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Research Article

Neutrophil Secretion Induced by an Intracellular Ca^{2+} Rise and Followed by Whole-Cell Patch-Clamp Recordings Occurs Without any Selective Mobilization of Different Granule Populations

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We have investigated calcium-induced secretion in human neutrophils, using a whole-cell patch-clamp technique. Mobilization of subcellular granules to the cell membrane was followed as the change in membrane capacitance (ΔC_m). Both the magnitude and the kinetics of the response differed between low and high concentrations of Ca^{2+}. A sustained secretion following a short lag phase was induced by high concentrations of Ca^{2+} (100 μM and higher). A stable plateau was reached after 5–7 minutes at ΔC_m values corresponding to values expected after all specific as well as azurophil granules have been mobilized. Capacitance values of the same magnitude could be obtained also at lower Ca^{2+} concentrations, but typically no stable plateau was reached within the measuring time. In contrast to previous studies, we were unable to detect any pattern of secretion corresponding to a distinct submaximal response or selective mobilization of granule subsets specified by their Ca^{2+}-sensitivity.

INTRODUCTION

Neutrophils constitute one of the major cell types of our innate immune system, with a main task to phagocytose and kill invading microbial pathogens [1]. To perform their duties, the neutrophils are equipped with subcellular granules, intracellular storage vesicles that contain their destructive weaponry (ie, microbicidal and proinflammatory agents), and a reserve pool of a variety of cell-surface receptors [2]. Neutrophils contain four granule/vesicle types, the azurophil granules, the specific granules, the gelatinase granules, and the secretory vesicles. These are formed (in the order listed) during neutrophil maturation in the bone marrow [3]. During neutrophil activation and diapedesis, the content of these granule/vesicle populations is secreted in a defined, hierarchic manner, in the reverse order of their formation [4].

A common model for membrane fusion events has been suggested [5, 6], and homologues to several (but not all) proteins of the synaptic secretory machinery have been found also in neutrophils [7–9]. Even though the precise molecular regulatory mechanism of exocytosis might differ between cell types, a common feature is the activation of the secretory system by an elevation of the cytosolic concentration of free calcium ions $[Ca^{2+}]_i$ [10], and the role of Ca^{2+} as a regulator of granule mobilization in neutrophils has been recognized for several years [11]. The regulatory system in neutrophils is particularly interesting, as these cells contain several different granule/vesicle populations that should be released sequentially [3]. There are several reports suggesting that the distinct neutrophil granule populations have different Ca^{2+} requirements for fusion with the plasma membrane [12–14]. Mobilization of the azurophil granule population bears resemblance to neuronal exocytosis in that it is characterized by low affinity for Ca^{2+} and a high degree of cooperativity. In contrast, fusion with the plasma membrane of the more easily mobilized granules was suggested to resemble that of endocrine exocytosis in that it is characterized by high affinity for Ca^{2+} and low cooperativity [15]. Based on results obtained from patch-clamp-capacitance studies a difference in calcium affinity among neutrophil granule population has

also been described [16]. This is a unique feature for the neutrophil granules and suggested to be the basis for Ca^{2+} induced selective granule secretion in neutrophils [15].

Neutrophil secretion is tightly regulated by the cytoskeleton [17] and it should be noticed that the conclusion that neutrophils are unique in the sense that they contain granule populations displaying distinct differences with respect to their Ca^{2+}-affinities was drawn from experiments in which cytoskeleton disrupting agents (cytochalasin B) were included. An intact cytoskeleton will most likely have profound impact on the process of granule release and these conclusions therefore need to be reevaluated. In this study, using whole-cell capacitance measurements of secretion in neutrophils with intact cytoskeletons, we found no evidence for the coexistence of two separate control mechanisms for granule mobilization.

METHODS

Isolation of phagocytic cells

Neutrophils were isolated [18] from heparinized whole blood obtained from apparently healthy adults. After dextran sedimentation, hypotonic lysis of the remaining erythrocytes, and centrifugation in a Ficoll-Paque gradient, the neutrophils were washed and resuspended in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG, pH 7.3). The cells were kept on melting ice and used within six hours.

