashed lines) are detectable. In this experiment a pipette solution containing 5 μM GTPγS, 500 μM EGTA (pCa ~8), and only 50 μM ATP was used to slow down the degranulation (see below) and to increase the capacitance resolution. The bulk capacitance of the cell was compensated (Cm = 3.5 pF, RA = 12 MΩ), and the patch-clamp amplifier was operated at high gain (100 mV/pA). Apparently continuous capacitance changes, as the part between the solid lines, were also seen. They may be due to the fusion of granules, which are too small to be resolved as stepwise changes by this method. Alternatively, these apparently continuous changes could also be a consequence of an initial flickering of the fusion pores (8). Flickering of the fusion pore which is faster than the time resolution of the measurement will lead to an apparent capacitance value between the unfused and fused state, corresponding to the time average of the fusion pore to be in the open or closed state, respectively. Fast flickering would thus mask the stepwise nature of the capacitance change.

The corresponding step size distribution (Fig. 2 b) was obtained from measuring 450 steps like those marked by dashed lines in Fig. 2 a. Five cells were used and the step size distribution from each single cell was not significantly different from that shown in Fig. 2 b. Parts showing gradual changes like that between the solid lines in a were discarded. Assuming a specific capacitance of Ca = 1 μF/cm² the capacitance change ΔC associated with the fusion of a granule having the diameter d is ΔC = Cₐπd². We have assumed the granule diameter to follow a gaussian distribution and have fitted the mean diameter d and the variance σ such that the corresponding calculated capacitance step size distribution fits the measured distribution. The fit (smooth line) revealed d = 280 nm and σ = 40 nm. These values are in good agreement with morphological data from purified azurophilic granules for which d = 300 nm was reported (28).

In the experiment in Fig. 1, the pipette solution contained 500 μM EGTA, 500 μM EGTA (pCa ~8) but with 2.5 mM ATP, the capacitance was nearly constant as long as the membrane conductance remained low.

**GTPγS Stimulates Exocytosis at Very Low (Ca²⁺)~**

In the experiment in Fig. 1, the pipette solution contained 500 μM EGTA. Since the membrane conductance was very low in this cell (lower trace of Fig. 1) the intracellular concentration of free calcium (Ca²⁺), was thus buffered well below the level of resting cells, which has been reported to be ~120 nM (26). At the EGTA concentration used here, only minor changes of (Ca²⁺) are expected (23) and virtually the same results were obtained when (Ca²⁺) was strongly buffered at ~30 nM using 5 mM EGTA and 1 mM CaCl₂ (not shown). In the experiment of Fig. 1 GTPγS-induced degranulation was not associated with significant changes in membrane conductance. The opening of calcium-activated ion channels which have recently been identified in a patch-clamp study on neutrophils (31) is apparently not an essential event for exocytosis stimulated by GTPγS.

**High (Ca²⁺), Stimulates Exocytosis with a Reduced Amplitude**

In control experiments without GTPγS and calcium (pCa ~8) but with 2.5 mM ATP, the capacitance was nearly constant as long as the membrane conductance remained low.

**Figure 2.** (a) High resolution capacitance measurement from a neutrophil stimulated with a pipette solution, supplemented with 5 μM GTPγS, 500 μM EGTA, and 50 μM ATP. (b) Step size distribution constructed from capacitance steps like those marked by dashed lines in a.
Figure 3. Time course of membrane capacitance ($C_m$) and membrane conductance ($G_m$) of a human neutrophil recorded with the standard pipette solution supplemented with 500 µM EGTA and 2.5 mM ATP.

($C_i = 3.45 \pm 0.31$ pF, $C_f = 3.05 \pm 0.30$, $n = 5$). In the experiment in Fig. 3, the capacitance was constant for ~6 min. At this time a large leakage conductance appeared, immediately followed by a small capacitance increase from 2.8 to 3.5 pF. This capacitance change could be due to fusion of a few granules induced by calcium influx from the external medium overwhelming the buffer capacity of the 500 µM EGTA. To test this hypothesis we perfused neutrophils with pipette solutions containing no GTPyS but with calcium buffered at 1.5-4.0 µM depending on the pH (7.0-7.2). This resulted in a similar small and fast capacitance increase in the absence of significant conductance changes (Fig. 4). In this experiment the capacitance increased from 2.9 to 3.4 pF within 2 min. This capacitance change corresponds to an extent of granule fusion ($C_f - C_i$) which is only ~10% of that observed in the GTPyS-stimulated cell. Comparison of Figs. 3 and 4 strongly suggests that the capacitance change after the large conductance increase was indeed due to calcium influx overwhelming the buffer capacity of the intracellular EGTA and other endogenous calcium buffers. We have also directly observed pronounced elevations of intracellular calcium in leaky cells using the calcium indicator fura 2 (unpublished data).

