

The Dynamics of Exocytosis in Human Neutrophils

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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) (GTP γS) 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 μm^2 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. GTP γS -stimulated exocytosis did not require pretreatment with cytochalasin B and the amplitude was independent of the intracellu-

lar-free calcium concentration between 10 nM and ~ 2.5 μM . In the absence of GTP γS elevation of intracellular-free calcium concentration to the micromolar range led to the fusion of only a limited number of granules. Degranulation stimulated with GTP γS 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.

NEUROPHILS (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)¹ (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 $\mu\text{F}/\text{cm}^2$, 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) (GTP γS) 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, 1.6 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

1. *Abbreviations used in this paper:* (Ca²⁺)_i, intracellular-free calcium concentration; G protein, guanine nucleotide-binding protein; GTP γS , guanosine-5'-O(3-thiotriphosphate); fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine.

mM glucose, 10 $\mu\text{g/ml}$ cytochalasin B (sometimes excluded as indicated) at pH 7.2–7.4). In some experiments glucose was replaced by 6 mM 2-deoxyglucose, and antimycin was included at 5 μM concentration. The cells were dialyzed in the whole-cell configuration with a pipette solution containing 125 mM K-glutamate, 10 mM NaCl, 10 mM HEPES/NaOH, 7 mM MgCl_2 , pH 7.0–7.2. CaCl_2 , EGTA, ATP, and GTP γS were varied as described in the text. EGTA, ATP, and 2-deoxyglucose were from Sigma Chemical Co. (St. Louis, MO), GTP γS and antimycin were from Boehringer Mannheim Biochemicals (Indianapolis, IN). For experiments at high intracellular-free calcium concentration ($[\text{Ca}^{2+}]_i$), the pipette solutions contained 5 mM EGTA and 4.5 mM CaCl_2 . The resulting free calcium concentrations were calculated to be in the range of 1.5–4.0 μM depending on the pH. These calculations were done using a computer program generously provided by Dr. B. D. Gomperts (see reference 4 for details). The program written by Sherwin Lee is based on the algorithm of Perrin and Sayce (25). For the patch-clamp recordings, we selected cells that adhered to the glass bottom of the recording chamber, but appeared spherical. All experiments were done at room temperature.

Time Course of Exocytosis

Voltage pulses of -20 mV and 2 ms duration were given every 3 s via the command input of an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, FRG) operating in the voltage-clamp mode (holding potential -10 mV). The potentials were corrected for the 10 mV liquid junction potential which is due to the different solutions in the pipette and the bath. The current responses were sampled by a PDP 11/73 minicomputer. Membrane conductance G_m , membrane capacitance C_m , and access resistance R_A were determined on-line from a single exponential fit of the current response as described (20). G_m , C_m , and R_A were displayed by the computer and stored on a magnetic disc.

For each cell the initial (C_i) and final (C_f) capacitance was determined. The time of patch rupture was always defined as $t = 0$. The delay was characterized by the time at which 10% of the total capacitance increase ($C_f - C_i$) had occurred (t_{10}). The subsequent rise time was characterized by the time between the 10 and 90% point of the capacitance change ($t_{10/90}$).

High Resolution Capacitance Measurements

For high resolution capacitance measurements a two-phase lock-in amplifier was built based on the circuit described by Lindau and Neher (20). The in-phase and out-of-phase outputs were averaged over one sine wave cycle by phase-locked integration as described by Breckenridge and Almers (9). A continuous 800 Hz sine wave of 20 mV_{rms} amplitude was applied via the command input of the EPC 7 operating in the voltage-clamp mode (holding potential -10 mV). The current output was filtered by the 10 kHz low pass filter of the EPC 7 and connected to the lock-in input. After attaining the whole-cell configuration the bulk capacitance of the cell was compensated. The phase setting ϕ of the lock-in was adjusted according to $\phi = [\arcsin(\omega C_m R_A) + \delta]$, where C_m and R_A were obtained from the compensation settings. The additional phase shift δ reflects the instrumental delay mainly arising from the 10 kHz low pass filter. The phase setting was checked simulating a 100 fF capacitance increase by decreasing the capacitance compensation. This simulation generated no change in the in-phase component and also provided the calibration for the capacitance trace. The compensation and calibration procedure was frequently repeated during an experiment. The lock-in outputs were filtered with 10 ms RC filters and sampled every 10 ms by the computer. Before the analysis the recordings were digitally filtered at 40 ms by averaging over four adjacent data points.