Whole-cell patch-clamp capacitance measurement of secretion

Patch-clamp recordings were performed according to standard procedures [19]. Coverslips were incubated with a bovine serum albumin (BSA) solution (1% w/v in distilled water) for 10 min and then washed with the standard bath solution (BS; see below) before use. Neutrophils were allowed to adhere to the slides for 5–10 min, and nonadherent cells were washed away with BS. Patch pipettes had pipette resistances between 4–7 MΩ. Capacitance changes were recorded using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). From a holding potential of −10 mV, the cells were exposed to a sine-shaped, alternating voltage pulse [20] with a frequency of 1.0 kHz and amplitude of 20 mV. From the resulting current, the capacitance was determined by the lock-in software-based phase-sensitive detector [21, 22] and sampled by the X-chart extension of pulse 8.41 (HEKA). The membrane capacitance was registered for 10 min or (if possible) longer. Only recordings during which the access resistance was well below 20 MΩ when the whole cell configuration was established, as well as during the major part of the recording, were regarded as reliable. Baseline or background secretion was determined using a pipette solution containing no added Ca^{2+} and 2 mM of the Ca^{2+} chelator HEDTA. This solution was regarded as Ca^{2+} free. The capacitance recordings were started when the membrane was ruptured and the whole cell configuration was established. The initial value (typically around 2.5 pF) of the capacitance for each cell has been subtracted in the recordings shown.

Data presentation and analysis

The time of membrane rupture and establishment of the whole cell configuration was set as time zero and initial capacitance at this time was about 2.5 pF (2.57 ± 0.05 pF, mean ± SEM, $n = 140$). X-chart data were imported into IGOR 3.03 for Macintosh (Wave Metrics Inc, Lake Oswego, OR) and analyzed. Statistical calculations were performed using Prism 3.02 (Graph Pad Software Inc, San Diego, CA) and $P < .05$ was considered significant.

Solutions and reagents

The standard bath solution (BS) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. The standard pipette solution (PS) contained 112 mM K-glutamate, 18 mM NaCl, 1.8 mM $MgCl_2$, 2.5 mM Na_2ATP, and 9 mM HEPES, pH 7.2. The pipette solution was supplemented with suitable concentrations of calcium ions and calcium chelator (citrate or HEDTA) to reach the desired concentration of free calcium, as determined by WinMaxChelator 1.78 (Chris Patton, Stanford, CA, USA), using the BERS constants [23]. Dextran and Ficoll-Paque were purchased from Pharmacia (Uppsala, Sweden).

RESULTS

Neutrophils respond to increased levels of $[Ca^{2+}]_i$ with exocytosis of granules (degranulation), in this system manifested as an increased membrane capacitance. The degree of granule mobilization depends on the concentration of free Ca^{2+} used in the pipette solution, which in the study ranged from nominally calcium-free solutions to 300 μM. The maximal ΔC_m values obtained at high $[Ca^{2+}]_i$ were 6–7 pF (Figure 1(a)), which correspond to an increase in total membrane area approaching 300%. The magnitude of the capacitance change as well as the kinetics of the secretory response differed between the systems with a low (10 μM or lower) or a high (100 μM or higher) concentration of Ca^{2+} in the pipette, (Figures 1(a) and 1(b), Table 1).

The secretory response induced by a high $[Ca^{2+}]_i$ started with a lag phase during which the capacitance increase was around 10 fF/sec (Figure 1(b)). The lag phase was followed by a more sustained phase of secretion after which the capacitance recordings reached a plateau, usually 5–7 min after rupture of the membrane. The maximal obtainable rate of secretion using a lower $[Ca^{2+}]_i$, (0, 1, and 10 μM) was around 10 fF/s, whereas this rate was around 50–60 fF/s for the higher concentrations (100 and 300 μM). No difference in maximal rate of secretion was found between $[Ca^{2+}]_i$ of 100 and 300 μM, respectively (Figure 2(a)). However, the time required to reach a capacitance value corresponding to half of the maximal (plateau) value was significantly ($P < .001$) shortened when the $[Ca^{2+}]_i$ concentration was increased (Figure 2(b)). The initial rate of secretion was higher with

