Granule-specific ATP requirements for Ca\(^{2+}\)-induced exocytosis in human neutrophils. Evidence for substantial ATP-independent release

Sten Theander\(^1\), Daniel P. Lew\(^2\) and Oliver Nüße\(^3\)

\(^1\)Division of Clinical Biochemistry, University of Geneva Medical School, 1211 Geneva 14, Switzerland
\(^2\)Division of Infectious Diseases, University Hospital Geneva, 1211 Geneva 14, Switzerland
\(^3\)Immunology Laboratory, Faculty of Sciences, University Nancy 1, 54506 Vandoeuvre-les-Nancy, France

*Author for correspondence (e-mail: Sten_Theander@hotmail.com)

Accepted 6 May 2002

Summary

Ca\(^{2+}\)-induced exocytosis in neuronal and neuroendocrine cells involves ATP-dependent steps believed to ‘prime’ vesicles for exocytosis. Primed, docked vesicles are released in response to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Neutrophils, however, do not possess voltage-gated Ca\(^{2+}\) channels and appear to have no docked vesicles. Furthermore, neutrophils have several types of granules with markedly different Ca\(^{2+}\) requirements for exocytosis. These differential Ca\(^{2+}\) dependencies were used as a tool to investigate the ATP dependence of different granule populations. Here we demonstrate distinct ATP requirements for release of neutrophil granule populations, with respect to rate as well as amplitude. Intracellular ATP was depleted to various levels, and exocytosis was stimulated with different Ca\(^{2+}\) concentrations and measured with the patch-clamp capacitance technique or by detecting enzyme release. Primary granule exocytosis displayed a distinct ATP dependence with an apparent K\(_m\) of \(-80\) \(\mu\)M ATP and no ATP-independent exocytosis. Release of secondary and tertiary granules displayed a more shallow ATP dependence (K\(_m\)=330 \(\mu\)M), and more than 50\% of secondary and tertiary granules appeared not to need ATP at all for their release. Individual granules in human neutrophils have distinct ATP requirements for exocytosis, suggesting that the ATP-sensitive elements are localised to the granules. Primary granule exocytosis has a very low affinity for ATP. Furthermore, substantial ATP-independent exocytosis of secondary and tertiary granule occurs despite the absence of docked granules. These characteristics should help neutrophils to fulfil their bactericidal functions at poorly irrigated sites of infection with low glucose supply.

Key words: Exocytosis, ATP affinity, Granule populations, Ca\(^{2+}\), Neutrophils

Introduction

Numerous physiological processes require the release of intracellular granules by exocytosis. The so-called SNARE-proteins (Rothman, 1994) are believed to form a universal fusion complex that is dissociated by the cytosolic ATPase NSF (Söllner et al., 1993). This would confer a high degree of ATP dependence on exocytosis. The ATP concentration that is required would be the same for all fusion processes using this pathway. However, ATP-requiring enzymes other than NSF such as protein and lipid kinases may also be involved in the exocytotic process (Burgoyne and Morgan, 1998; Klenchin and Martin, 2000; Martin, 1997). Furthermore, functional studies revealed both ATP-dependent and ATP-independent exocytosis (Eliasson et al., 1997; Holz et al., 1989; Parsons et al., 1995). In some cells, the small pool of vesicles that are releasable in an ATP-independent manner correlates well with the number of vesicles which, in electron microscopy micrographs, appear docked to the plasma membrane (Parsons et al., 1995). In general, exocytosis requires the attachment of the vesicle to the membrane, often called docking, and ATP-dependent maturation, called priming. ATP-dependent events before and after docking have been described (reviewed in Burgoyne and Morgan, 1998; Robinson and Martin, 1998). Once the vesicle is docked and primed, it can be exocytosed, without further consumption of ATP, in response to a local increase in Ca\(^{2+}\) concentration in the vicinity of voltage-gated Ca\(^{2+}\) channels (Martin, 1997).

The current model of the exocytotic mechanism is mainly based on work in excitable cells. Numerous cells of the immune system, such as mast cells, neutrophils or cytotoxic T-lymphocytes, also use regulated granule exocytosis to fulfil their physiological role. Taking the example of the neutrophil, some proteins belonging to the SNARE family have been identified, whereas others, among them NSF, have not been found in neutrophils (reviewed in Ligeti and Mocsai, 1999). Furthermore, neutrophils lack voltage-gated Ca\(^{2+}\) channels (Krause and Welsh, 1989), and docked vesicles are not found in unstimulated neutrophils (Borregaard et al., 1993).

In view of these differences between excitable cells and neutrophils we wanted to assess the role of ATP in Ca\(^{2+}\)-induced exocytosis of neutrophils and to determine the dose-response curve between intracellular ATP and exocytosis. Previous investigations on permeabilized mast cells and neutrophils revealed both ATP-dependent and -independent
pathways of exocytosis (Churcher and Gomperts, 1990; Cockcroft, 1991; Rosales and Ernst, 1997).