Figure 4. Time course of membrane capacitance ($C_m$) and membrane conductance ($G_m$) of a human neutrophil stimulated with a pipette solution supplemented with 4.5 mM CaCl$_2$, 5 mM EGTA (pH 7.2, Ca$^{2+}$-free ~1.7 µM), and 2.5 mM ATP.

The Amplitude of Exocytosis

The average total number of granules per cell can be estimated from morphological data (2, 28). Using the formula given by Elias et al. (11) we obtained a total number of ~1,300 primary granules per cell and ~4,600 secondary granules per cell. If all these granules fuse with the plasma membrane, the capacitance should increase by ~3.6 pF (primary granules) plus about 2.4 pF (secondary granules). Fig. 5 shows the average values of $C_i$ and $C_f$ for various conditions. The mean initial capacitance of resting human neutrophils that were not treated with cytochalasin B was 2.96 ± 0.13 pF (solid line). If all granules fuse with the plasma membrane the capacitance should increase by ~6 pF, giving an average final capacitance $C_f$ ~ 9 pF. This value is marked by the dashed line in Fig. 5. The final capacitance of cells stimulated by GTPyS in the presence of 2.5 mM ATP was very close to this value. These results strongly suggest that virtually all granules of both types fused with the plasma membrane in response to intracellular application of GTPyS. $C_i$ was independent of the intracellular calcium concentration and also independent of the pretreatment with cytochalasin B. The mean initial capacitance of cells pretreated with cytochalasin B was slightly higher (3.22 ± 0.08 pF), which could reflect exocytosis of a few percent of neutrophil granules in response to cytochalasin B.

In control experiments using pipette solutions containing 500 µM EGTA, 2.5 mM ATP, but no GTPyS the capacitance was nearly constant. In cytochalasin B–treated cells the capacitance values were $C_i = 3.45 \pm 0.31$ pF and $C_f = 3.05 \pm 0.30$ pF ($n = 5$). Here $C_f$ was defined as the capacitance after ~15 min. In cells that had not been pretreated with cytochalasin B, the corresponding values were $C_i = 3.41 \pm 0.25$ pF and $C_f = 3.64 \pm 0.27$ pF ($n = 5$).
When intracellular Mg-ATP was reduced from 2.5 mM to 50 μM, GTPγS stimulation resulted in significantly lower amplitudes of exocytosis ($C_i = 6.5 \text{ pF}$), suggesting a partial ATP dependence. It should be noted that a value of $\sim 6.5 \text{ pF}$ is expected if only primary granules are exocytosed. To test if this part of exocytosis is completely independent of ATP we also did experiments in the absence of ATP. To deplete the cells of endogenous ATP they were perfused with a solution containing no glucose, 6 mM 2-deoxyglucose, and 5 μM antimycin several minutes before the experiment. Under these conditions GTPγS stimulated generally similar capacitance changes ($C_i = 6.0 \pm 0.3 \text{ pF}$, $n = 10$) in the absence of ATP, although the time course was usually slower (see below). ATP alone did not stimulate exocytosis as long as the intracellular calcium concentration was low (see Fig. 3, 0–6 min and legend to Fig. 5). The combination of high calcium and high ATP, however stimulated a capacitance increase by $\sim 1.3 \text{ pF}$. Among the cells in this group those with lower (Ca$^{2+}$) displayed smaller capacitance changes than those with higher (Ca$^{2+}$). The comparably low amplitude of the experiment shown in Fig. 4 (although within the range of mean ± SEM given in Fig. 5) may correspond to the low calcium concentration compared with other cells in this group. When the ATP concentration was only 50 μM, exocytosis in response to high (Ca$^{2+}$), was further reduced ($C_i - C_i \sim 0.4 \text{ pF}$). This small increase is hardly distinguishable from control values and may reflect spontaneous fusion events in the absence of a specific stimulus. Unfortunately exocytosis induced by the combination calcium + ATP is very fast and we were not yet able to use the phase-sensitive detection method with calcium + ATP-stimulated cells. Thus we do not know if this type of stimulation activates only one particular type of granules.