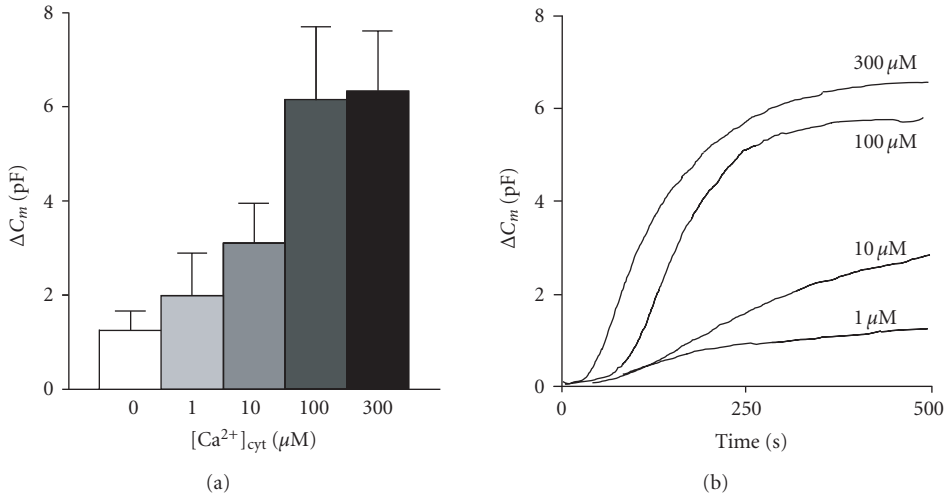

Figure 1: Characteristics of calcium-induced secretion in neutrophils. (a) Change in membrane capacitance induced by increasing concentrations of $[Ca^{2+}]_i$. Values were determined when a plateau level was reached or 10 min after breakthrough with the patch pipette. (b) Time-course of calcium induced neutrophil exocytosis. The recordings are from representative experiments.

Table 1: Basic characteristics of the secretory process in human neutrophils.

$[Ca^{2+}]$ (μM)	n	$C_{breakthrough}$ (pF)[a]	ΔC_m (pF)[b]	$Rate_{max}$ (fF/s)[c]	Point of inflection (pF)[c]	Half-time rise (s)[d]
0	13	2.42 ± 0.36	1.10 ± 0.44	7.38 ± 2.69	nd[e]	nd
1	7	2.20 ± 0.24	1.39 ± 0.48	6.63 ± 1.86	nd	nd
10	17	2.40 ± 0.35	1.82 ± 0.59	5.27 ± 3.06	nd	nd
100	23	2.62 ± 0.50	5.95 ± 1.35	51.55 ± 18.52	0.57 ± 0.32	142.2 ± 52.0
300	27	2.71 ± 0.73	6.30 ± 1.29	58.90 ± 22.73	0.35 ± 0.17	95.8 ± 31.4

Secretion was induced by an introduction through the patch pipette of pipette solutions containing increased concentrations of free Ca^{2+}. The concentration of Ca^{2+} was varied from 0 to 300 μM and all numerals presented represent mean values ±S.D. The number of cells studied (n) is given for each concentration of Ca^{2+}.

[a]Values represent the initial capacitance values of the cell, determined upon the establishment of the whole-cell configuration.

[b]Values represent the change in capacitance reached at the plateau (for calcium concentrations of 100 and 300 μM), and for comparison with those that did not reach a plateau (calcium concentrations 1 and 10 μM) 5 minutes after breakthrough, a time point that corresponds to the time required to reach a plateau at the higher calcium concentrations.

[c]Values represent the maximal rate of capacitance change and the capacitance value (point of inflection) corresponding to the time point at which the maximal of secretion was reached, respectively.

[d]Values represent the time required to reach a ΔC_m corresponding to 50% of the plateau value.

[e]Not determined.

a $[Ca^{2+}]_i$ of 300 μM compared to 100 μM and the time required to reach a maximal response was shorter. The ΔC_m value at which the maximal rate of secretion reached was somewhat lower for the responses induced at a $[Ca^{2+}]_i$ of 300 μM as compared to 100 μM. These values were typically below 0.5 pF and almost always (48 of 50 cells, 96%) below 1.0 pF for both concentrations (Table 1, point of inflection).