Here, we used chemical depletion of intracellular ATP and determined the resulting time-course of ATP depletion by using the luciferase assay (Stanley and Williams, 1969). We studied Ca\(^{2+}\)-induced exocytosis, using both patch-clamp capacitance measurements (Lollike and Lindau, 1999) and the β-glucuronidase release assay (Nüße et al., 1997), over a wide range of intracellular ATP concentrations. Since different granule populations in neutrophils have different Ca\(^{2+}\) sensitivities for exocytosis (Borregaard et al., 1993; Lew et al., 1986; Nüße et al., 1998), it was, furthermore, possible to address the ATP dependence of these granule populations. This approach gave for the first time an estimated K_m for ATP dependence of exocytosis of different granules in the same cell. Our results show different ATP requirements for the release of two main granule population and substantial release of apparently undocked granules in the absence of ATP.

Materials and Methods

Material and solutions

Medium 199 was obtained from Gibco, and capillaries were from Clark Instruments (Reading, UK). All other chemicals were purchased from Sigma or Fluka. The extracellular solution (ES) contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 2 mM CaCl_2, 10 mM HEPES, pH 7.4 and 8.9 mM glucose. The ATP-depleting medium contained ES without glucose + 6 mM 2-deoxyglucose + 5 μM antimycin. The standard pipette solution, internal solution (IS), contained 125 mM K-glutamate, 10 mM NaCl, 2 mM MgCl_2, 1 mM MgATP and 10 mM HEPES, pH 7.2. MgATP was omitted in ATP-depleted cells except for Fig. 3C. The standard IS was used as a base to which Ca\(^{2+}\)-buffered pipette solutions were constructed using the program WINMAX version 1.8 (Chris Patton, Stanford, CA). Pipette solutions buffered to 300 μM Ca\(^{2+}\) contained 10 mM NaCl, 10 mM HEPES, 120 mM K-glutamate, CaCl_2 and 10 mM K-citrate serving as a Ca\(^{2+}\) buffer. The 10 μM Ca\(^{2+}\) buffers contained 10 mM NaCl, 10 mM HEPES, 125 mM K-glutamate, CaCl_2 and 5 mM HEDTA serving as a Ca\(^{2+}\) buffer. Both buffers also contained either 2 mM MgCl_2 and 1 mM MgATP or 3 mM MgCl_2 for the ATP-free buffers. All pipette solutions had a pH at 7.2.

Neutrophil preparation

Citrated blood was drawn from healthy volunteers. Most of the red blood cells were removed by dextran sedimentation. The leukocytes were then separated by centrifugation on a Ficoll layer, and the remaining red blood cells were lysed by hypoosmotic shock. Cells were kept in Medium 199 on ice and used within 8 hours.

Measurements of intracellular ATP

Cell extract preparation

A neutrophil suspension of 10^6 cells per ml of ES was kept on ice. Just before further preparations, the cell suspension was warmed to room temperature. For incubations, 0.5 ml of cell suspension was centrifuged for 3 minutes at 224 g. The supernatant was then removed, replaced with ATP depletion medium and left to incubate for various times. Aliquots were then centrifuged again for 3 minutes at 224 g (for incubation times less than 2 minutes the time of centrifugation had to be shortened appropriately), and the supernatant was quickly removed and replaced with ice cold ES + 0.01% Triton X-100 for lysis of cells. After lysis for 5 minutes the aliquots were centrifuged for 3 minutes at 2012 g. The supernatant was taken and quickly stored at −20°C for later analysis. For control samples only the lysis step of the procedure was applied.

ATP measurements

For measurements of intracellular ATP, the luciferase bioluminoncence assay (Stanley and Williams, 1969) was used. Firefly luciferase extract (FLE50 Sigma) was dissolved in water (10 mg/ml) and 1 ml of the dissolved extract was added to 200 ml arsenate buffer (100 mM Na_2HAsO_4, 20 mM MgSO_4, pH 7.4 with H_2SO_4) and mixed in the dark at 4°C for 90 minutes. An ATP standard was constructed by adding various amounts of MgATP salt to a glycine buffer (glycine 50 mM, pH 11 with NaOH). For the bioluminoncence assay of ATP, 10 μl cell extract or ATP standard was added to 5 ml enzymatic mixture, and light emission was measured with a LS 8000 beta counter (Beckman). After calibration against the ATP standard, the ATP content of the cell extract was determined. For the calculation of intracellular ATP concentrations, the cell count (10^6 per ml) and an estimate of the neutrophil volume (Nüße and Lindau, 1988) to 250 μm^3 (=250 fl) was used to calculate the dilution factor for the cell extract.