Estimation of the Total Granule Membrane Area

The mean number of granules in a thin section through the middle of a neutrophil was taken to be 75 primary and 150 secondary granules (2). Under the microscope we determined the mean diameter of a spherical neutrophil (as used for our experiments) to be ~ 8 μm . From May-Gruenwald/Giemsa (Aldrich Chemical Co., Milwaukee, WI) stained light micrographs the area of the nucleus was estimated to cover $\sim 20\%$ of the cell profile in agreement with electron microscopy (2). This part was subtracted from the area of a mid profile when the number of granules per unit area of cytoplasm in a thin section (n_s) was estimated giving ~ 1.9 primary granules/ μm^2 and ~ 3.7 secondary granules/ μm^2 . The volume of the nucleus was approximated assuming four spheres and was thus estimated to occupy $\sim 5\%$ of the cell volume. The total number n of granules of each type was estimated using the formula given by Elias et al. (11):

$$n = V \times n_s / (\bar{d} + T),$$

where \bar{d} = mean granule diameter (primary 0.3 μm , secondary 0.13 μm [28]), T = thickness of the section (75 nm), and V = mean cytoplasmic volume (255 μm^3). The total granule membrane area was accordingly estimated to be ~ 360 μm^2 for primary and ~ 240 μm^2 for secondary granules.

Results

Intracellular GTP γS 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 GTP γS , 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 $\mu\text{g/ml}$) 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 Measurements Reveal Discrete Fusion Events

The fusion of a secretory granule is a discrete event and

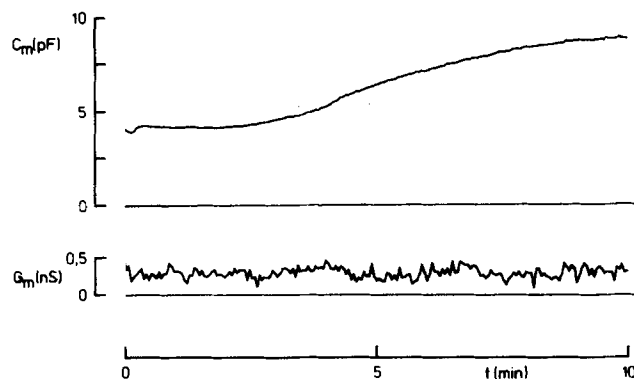


Figure 1. Time course of membrane capacitance (C_m) and membrane conductance (G_m) of a human neutrophil stimulated with a pipette solution supplemented with 20 μM GTP γS , 500 μM EGTA (Ca^{2+} -free ~ 10 nM), and 2.5 mM ATP.

should result in a stepwise capacitance increase as previously demonstrated in chromaffin 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 GTP γ S. In Fig. 2 *a* stepwise capacitance changes of 2–4 fF (dashed lines) are detectable. In this experiment a pipette solution containing 5 μ M GTP γ S, 500 μ M EGTA (pCa \sim 8), and only 50 μ M ATP was used to slow down the degranulation (see below) and to increase the capacitance resolution. The bulk capacitance of the cell was compensated ($C_m = 3.5$ pF, $R_A = 12$ M Ω), 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