**The Time Course of Exocytosis**

The time course of exocytosis was affected by the different conditions in a more complicated manner. In the cytochalasin B–treated cells the time course in response to GTPγS at low (Ca$^{2+}$), was roughly similar to that shown in Fig. 1. At high (Ca$^{2+}$), and without the cytochalasin B treatment the time courses were more variable. To give a quantitative description of the time course we have used the time from breaking into the cell to the point where 10% of the capacitance change had occurred ($t_{10}$) and the time required for the capacitance change to go from 10 to 90% ($t_{90}$). These values are shown in Fig. 6. Both values show a similar dependence on the various conditions used. Exocytosis in response to GTPγS was much faster in cytochalasin B–treated cells than in untreated cells. Reducing ATP from 2.5 mM to 50 μM in cytochalasin B–treated cells slowed down the degranulation. Omitting ATP in metabolically inhibited cells led to very variable time courses. In these cells degranulation stimulated by GTPγS sometimes took up to 30 min. At low (Ca$^{2+}$), exocytosis was also somewhat slower than at high (Ca$^{2+}$). The capacitance change observed in response to high (Ca$^{2+}$), + high ATP was much faster than that in response to GTPγS and was not significantly affected by the pretreatment with cytochalasin B. The capacitance change in response to high (Ca$^{2+}$), at 50 μM ATP was very fast, but had a very small amplitude (see Fig. 5).

**Discussion**

Our results show that exocytosis can be studied quantitatively in single human neutrophils by time-resolved patch-clamp capacitance measurements. During degranulation we have observed capacitance steps of the size expected for the fusion of single granules which demonstrates that the observed capacitance changes indeed reflect the plasma membrane area expansion associated with granule fusion. In the whole-cell configuration the composition of the cytoplasm is tightly controlled by the pipette solution. The method thus enables us to interact biochemically with the postreceptor events playing a role in neutrophil activation and at the same time measure the exocytotic response at extremely high resolution. Contrary to conventional methods of cell permeabilization the whole-cell patch-clamp technique has the advantage that the composition of the intracellular and extracellular media can be controlled independently.

The data presented here demonstrate that introduction of GTPγS and ATP into the cytoplasm of single neutrophils stimulates complete degranulation. In contrast to receptor-mediated exocytosis (1) stimulation by intracellular application of GTPγS did not require pretreatment with cytochalasin B. The primary and secondary granules of neutrophils may have distinct functions (12) and have been demonstrated to be differently controlled. In permeabilized neutrophils stimulated with Ca$^{2+}$ primary granule discharge required ATP or other nucleotides, whereas exocytosis of secondary granules was strictly ATP dependent (4). The reduced amplitudes which we measured at low (50 μM) ATP concentration as well as in the complete absence of ATP could accordingly reflect the absence of secondary granule exocytosis. Interestingly, the change in membrane area under these conditions is very close to that which would be expected if only primary granule exocytosis occurs.

The time course of exocytosis in response to GTPγS was affected by cytochalasin B, ATP, and calcium. Cells pretreated with cytochalasin B and stimulated with GTPγS in the presence of 2.5 mM ATP and micromolar (Ca$^{2+}$), degranulate completely within 5–10 min. The time course is somewhat slower when the calcium concentration is low. If the cytochalasin B treatment is omitted, degranulation is
slow down more than twofold. This result supports the view that the intact cytoskeleton may normally reduce granule motility (1) resulting in a reduced fusion rate. The effect of reducing ATP to 50 μM on the time course is intermediate. The lag phase (t₀₀) is only marginally affected whereas the rise time (t₁₀₀₀) increases by ~60%. In metabolically inhibited cells without ATP, degranulation was even slower. However, the time courses measured in individual cells scattered widely which suggests that these effects may be due to intracellular changes secondary to the metabolic inhibition (e.g., dephosphorylation). Cells without cytochalasin B, low ATP, or high calcium frequently degranulated in phases with high and low fusion rates. The mechanisms regulating the rate of granule fusion are still to be elucidated.