Capacitance measurement of secretion

For patch-clamp recordings, the neutrophils were placed on glass coverslips. After 3-5 minutes, non-adherent cells were washed away with ES or ES lacking glucose for ATP depletion experiments. The adherent cells were incubated with ES containing 5 μg/ml cytochalasin B or, for ATP depletion experiments, with ES without glucose supplemented with 6 mM 2-deoxyglucose, 5 μM antimycin and 5 μg/ml cytochalasin B. Treatment with cytochalasin B, an inhibitor of actin polymerization, enhances Ca\(^{2+}\)-induced exocytosis of primary, secondary and tertiary granules (Lew et al., 1986) and is required for FMLP-stimulation of primary granule exocytosis from human neutrophils. All experiments were done at room temperature. For ATP depletion experiments, the time between incubation and patch rupture was carefully noted and registered as the incubation time. Experiments were performed with an inverted microscope (Nikon, Diaphot) with a 40x oil immersion objective. Patch pipettes were pulled from borosilicate glass capillaries (GC150F-10, Clark Instruments, Reading, UK) on a Model P-97 puller from Sutter Instruments (Novato, CA). Pipette resistance was between 4 and 10 MOhm. Patch-clamp recordings were performed with a List EPC 9 amplifier (HEKA, Darmstadt, Germany) in voltage-clamp mode. Capacitance measurements were performed after applying a 1-kHz, 50-mV peak to peak sinusoid stimulus from a dc holding potential of −10 mV. The ‘sine+dc’ mode of the software lock-in extension of the PULSE software was used to calculate the equivalent circuit parameters C_m (membrane capacitance), membrane conductance (G_m) and access resistance (R_A) from the current recordings.

Measurement of exocytosis in suspension

1.25×10^6 cells were suspended in 500 μl of ice-cold medium. Aliquots were then warmed to 22°C and either glucose (controls) or 6 mM deoxyglucose plus 5 μM antimycin were added. The aliquots were then left to incubate for various times (controls for 30 minutes). 5 minutes prior to stimulation, 5 μg/ml cytochalasin B was added to the aliquots to enhance exocytosis (see above). Stimulation of exocytosis was elicited by adding 1 μM ionomycin, 1 μM fMLP or the DMSO vehicle only. Incubation was terminated by rapid cooling with an equal volume of ice-cold buffer and further cooled on ice before centrifugation (800 g for 10 minutes). β-glucuronidase in the supernatant was measured fluorimetrically with 4-methylumbelliferyl-glucuronide as described previously for β-glucosaminidase (Nüße et al., 1997). The results were calculated as a percentage of initial total cellular content. Background exocytosis (5
Measurement of intracellular Ca2+ after ATP depletion

The intracellular free Ca2+ concentration ([Ca2+]i) was measured using the fluorescent Ca2+ indicator fura-2/AM. Cells (2×10^7 per ml) suspended in ES containing 0.1% BSA were loaded for 45 minutes at room temperature with 2 μM fura-2/AM. Just before measurement, 0.5 ml of the cell suspension was taken and centrifuged for 3 minutes at 224 g. The supernatant was taken off, and the cells were resuspended in 2.5 ml glucose free ES and transferred to a cuvette for measurements. The excitation wavelength was alternating between 340 and 380 nm, and emission was recorded at 509 nm at 0.26 Hz in a Perkin Elmer LS-50B spectrofluorimeter. 5 μg/ml cytochalasin B was added at time zero when the recordings started. For the ATP depletion protocol it was found that antimycin displayed too much autofluorescence to be used with fura-2. Hence, it was replaced by 2 μM FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), another blocker of the mitochondrial ATP production. 6 mM of deoxyglucose and 2 μM FCCP were added to the cuvette at 10 and 20 minutes, respectively, after which measurements proceeded for another hour. Calibration was performed for each cuvette by sequential addition of 1 μM ionomycin to measure Ca2+-saturated fura-2 signal (Rmax), followed by the simultaneous addition of 8 mM EGTA and 3 mM Tris (pH 9.3). Finally, 0.1% Triton X-100 was added to measure the Ca2+-free fura-2 signal (Rmin). [Ca2+]i was calculated from the 340/380 nm ratio (R) according to the formula (Grynkiewicz et al., 1985): [Ca2+]i=[Kr](F2min/F2max)((R-Rmin)/(Rmax-R)) where F2min and F2max are the fluorescence at 380 nm at times corresponding to Rmin and Rmax, respectively, and Kr=224 nM.

Analysis

The plot of intracellular ATP concentration, [ATP], over time of ATP depletion (t) was fitted to a sum of two exponential functions: [ATP]=2111 μM×e^(-t/3.5)+250 μM×e^(-t/27). The value of [ATP] has three potential sources of error, the measurement of ATP, the fitting and the estimation of the cytoplasmic volume, which was based on electron microscopy micrographs. The absolute value of [ATP] may have a significant error margin; however, the relative decline of [ATP] over time is well represented by the fit function. From capacitance recordings, the amplitude of exocytosis, ΔCm, was calculated as final capacitance minus initial capacitance immediately after patch rupture. Cells with the same intracellular solution and similar times of ATP depletion prior to patching were grouped in 20 minutes intervals. All cells with more than 60 minutes of ATP depletion were pooled in one group. Choosing smaller time intervals for the groups of cells did not change the overall shape of the dose response curves (data not shown).