When the patch pipette contained lower Ca^{2+} concentrations (up to 10 μM), no distinct shift in the rate of secretion could be observed (Figure 1(b)). The secretory response induced at lower $[Ca^{2+}]_i$ also differed from that induced with higher $[Ca^{2+}]_i$ in that it seldom reached a plateau during the 10 min measurement time. In fact, four individual measurements that maintained the required values of seal and access resistance for around 15 minutes (875 ± 171s, $n = 4$) still showed increasing capacitance that could reach ΔC_m values of up to 5 pF (mean = 4.27 ± 0.85 pF, $n = 4$) without any sign of decrease in the secretion rate (Figure 3). Perfusion with nominally calcium-free solutions resulted in an increase in capacitance during a 3–5 min period, and this phase typically reached a ΔC_m value of around 1 pF and was followed by a stable and prolonged plateau level. The outcome of experiments with a $[Ca^{2+}]_i$ of 60 μM was highly unpredictable. Such an experiment could result in either of the two secretory patterns described (high or low $[Ca^{2+}]_i$), suggesting that there is a threshold concentration somewhere around 60 μM.

Obtaining the rate of capacitance increase directly by plotting the derivate of the capacitance traces against time revealed a unimodal pattern of response with only one distinct peak, for both concentrations (Figure 4). Furthermore, all the traces from both 100 μM and 300 μM $[Ca^{2+}]_i$ could be well fitted (R2 = 0.996 ± 0.001, mean ± SEM, $n = 50$) to a simple logistic equation describing a monophasic curve with sigmoid appearance.

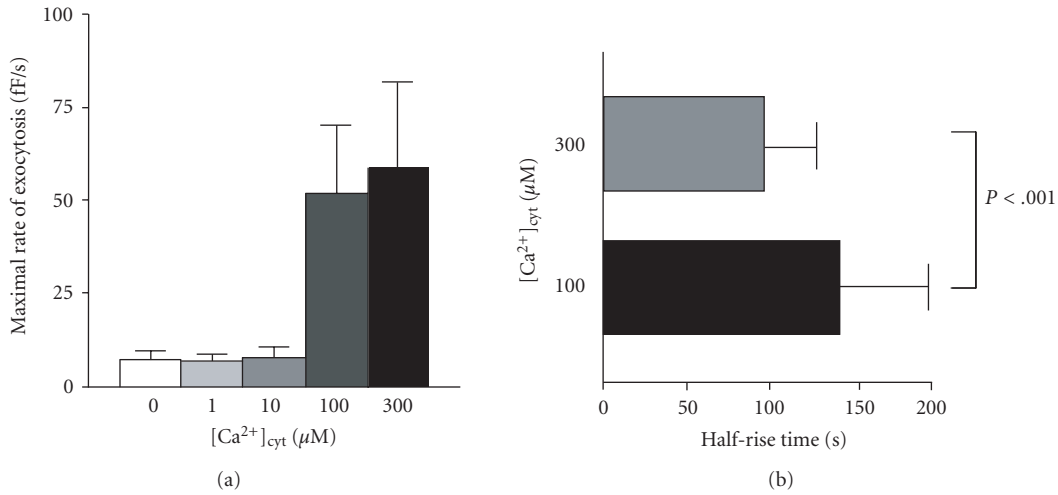

FIGURE 2: Dynamics of secretion at different $[Ca^{2+}]_i$. (a) Maximal rates of capacitance increase during neutrophil exocytosis. The capacitance values were recorded during a 5–10 min period after breakthrough with the patch pipettes, and the maximal rate of exocytosis was determined from each capacitance trace. (b) Higher $[Ca^{2+}]_i$ gives shorter half-rise time. The time required for reaching half of the maximal capacitance increase is induced by 100 μM and 300 μM $[Ca^{2+}]_i$, respectively.

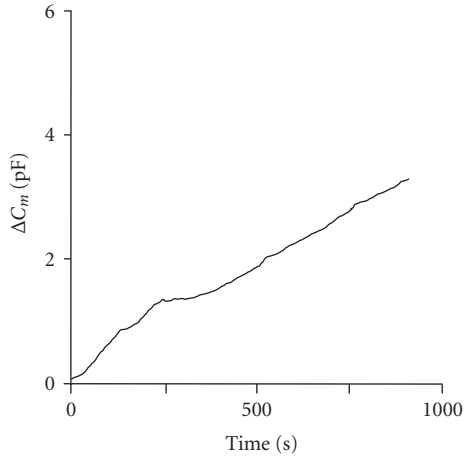

FIGURE 3: Long exposure to low levels of $[Ca^{2+}]_i$ can induce secretion of all granules. The change in membrane capacitance induced with a pipette solution containing 10 μM of $[Ca^{2+}]_i$. The curve is from one of the four recordings that could be followed for more than 15 min.