The results that we obtained from human neutrophils share several similarities with those obtained previously in rat mast cells. Intracellular stimulation of GTPγS to mast cells is also an effective stimulus to induce complete degranulation (14). The time course of exocytosis in mast cells is also characterized by a lag phase which is ~1 min at 20 μM GTPγS if calcium is weakly buffered (13). In the presence of 500 μM EGTA, however, the degranulation of mast cells is several-fold slower (21, 23). The calcium dependence of the time course is thus markedly stronger in mast cells than in neutrophils indicating that the mechanism affecting the rate of granules fusion may be different in both cell types. In mast cells the cytoplasmic space is densely packed with granules that are already in the right place to fuse among each other and with the plasma membrane. In neutrophils the situation is different. The granules are separated by comparably large distances and they must move to the plasma membrane before fusion can occur. This additional step may be responsible for the slower time course of exocytosis in neutrophils. The weaker calcium dependence of the time course in neutrophils could be explained if a calcium-independent time required for granule movement is simply added to a strongly calcium-dependent time course of granule fusion. In mast cells complete degranulation is observed in the presence of 100 μM ATP (13, 14). If the different amplitudes that we observed in neutrophils at high and low ATP concentrations reflect an ATP requirement for secondary granule fusion, then exocytosis of mast cell granules and primary neutrophil granules appear to be similarly controlled, whereas secondary granule exocytosis involves an additional ATP-dependent step.

In intact neutrophils exocytosis of primary granules in response to stimulation with fMet-Leu-Phe is blocked if (Ca²⁺)₂ is <50 nM and is half maximal at (Ca²⁺)₂ = 200 nM (19). Previous experiments using GTPγS in permeabilized neutrophils led to conflicting results with respect to the calcium dependence. The release of β-glucuronidase, a primary granule marker, was observed when GTPγS was introduced using Sendai Virus (3). With this method maximal exocytosis required the presence of exceedingly high concentrations of the calcium chelator EGTA (3). When the cells were permeabilized using streptolysin O, however, half-maximal release of β-glucuronidase in response to GTPγS required the presence of ~1 μM free Ca²⁺ in HL-60 cells (23) and human neutrophils (O. Nüüsre, unpublished data). In the experiments described here, we have observed exocytosis of the corresponding granule type in response to intracellular GTPγS application with a patch pipette at pCa ~8. Degranulation is virtually complete and independent of (Ca²⁺)₂. In contrast to the conventional cell permeabilization techniques, loading the cells with GTPγS via the patch pipette can be done in the presence of normal (2 mM) extracellular calcium. The calcium dependence observed in experiments with streptolysin O may thus reflect a calcium requirement at the extracellular side of the membrane. In addition, for permeabilization experiments cells are prepared and stored in calcium-free solutions which may cause extensive calcium depletion at intracellular sites relevant to the mechanisms of exocytosis. The existence of a regulatory role of calcium is also evident from the effect on the time course observed in our experiments.

The involvement of a guanine nucleotide-binding protein (G protein) in neutrophil activation has been implicated from the inhibitory effect of pertussis toxin on the response stimulated by fMet-Leu-Phe (18, 30). This G protein could have been directly activated by GTPγS in our experiments. The results presented here strongly suggest that fusion of both granule types is mediated by a G protein. The direct activation of the yet unknown G protein bypasses the Ca²⁺ dependence and the requirement for pretreatment with cytochalasin B.

A second G protein (Go) has been proposed to be the target of GTPγS in neutrophils (3) and mast cells (10) and we have presented evidence that the G protein activated by GTPγS is not identical with the pertussis toxin-sensitive G protein in mast cells (21). Recently, it has been reported that pertussis toxin inhibits exocytosis from mast cells stimulated by compound 48/80 and from human basophils stimulated by fMet-Leu-Phe. However, in both cell types exocytosis in response to anti-IgE was not significantly affected by the pertussis toxin treatment (27). Intracellular stimulation of rat mast cells with GTPγS, however, mimicks the effect of antigen rather than compound 48/80 (13). Taken together, these results suggest that in the same cell different G proteins may couple the different receptor systems to the exocytotic response. The pertussis toxin-sensitive G protein mediating the fMet-Leu-Phe response in neutrophils is thus not necessarily the target of GTPγS in the experiments described here.

The identity of the G proteins mediating exocytosis in neutrophils remains to be elucidated. Several different G proteins have recently been identified in this cell type (6, 15, 17) and these are candidates to be tested for their functional role. We wish to thank Dr. W. Almers who supplied us with the design of the patch pipette.

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