The mean ATP depletion time and the mean ΔCm were calculated. For each group, the mean [ATP] was calculated from the mean ATP depletion time. The plots of ΔCm vs [ATP] were fitted with a logistic equation: ΔCm=ΔC0/[1+(Km-1/[ATP])^n] where ΔC0 was the capacitance change at [ATP]=0, Km was the apparent affinity, and n was the cooperativity factor. Data are presented as mean±s.e.m.

Results

ATP depletion during metabolic inhibition

Most studies on the role of ATP in exocytosis compare ATP-depleted cells with controls. We describe a new approach depleting cells to various levels of intracellular ATP to establish a dose response curve for ATP. To this end, the cellular contents of ATP were measured after different times of ATP depletion by incubation with 6 mM deoxyglucose and 5 μM antimycin. The luciferase assay for ATP was linear over at least two orders of magnitude in the relevant range for our experiments (Fig. 1A). Using the cell count of the samples together with an estimate of the cell volume, the intracellular concentration of ATP was calculated for various times of incubation as shown in Fig. 1B. It is evident that the time-course of ATP depletion contained both fast and slow components. Starting from a value of ~2 mM, the ATP concentration fell to about 7% of its initial value in 15 minutes. This fast phase was followed by a phase with much slower kinetics. The time-course of ATP depletion could be fitted using a sum of two exponentials with time constants of 3.5 and 27 minutes, respectively. The fast phase of decline in ATP is an expected consequence of inhibition of ATP production and consumption of free ATP. The slow phase may partially represent slow dissociation of bound ATP from cytosolic proteins (Parsons et al., 1995). Below, the double exponential function will be used to calculate the intracellular ATP concentration at any given time of ATP depletion.

ATP requirements of different granule populations

We used two complementary techniques to stimulate neutrophil exocytosis by Ca2+, the Ca2+ ionophore ionomycin and the perfusion of Ca2+ buffers through the patch pipette.
Patch-clamp capacitance measurements monitor the increase in plasma membrane surface during exocytosis. The patch pipette provides a direct control of the cytosolic Ca\(^{2+}\) concentration independently of cellular Ca\(^{2+}\) regulation. Furthermore, the amplitude and the kinetics of exocytosis are recorded, and two granule populations are released by quite different Ca\(^{2+}\) concentrations (Nüße et al., 1998). Infusion of a 10 \(\mu\)M Ca\(^{2+}\) pipette solution elicited the release of peroxidase-negative granules (secondary and tertiary granules), whereas 300 \(\mu\)M elicited exocytosis also of primary granules (Nüße et al., 1998). Under these experimental conditions, almost all neutrophil granules are released. The differences in Ca\(^{2+}\) sensitivities make it possible to investigate the ATP dependence of different granule populations by infusion of the appropriate Ca\(^{2+}\) buffers.

In Fig. 2, representative examples of capacitance traces from cells in control conditions (0 minutes ATP-depletion) or after various times of incubation in an ATP-depleting medium and stimulated with either 10 \(\mu\)M (A) or 300 \(\mu\)M (B) Ca\(^{2+}\) in the pipette are displayed. Under control conditions, cells were in glucose-containing medium without metabolic inhibitors, and the patch-pipette contained 1 mM ATP. For cells in ATP-depleting medium, the pipette solution did not contain ATP. 10 \(\mu\)M Ca\(^{2+}\) in the pipette stimulated an essentially monophasic increase in capacitance. The traces (control and 10 minutes ATP depletion) obtained using 300 \(\mu\)M Ca\(^{2+}\) in the pipette displayed typically biphasic secretion. ATP depletion for more than \(~\)15 minutes resulted in a progressive loss of secretion. For both Ca\(^{2+}\) concentrations, however, the cells display robust exocytosis also after very long (>60 minutes) incubations in ATP-depleting media. The initial capacitance of the neutrophils was unaffected by incubation in the ATP-depleting medium (Fig. 2C), demonstrating that the ATP depletion itself did not induce exocytosis.