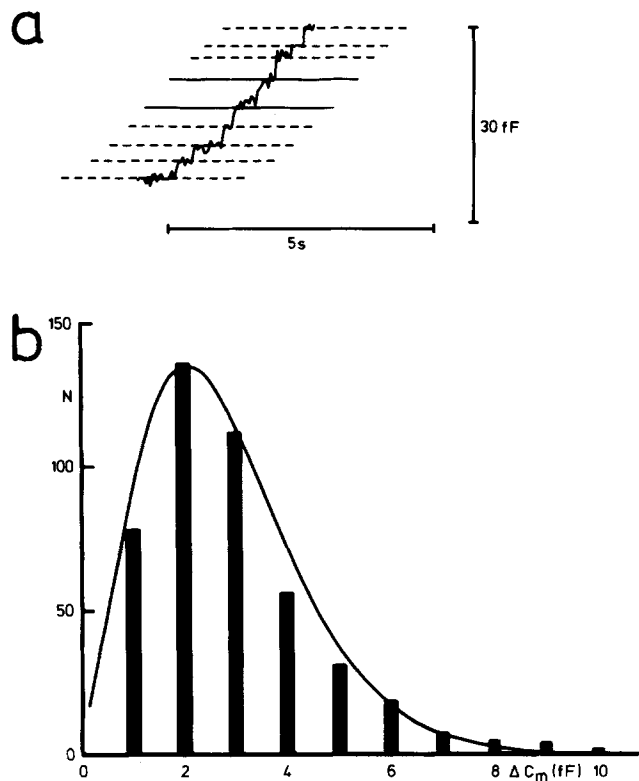


Figure 2. (a) High resolution capacitance measurement from a neutrophil stimulated with a pipette solution, supplemented with 5 μ M GTP γ S, 500 μ M EGTA, and 50 μ M ATP. (b) Step size distribution constructed from capacitance steps like those marked by dashed lines in *a*.

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 $c_s = 1$ μ F/cm 2 the capacitance change ΔC associated with the fusion of a granule having the diameter d is $\Delta C = c_s \pi d^2$. We have assumed the granule diameter to follow a gaussian distribution and have fitted the mean diameter \bar{d} and the variance σ such that the corresponding calculated capacitance step size distribution fits the measured distribution. The fit (smooth line) revealed $\bar{d} = 280$ nm and $\sigma = 40$ nm. These values are in good agreement with morphological data from purified azurophilic granules for which $\bar{d} = 300$ nm was reported (28). In transmission electron micrographs (2) primary granules of 0.19–0.36 μ m diam can be seen. This agrees very well with the distribution width $\bar{d} \pm 2\sigma$ which we obtained from the capacitance steps. The other major type of neutrophil granules, the secondary granules, are much smaller, having diameters between 0.1 and 0.24 μ m (2) with a mean value of 0.13 μ m (28). As the limit of resolution of the phase-sensitive detection method is ~ 1 fF which corresponds to a granule diameter of 0.18 μ m, most of the secondary granules are too small to be detected as single capacitance steps. The fusion of these granules with the plasma membrane would thus lead to apparently continuous capacitance changes. The relative contribution of stepwise and continuous changes has not been determined since the stepwise changes could only be detected during recording periods exhibiting extremely low noise. Frequently the noise level exceeded the size of the capacitance steps due to spontaneous fluctuations of the pipette or membrane resistance or due to flickering of the fusion pores. In addition, when subsequent fusion events follow each other at intervals approaching the time constant of the measurement the steps also escape detection. Clearly identified steps thus always presented a minor fraction of the total capacitance change (<20%) even at 50 μ M ATP.

GTP γ S Stimulates Exocytosis at Very Low (Ca^{2+})_i

In the experiment in Fig. 1, the pipette solution contained 500 μ M EGTA. Since the membrane conductance was very low in this cell (lower trace of Fig. 1) the intracellular concentration of free calcium (Ca^{2+})_i 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 (Ca^{2+})_i are expected (23) and virtually the same results were obtained when (Ca^{2+})_i was strongly buffered at ~ 30 nM using 5 mM EGTA and 1 mM CaCl $_2$ (not shown). In the experiment of Fig. 1 GTP γ S-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 GTP γ S.

High (Ca^{2+})_i Stimulates Exocytosis with a Reduced Amplitude

In control experiments without GTP γ S and calcium (pCa \sim 8) but with 2.5 mM ATP, the capacitance was nearly constant as long as the membrane conductance remained low

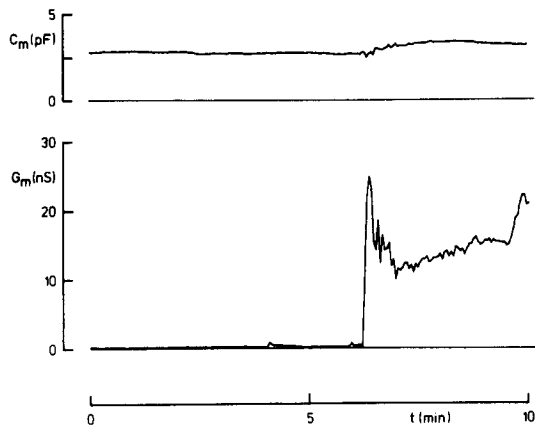


Figure 3. Time course of membrane capacitance (C_m) and membrane conductance (G_m) of a human neutrophil recorded with the standard pipette solution supplemented with 500 μ M EGTA and 2.5 mM ATP.