DISCUSSION

The level of cytosolic Ca^{2+} is a key regulatory element for the release of neutrophil granule markers and exposure of new membrane proteins [11]. The hierarchy in neutrophil vesicle and granule mobilization has been well established in vitro as well as in vivo [4], and as a general rule the degree of mobilization is inversely proportional to the size of the granules. A fairly simple model for how this hierarchy is achieved has been presented [24]. According to this model the densest (and largest in size) granule population (the azurophil granules) is also the hardest to mobilize because a greater stimulation or pulling force is required for their fusion with the plasma membrane. Such a pulling force can be manifested either in the form of highly elevated Ca^{2+} concentrations or prolonged exposure to lower levels. We achieve a ΔC_m of 6–7 pF through microperfusion of neutrophils with $[Ca^{2+}]_i$ in the 100–300 μM range corresponding to earlier published values for complete degranulation [15, 25] where specific granules are suggested to account for 2.4 pF and azurophil granules for 3.6 pF, respectively [26].

We have chosen to focus on the two secretion patterns obtained with high (>100 μM) and low (10 μM) $[Ca^{2+}]_i$. Results obtained from measurements with intermediate calcium concentrations also show one or another of the two secretion patterns. This variability probably depends on whether or not the specific cell experiences a concentration of calcium that is high enough to induce a rapid secretory response, but more importantly, the high degree of mobilization was never associated with a biphasic pattern of capacitance change as previously reported [15], as determined by the derivation of the capacitance traces. The high- and low-Ca^{2+}-affinity model proposed to be the regulatory base for the hierarchical neutrophil granule secretion [15] implies that the release of azurophil granules occurring at a high rate (>50–60 fF/s) cannot be achieved until all peroxidase negative granules have been mobilized. This suggests that high rates of secretion should not occur until ΔC_m exceeds 2.4 pF, a value corresponding to the membrane area of all the specific granules. We found, however, that a high rate (>70 fF/s) of secretion could be obtained long before ΔC_m had reached 2.4 pF. In fact, the point of inflection was almost always (48 out of 50 cells, 96%) found to be below 1 pF and could even be as low as 0.1 pF. Our data thus suggest that also the specific granules can be secreted at a high rate.

Furthermore, our data also imply that the rate of secretion is not directly linked to the type of granule involved