Unlike the situation in many other cell types (Henkel and Almers, 1996), exocytosis in patch-clamped neutrophils does not appear to be accompanied by concomitant endocytosis, and the capacitance traces usually reach a stable plateau value. The difference between the capacitance value at the plateau and the initial capacitance, \(\Delta C_m\), therefore, directly reflects the amplitude of exocytosis (Nüße et al., 1998; Nüße and Lindau, 1988). Fig. 3 shows \(\Delta C_m\) as a function of time of incubation in ATP-depleting medium for cells stimulated with 10 \(\mu\)M Ca\(^{2+}\) or with 300 \(\mu\)M Ca\(^{2+}\). In both cases, exocytosis was reduced by ATP depletion but reached similar stable amplitudes after more than 40 minutes. The decline of exocytosis was delayed with higher Ca\(^{2+}\) stimulation, suggesting different ATP requirements for different granule populations. By calculating the intracellular ATP concentration at the time of patch rupture for each experiment (see Materials and Methods), a dose-response curve for exocytosis versus intracellular ATP could be constructed (Fig. 3B). The \(\Delta C_m\) obtained with 10 \(\mu\)M Ca\(^{2+}\) could be well fitted by a logistic function with an apparent \(K_m\) of 328 \(\mu\)M ATP and a cooperativity factor of 2.0. Furthermore \(~\)56\% of the granules were released in an ATP-independent manner. Secondary and tertiary granules have an average capacitance of 1.1 fF (Lolli et al., 1995), thus some 1250 granules were released at very low intracelluar ATP concentrations. The dose-response curve for stimulation with 300 \(\mu\)M Ca\(^{2+}\) shows that exocytosis remained unaltered down to concentrations of \(~\)200 \(\mu\)M ATP. At lower values, however, exocytosis dropped steeply down (\(K_m\) of 95 \(\mu\)M, cooperativity 3.7) to a level that was, interestingly, similar to that obtained with 10 \(\mu\)M Ca\(^{2+}\). This suggests that the secretion of granules that require more than 10 \(\mu\)M Ca\(^{2+}\) was completely abolished at low ATP concentrations.

To exclude the possibility that ATP depletion caused irreversible damage to the exocytotic machinery of the cells, we re-introduced ATP, using the same pipette solutions as for the controls (1 mM ATP), after prolonged depletion. On the basis of Fig. 3A, cells were treated with metabolic inhibitors for at least 25 and 40 minutes before stimulation with 10 \(\mu\)M Ca\(^{2+}\) and 300 \(\mu\)M Ca\(^{2+}\), respectively, to lower the intracellular ATP concentration to levels that affect granule exocytosis. Reperfusion of these cells with 1 mM ATP in the pipette solution completely reversed the effect of ATP depletion with both Ca\(^{2+}\) concentrations (Fig. 3C).

ATP may not only influence the amplitude of exocytosis but also its speed. We measured the maximal rate of secretion by

![Fig. 2. Ca\(^{2+}\)-induced exocytosis after ATP depletion. Representative patch-clamp capacitance traces in control conditions, using pipette solutions containing 1 mM ATP, and after various times of incubation in an ATP-depleting medium using ATP-free pipette solutions. The incubation times until patch rupture in minutes are indicated next to the traces. In A pipette solutions contained 10 \(\mu\)M Ca\(^{2+}\) and in B 300 \(\mu\)M Ca\(^{2+}\). Time is given from the time of patch rupture. Initial capacitances have been subtracted for clarity. (C) Initial capacitance (\(C_i\)) versus time of ATP depletion with linear regression \(C_i(t)=0.0008\) pF/minute\(\times t+2.692\) pF.](image-url)
taking the maximum derivative of the capacitance traces. The results, plotted as a function of intracellular ATP at the time of patch rupture, are given in Fig. 4. The maximum rates of capacitance increase displayed a strikingly similar ATP dependence, as did the measurements of total increase in capacitance. The kinetic analysis confirmed the different Km values and the cooperativity for stimulation with 10 μM and 300 μM Ca²⁺. In an ATP-consuming enzymatic process, the concentration of ATP should affect the rate of secretion. It is unlikely that ATP was completely used up during secretion. Therefore, the amplitude of secretion should only be affected if the ATP concentration had an influence on the number of granules that are capable of fusion. This phenomenon, called submaximal secretion in response to submaximal stimulation (Nüße et al., 1998), resembles the effect of different Ca²⁺ concentrations on the kinetics and amplitude of exocytosis in neutrophils. Like Ca²⁺, the ATP concentration appears to influence the size of the granule pool that is available for exocytosis. For each granule in this pool of available granules, the probability of fusion may be the same. Therefore, the rate of exocytosis would be directly linked to the number of fusing granules.

Primary granule exocytosis following ATP depletion
In order to have an independent assessment of the ATP dependence of primary granule exocytosis we used the β-glucuronidase release assay for the detection of exocytosis in

![Fig. 3. ATP dose-response curve. (A) Relationship between total increase in capacitance ΔCₘ (measured as the difference between the final plateau value and the initial capacitance) and time in ATP-depleting medium. The time was counted until the time of patch rupture. Open squares and filled circles represent mean values using 300 μM Ca²⁺ (n=3-13) and 10 μM Ca²⁺ (n=5-10) in the pipette, respectively. (B) The same data sets as in A are shown but plotted as a function of intracellular ATP, as calculated using the fitting function from Fig. 1B, at the time of patch rupture. Solid lines in B are logistic functions fitted to the data. For 10 μM Ca²⁺ Kₘ=328 μM and the cooperativity factor P=2.0, for 300 μM Ca²⁺ Kₘ=95 μM and P=3.7. (C) Reversibility of ATP depletion. Cells were ATP-depleted for 39±6.5 minutes or 65±8 minutes to lower intracellular ATP to the critical level for secondary/tertiary granules and primary granules, respectively. These cells were then stimulated with 10 μM Ca²⁺ or 300 μM Ca²⁺, respectively, including 1 mM ATP in the patch pipette (reperfusion). Mean capacitance increase from control cells (no ATP depletion) or cells reperfused with ATP after depletion, n=4-8.