($C_i = 3.45 \pm 0.31$ 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 overcoming the buffer capacity of the 500 μ M EGTA. To test this hypothesis we perfused neutrophils with pipette solutions containing no GTP γ S but with calcium buffered at 1.5–4.0 μ M 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 $\sim 10\%$ of that observed in the GTP γ S-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).

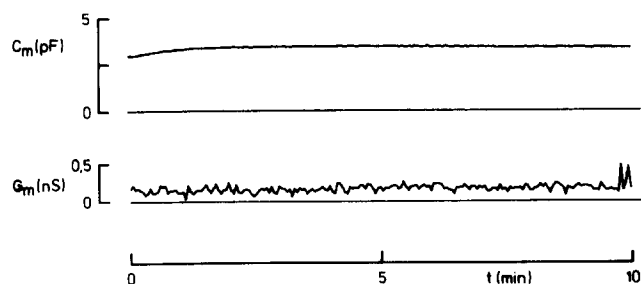


Figure 4. Time course of membrane capacitance (C_m) and membrane conductance (G_m) of a human neutrophil stimulated with a pipette solution supplemented with 4.5 mM CaCl_2 , 5 mM EGTA (pH 7.2, Ca^{2+} -free ~ 1.7 μ M), and 2.5 mM ATP.

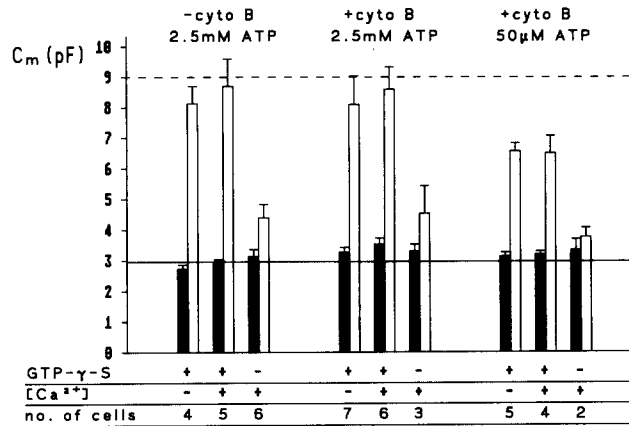


Figure 5. Mean values of initial (C_i) and final (C_f) capacitance of neutrophils under various experimental conditions (error bars are SEM). The average C_i of resting neutrophils is marked by the solid line. The expected C_f for complete degranulation (see text) is marked by the dashed line. 20 μ M GTP γ S (+), no GTP γ S (-); 1.5–4.0 μ M Ca^{2+} (+), 500 μ M EGTA (-); pretreatment with 10 μ g/ml cytochalasin B (+), no pretreatment (-). In control cells (no GTP γ S, 500 μ M EGTA, 2.5 mM ATP) the capacitance values were $C_i = 3.45 \pm 0.31$ pF and $C_f = 3.05 \pm 0.30$ pF ($n = 5$) with cytochalasin B and $C_i = 3.41 \pm 0.25$ pF and $C_f = 3.64 \pm 0.27$ pF ($n = 5$) without cytochalasin B. In these control cells C_f was defined as the capacitance value after ~ 15 min. ■, C_i ; □, C_f .

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 $\sim 1,300$ primary granules per cell and $\sim 4,600$ 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 $C_f \sim 9$ pF. This value is marked by the dashed line in Fig. 5. The final capacitance of cells stimulated by GTP γ S 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 GTP γ S. C_f 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 μ M EGTA, 2.5 mM ATP, but no GTP γ S 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 ($n = 5$).