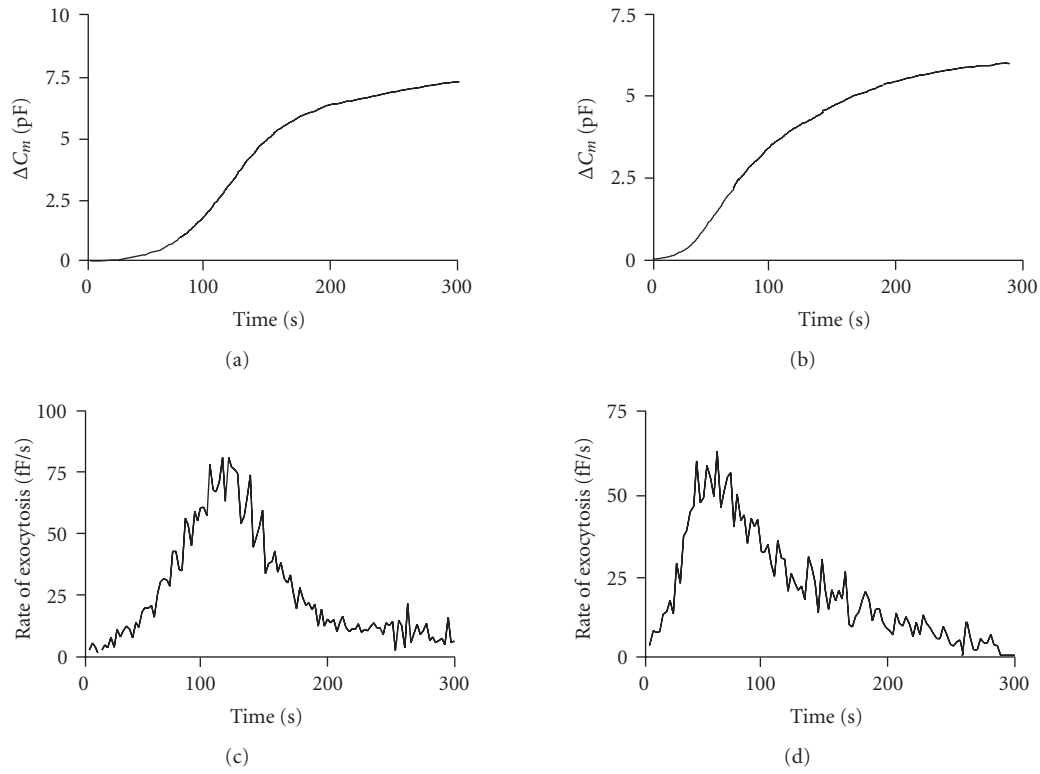

FIGURE 4: Neutrophil secretion is monophasic. The rate of capacitance increase during neutrophil exocytosis is induced by $[Ca^{2+}]_i$ of 100 μM (left) and 300 μM (right), respectively. (a) and (b) show the capacitance recordings of two representative experiments, and (c) and (d) show the rates of exocytosis, determined as the derivatives of these capacitance curves. No cells ($n = 50$) showed two peaks in the derived traces. No direct comparisons were made between the two pairs of traces (a and c versus b and d) making it possible to use scales that accurately show each individual trace.

in the secretory process. This suggestion gains support from the fact that ΔC_m values close to those corresponding to a mobilization of all granules (including at least 50% of the azurophil granules) can be obtained with an experimental setup in which the rate of secretion is fairly low (<10 fF/s). Based on these data it also seems reasonable to believe that the azurophil granules can be mobilized without the prior degranulation of all peroxidase-negative/specific granules. Accurate measurements of the release of distinct granules would help clarify this issue better. Attempts have been made but the technique used has not the resolution required and the results obtained have (according to the authors) to be considered with caution [15].

Taken together, our data reveal no granule subsets differing in calcium affinities, making the concept of hierarchical exocytosis based on this property not applicable. The results presented here differ in certain respects from earlier published data regarding the basic regulatory mechanisms in neutrophil granule secretion [12, 13, 15, 16]. Several factors can underlie these discrepancies. For example, the studies were performed in different cell types, different methods were used to elevate $[Ca^{2+}]_i$ and/or induce secretion, and the duration of the measurements was also different. More importantly, the basic finding that not all specific granules must be released before secretion of azurophil granules may commence also finds support in previous investigations [13, 27]. The precise mechanism(s) underlying the different kinetics of the responses is not clear at this time, although both surface-to-mass ratio [24] or differential expression of SNARE proteins [7–9] are attractive alternatives. Furthermore, also the membrane tension of a vesicle, being higher in smaller vesicles and lower in larger vesicles, may play a part in the process. Studies of fusion events occurring in artificial membrane systems indicate a similar behavior in that the smaller membrane vesicles are undergoing fusion faster, as compared to larger vesicles [28]. As such, the mere size of the vesicle may serve as a control element of the fusion event. In all cases, more calcium makes more granules eligible for release faster, resulting in a faster kinetics at higher $[Ca^{2+}]_i$.

In living cells, the cytoskeleton is essential for the control of activity and function [17, 29]. Therefore, data obtained and conclusions drawn from experimental systems with disrupted cytoskeleton (eg, systems using cytochalasin B to increase secretion) are, in our view, difficult to relate to the actual in vivo situation. In addition, we found it exceedingly hard to achieve good seals and whole-cell recordings in the presence of cytochalasin B. We were thus not able to investigate the direct effects of cytochalasin B on the secretory process under study.

In summary, we were unable to detect biphasic capacitance increases as has previously been described and suggested to reflect distinct high- as well as low-affinity granule

populations in human neutrophils. These findings extend and modulate previous knowledge regarding neutrophil granule mobilization. Recent studies on both eosinophils and neutrophils also reveal several distinct types of granule fusion events including not only fusion of single granules with the plasma membrane but also intracellular granule-granule fusion and fusion of large compounds of prefused granules with the plasma membrane [30]. It is obvious that such a prefusion process may involve granules of different types [31] and this may have implication for the data suggesting that not all specific granules have to be mobilized before release of azurophil granules will commence. The coexistence of distinct modes of exocytosis in which granule content from different granule subsets interact may be a mechanism for efficient targeting of release during exocytosis and further tune neutrophil function in the inflammatory response.

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