![Fig. 4. ATP dependence of the rate of exocytosis. Relationship between the maximum rate of exocytosis, measured as the maximum value of the derivative of capacitance traces, and intracellular ATP at the time of patch rupture. A represents mean values obtained using 10 μM Ca²⁺ (n=5-10) and (B) using 300 μM Ca²⁺ (n=3-13) in the pipette. Both sets of values are normalised to their respective value in the control situation. The solid line represents logistic functions fitted to the data. In A Kₘ=476 μM and a cooperativity factor P=2.6 was obtained. Corresponding values in B were Kₘ=99 μM and P=7.9.]}
Fig. 5. ATP dependence of primary granule release. (A) ATP dependence of primary granule exocytosis elicited by either 1 μM ionomycin (squares, mean of two to six experiments) or 1 μM fMLP (triangles, mean of two to four experiments). Exocytosis was measured using the β-glucuronidase release assay. Both sets of data were normalised to their value in the control situation. The ionomycin data were fitted to a logistic function having a $K_m =$55 μM and a cooperativity factor $P=4$. (B) Comparison between the ATP dependence of primary granule exocytosis as measured by the β-glucuronidase release assay (squares, same data as in A) and as calculated from the patch-clamp measurements (solid line). The solid line was obtained as the difference between the logistic functions (in Fig. 3B) fitted to the data points obtained using 300 μM Ca$^{2+}$ and 10 μM Ca$^{2+}$ in the pipette. (C) Ratiometric measurements of intracellular Ca$^{2+}$ concentrations after ATP depletion using fura-2/AM. The trace, an average of four experiments, is the ratio of fura-2 fluorescence at 340/380 nm excitation. Standard errors of the mean are represented by grey shading. The thick bars represent the sequential addition of 6 mM deoxyglucose, 2 μM FCCP and 1 μM ionomycin. In the calibration scaling to the right, each scale step represents an increase of 200 nM Ca$^{2+}$.

Effects of ATP depletion on intracellular Ca$^{2+}$ concentrations

In the patch-clamp situation, the intracellular solute composition is clamped by the pipette solution, whereas in the conditions of ATP depletion. Fig. 5A shows the results of ionomycin-induced secretion as a function of intracellular ATP at the beginning of stimulation, as calculated from the time-course of ATP depletion. Also shown is the response when exocytosis was elicited by receptor stimulation with 1 μM fMLP. All values were normalised to their respective value in the control situation. Ionomycin-induced primary granule release had an ATP affinity of 55 μM and an apparent cooperativity of four. The result obtained by receptor stimulation, however, displayed a very high sensitivity to decreases in intracellular ATP. The high ATP dependence of receptor stimulation may reflect the need for phosphorylated compounds, which are essential in the stimulus response coupling. The direct stimulation with Ca$^{2+}$ ionophores or Ca$^{2+}$ buffers in the patch pipette probably bypassed these requirements.

As shown previously (Nüße et al., 1998), 10 μM Ca$^{2+}$ in the patch pipette stimulates mainly the complete release of secondary and tertiary granules, and 300 μM Ca$^{2+}$ stimulates the release of primary as well as secondary and tertiary granules. The difference between the amplitude of secretion with 300 μM Ca$^{2+}$ and 10 μM Ca$^{2+}$ should, therefore, reflect primary granule release alone. Thus we could obtain the ATP dependence of primary granule release under patch-clamp conditions by subtracting the curve fitted to the ATP dose-response relationship (from Fig. 3B) obtained for 10 μM Ca$^{2+}$ from the one obtained for 300 μM Ca$^{2+}$.

The direct comparison of the ATP dependence of primary granule exocytosis induced by ionomycin or by Ca$^{2+}$ buffers in the patch pipette is shown in Fig. 5B. Both methods revealed a sharp fall in exocytosis with a midpoint around 60-80 μM ATP. It is interesting that two rather different approaches to measuring primary granule release give, essentially, the same result.

The capacitance measurements suggested that essentially no primary granule exocytosis is possible in the absence of ATP, whereas the β-glucuronidase release assay indicated some ATP-independent secretion. Possibly, this discrepancy arose as a consequence of some overlap in vesicle contents such that a small amount of β-glucuronidase may have been derived from vesicles that are less sensitive to ATP depletion than the primary granules. In fact, secondary or tertiary granules may contain low amounts of β-glucuronidase (Edwards, 1994). In control cells, ionomycin released about 45% of the total cellular β-glucuronidase content. The fraction of ATP-independent granules (0.2) therefore represented less than 10% of the total. We conclude that no or few primary granules were released in the absence of ATP.