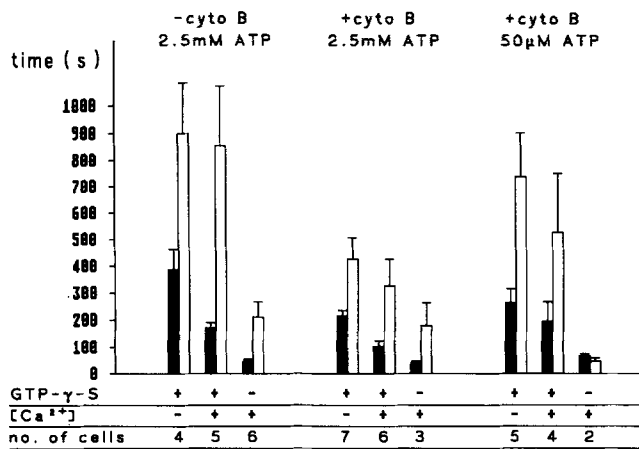


Figure 6. Mean delay (t_{10}) and rise time ($t_{10/90}$) of the capacitance change recorded under various experimental conditions as in Fig. 5. ■, t_{10} ; □, $t_{10/90}$.

When intracellular Mg-ATP was reduced from 2.5 mM to 50 μ M, GTP γ S stimulation resulted in significantly lower amplitudes of exocytosis ($C_f = 6.5$ pF), suggesting a partial ATP dependence. It should be noted that a value of ~ 6.5 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 μ M antimycin several minutes before the experiment. Under these conditions GTP γ S stimulated generally similar capacitance changes ($C_f = 6.0 \pm 0.3$ 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 ~ 1.3 pF. Among the cells in this group those with lower (Ca^{2+})_i displayed smaller capacitance changes than those with higher (Ca^{2+})_i. The comparably low amplitude of the experiment shown in Fig. 4 (although within the range of mean \pm 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 μ M, exocytosis in response to high (Ca^{2+})_i was further reduced ($C_f - C_i \sim 0.4$ 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 GTP γ S at low (Ca^{2+})_i was roughly similar to that shown in Fig. 1. At high (Ca^{2+})_i 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% ($t_{10/90}$). These values are shown in Fig. 6. Both values show a similar dependence on the various conditions used. Exocytosis in response to GTP γ S was much faster in cytochalasin B-treated cells than in untreated cells. Reducing ATP from 2.5 mM to 50 μ M 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 GTP γ S sometimes took up to 30 min. At low (Ca^{2+})_i exocytosis was also somewhat slower than at high (Ca^{2+})_i. The capacitance change observed in response to high (Ca^{2+})_i + high ATP was much faster than that in response to GTP γ S and was not significantly affected by the pretreatment with cytochalasin B. The capacitance change in response to high (Ca^{2+})_i at 50 μ M 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 GTP γ S and ATP into the cytoplasm of single neutrophils stimulates complete degranulation. In contrast to receptor-mediated exocytosis (1) stimulation by intracellular application of GTP γ S 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 μ M) 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 GTP γ S was affected by cytochalasin B, ATP, and calcium. Cells pretreated with cytochalasin B and stimulated with GTP γ S in the presence of 2.5 mM ATP and micromolar (Ca^{2+})_i 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

slowed 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 (t_{10}) is only marginally affected whereas the rise time ($t_{10/90}$) increases by $\sim 60\%$. 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 GTP γ S 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 GTP γ S 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 $(\text{Ca}^{2+})_i$ is < 50 nM and is half maximal at $(\text{Ca}^{2+})_i = 200$ nM (19). Previous experiments using GTP γ S 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 GTP γ S 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 GTP γ S required the presence of ~ 1 μM free Ca^{2+} in HL-60 cells (23) and human neutrophils (O. Nüße, unpublished data). In the experiments described here, we have observed exocytosis of the corresponding granule type in response to intracellular GTP γ S application with a patch pipette at $p\text{Ca} \sim 8$. Degran-

ulation is virtually complete and independent of $(\text{Ca}^{2+})_i$. In contrast to the conventional cell permeabilization techniques, loading the cells with GTP γ S 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 GTP γ S 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 Ca^{2+} dependence and the requirement for pretreatment with cytochalasin B.

A second G protein (G_E) has been proposed to be the target of GTP γ S in neutrophils (3) and mast cells (10) and we have presented evidence that the G protein activated by GTP γ S 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 GTP γ S, 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 GTP γ S 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.

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