The β-glucuronidase release assay gave values that are somewhat left-shifted with respect to those obtained using capacitance measurements. This may reflect ATP washout into the patch pipette that we have neglected in the calculation of the intracellular ATP concentration. Consequently we slightly overestimated the true ATP concentration sensed by the exocytotic machinery. Nevertheless, the two rather different techniques provided very similar results.
β-glucuronidase release assay, changes secondary to ATP depletion such as alterations of ionic composition and, in particular, intracellular Ca²⁺, may well occur. In order to investigate this we measured [Ca²⁺]ᵢ after depletion of intracellular ATP. Fig. 5C shows that [Ca²⁺]ᵢ increased slowly after ATP depletion and that most of the increase occurs after 40 minutes of ATP depletion. After 1 hour of ATP depletion, [Ca²⁺]ᵢ rose to ~800 nM, a level that stimulates very little exocytosis of secondary and tertiary granules in patch-clamp experiments (Nüße et al., 1998) and is well below the EC₅₀ for release of primary granules in non-permeabilised cells (Lew et al., 1986). These results are therefore in good agreement with the absence of any increase in initial capacitances (Fig. 2C) and with the stable baseline during ATP depletion in the β-glucuronidase release assay. Addition of ionomycin even after 1 hour of ATP depletion caused a rapid and sharp rise in [Ca²⁺]ᵢ, which is known to induce exocytosis in human neutrophils (Lew et al., 1986). We conclude that the slow rise in [Ca²⁺]ᵢ during ATP depletion did not cause significant exocytosis and did not interfere with the stimulation with ionomycin.

**Discussion**

To obtain an ATP dose-response curve for exocytosis of several granules populations, we measured secretion at different degrees of ATP depletion. The approach gives for the first time an estimated Kₘ for ATP dependence of exocytosis of different granules in the same cell.

**ATP-independent exocytosis without docking?**

It has been claimed that ‘it is dogma that neutrophil granules are not docked’ (Lollike et al., 1999), and it is indeed a puzzling circumstance that granules of resting neutrophils do not appear to be docked to the plasma membrane when seen in electron microscopy micrographs (e.g. Borregaard et al., 1993). Docking could be very brief and immediately precede fusion. However, our measurements detected over 1000 granules that were released in the absence of ATP. A pool of this size should not go unnoticed if they were accumulated just beneath the plasma membrane. We have to conclude that these granules can dock and fuse with the plasma membrane without using ATP. A similar phenomenon has indeed been described for the ‘reserve’ granules in sea urchin eggs (Chestkov et al., 1998). The granules of unstimulated neutrophils may be already primed. However, priming is reversible (Klenchin and Martin, 2000) with a half-life of ~4 minutes in permeabilized chromaffin cells (Holz et al., 1989). Most probably, primed granules would be unprimed during the prolonged ATP depletion in our experiments. Alternatively, only a few granules may fuse directly with the plasma membrane. Then, more granules fuse with the membrane of granules that are already inserted into the plasma membrane. Such cumulative fusion occurs in other cells such as eosinophils (Scepek and Lindau, 1993) and pancreatic β-cells (Bokvist et al., 2000). However, there is no evidence, that neutrophil granules are chained up or otherwise associated prior to exocytosis. Thus the problem of how the granules attach (dock) to and fuse with the target membrane without ATP remains to be solved.

**The nature of the ATP-requiring steps**

Which ATP-binding proteins could explain the apparent Kₘ for ATP of neutrophil exocytosis? Multiple ATP-dependent steps may be involved. The Kₘ of 80 µM ATP for the primary granule release is much lower than has been reported for NSF and for other cells. Holz et al. found a half-maximal activation of exocytosis in chromaffin cells at 400 to 600 µM ATP (Holz et al., 1989), compatible with the Kₘ of 650 µM that was determined for NSF in vitro (Tagaya et al., 1993). It therefore appears unlikely that NSF is causing the ATP dependence of primary granule exocytosis. The apparent Kₘ for release of secondary and tertiary granules (330 µM) is closer to the reported Kₘ of NSF and might be compatible with NSF participating in the release machinery for one or both granule types.

NSF was not found when screening a cDNA library from HL-60 cells, a cell line derived from neutrophil precursors (Ligeti and Mocsai, 1999). A possible explanation for the absence of NSF in neutrophils is that the hydrolysis of ATP by the NSF serves mainly to dissolve the fusion complex and thereby prepare for a second round of fusion events (Burgoine and Morgan, 1998; Robinson and Martin, 1998). Neutrophils are terminally differentiated cells with a very short life span and may not be capable of repeated rounds of fusion events (Ligeti and Mocsai, 1999), possibly making the NSF unnecessary.

**Localisation of the ATP-sensitive element to the granules?**

Our results show that primary granule release has a strong dependence on ATP, with a apparent Kₘ in the range of 80 µM and a high degree of cooperativity (n=4). A similarly high degree of cooperativity was also found for the Ca²⁺ dependence of primary granules (Nüße et al., 1998). The high cooperativity for ATP dependence as well as Ca²⁺ dependence may indicate that complexes of several primary granules have to be formed prior to or in the process of exocytosis.

The granules released at lower Ca²⁺ concentration probably represent a mixture of secondary, tertiary granules and a small contribution from secretory vesicles (Nüße et al., 1998). This heterogeneous granule population showed a more shallow ATP dependence than the primary granules, perhaps reflecting several underlying processes. The apparent Kₘ for release of this population was higher than for primary granules. In contrast to the primary granules, the release process is not absolutely dependent on ATP as more than half of this population can be released also in the absence of ATP. Our experimental approach does not permit us to further distinguish the ATP dependence of the different subpopulations. It is possible that those vesicles, which are released in an ATP-independent manner, represent a single subpopulation. The observation that different granule populations display different ATP affinities suggests that the ATP-dependent mechanism is granule specific, at least in part. This is obviously in contradiction to the idea of a universal ATP-dependent step in granule exocytosis. Our data do not exclude a common ATP-dependent mechanism, but this mechanism is not rate limiting in neutrophils. They do, however, indicate that the ATP-sensitive elements are granule specific and may, therefore, be localised to the granules.
Numerous other ATP-binding proteins may be involved in exocytosis. The ATPase p97 is a homolog of NSF and could take up some of its function (Robinson and Martin, 1998). Many protein serine/threonine kinases have a $K_m$ below 100 $\mu$M ATP (Edelman et al., 1987). Protein kinase $C$ requires only 6 $\mu$M ATP for half-maximal activity. Protein kinase $A$ ($K_m$ of 40 $\mu$M) has recently been implicated in insulin secretion from $\beta$-cells (Takahashi et al., 1999). Phosphoinositides play an important role in exocytosis (Klenchin and Martin, 2000; Pinxteren et al., 2001). The phosphoinositide kinases, phosphoinositide 3-kinase ($K_m$<10 $\mu$M), phosphoinositide 4-kinase [$K_m$ 18 $\mu$M (Porter et al., 1988)] and phosphoinositide 5-kinase [0.5 $\mu$M (Singhal et al., 1994)] generate important signalling molecules.

Proteins with a $K_m$ for ATP significantly higher than 80 $\mu$M and 330 $\mu$M are unlikely to represent the critical steps in primary and secondary/tertiary granule exocytosis, respectively. Furthermore, the reversibility by reperfusion with ATP (Fig. 3C) suggests that the ATP-sensitive element acts rapidly and is either constitutively active or is activated by Ca$^{2+}$ infusion through the patch pipette.

**Why neutrophils may be different**

Immune cells appear to have their own way of regulating exocytosis, namely stimulation by either Ca$^{2+}$ or guanine nucleotides alone (Pinxteren et al., 2000), absence of voltage-gated Ca$^{2+}$ channels, lysosome-like granules that undergo regulated secretion (Griffiths, 1996), little evidence for docked vesicles and low numbers or absence of SNARE proteins (Ligeti and Mocsai, 1999). The neutrophil has the additional feature of several populations of releasable granules with distinct requirements for exocytosis.

Infection often occurs in poorly irrigated regions of the body. Neutrophils are designed to combat such infections. They have few mitochondria and therefore use mainly oxygen-independent glycolysis to derive energy (Edwards, 1994). However, glucose supply may be low at sites of infection (Chavalittamrong et al., 1997). A low $K_m$ for ATP dependence of the secretory machinery may be needed to keep the cells functional in such situations. Docking and priming of granules before reaching the site of infection could solve the energy requirement for exocytosis. However, it must be remembered that those cells in which docking has been observed are part of a fixed geometric arrangement within the tissue, whereas the neutrophil is a highly mobile cell for which the release target(s) are not defined by any tissue geometry. Apparently, stimulation of neutrophils with chemoattractants does not cause docking (Lollike et al., 1999). Furthermore, it would be rather hazardous to have primary granules docked and ready to fuse in large numbers given their toxic contents.

In conclusion, the ATP requirement for neutrophil exocytosis appears to be a feature of individual granules instead of being a general mechanism for all granules within the cell. This suggests that the rate limiting ATP-dependent step in exocytosis involves granule-specific elements, such as protein or lipid kinases or their substrates, located on the granules. In addition, about 50% of all secondary and tertiary granules are secreted at very low ATP concentrations despite the fact that they do not appear to be docked to the plasma membrane. For comparison, only 4% of the vesicles in chromaffin cells undergo exocytosis in the absence of ATP (Holz et al., 1989). Thus, a reversible priming step either before or after docking is not obligatory for a substantial number of neutrophil granules. Our results strengthen the idea that immune cells have adapted the mechanism of exocytosis to their particular physiological needs.

We thank Elzbieta Hugger and Antoinette Monod for skilled technical assistance, Pierre Maecheler for help with the calcium measurements and Jochen Lang and Claes Wollheim for comments on the manuscript. This work was supported by grants from the Swiss National Fund No 31-50786.97 and 31-55344.98, the Foundation Caritis and Elsie de Reuter and the Société Académique de Genève. S.T. was supported by the Swedish Medical Research Council, The Swedish Institute and The Novartis Foundation.

**References**


A TP dependence of neutrophil exocytosis


