Ca²⁺-induced exocytosis in individual human neutrophils: high- and low-affinity granule populations and submaximal responses

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We have investigated Ca²⁺-induced exocytosis from human neutrophils using the whole cell patch-clamp capacitance technique. Microperfusion of Ca²⁺ buffer solutions (<30 nM to 5 mM free Ca²⁺) through the patch-clamp pipette revealed a biphasic activation of exocytosis by Ca²⁺. The first phase was characterized by high affinity (1.5–5 μM) and low apparent cooperativity (≤2) for Ca²⁺, and the second phase by low affinity (~100 μM) and high cooperativity (>6). Only the second phase was accompanied by loss of myeloperoxidase, suggesting that the low-affinity exocytosis reflected release of peroxidase-positive (primary) granules, while the high-affinity exocytosis reflected release of peroxidase-negative (secondary and tertiary) granules. At submaximal Ca²⁺ concentrations, only a fraction of a given granule population was released. This submaximal release cannot simply be explained by Ca²⁺ modulation of the rate of exocytosis, and it suggests that the secretory response of individual cells is adjusted to the strength of the stimulus. The Ca²⁺ dependence of the high- and low-affinity phases of neutrophil exocytosis bears a resemblance to endocrine and neuronal exocytosis, respectively. The occurrence of such high- and low-affinity exocytosis in the same cell is novel, and suggests that the Ca²⁺ sensitivity of secretion is granule-, rather than cell-specific.

Keywords: Ca²⁺/capacitance/exocytosis/granule populations/neutrophil

Introduction

Regulated exocytosis is involved in a large variety of physiological functions, including processes as different as insulin secretion from β-cells, neurotransmitter release from nerve terminals and histamine secretion from mast cells. A widely conserved, universal molecular mechanism of exocytosis has been proposed [SNARE hypothesis (Söllner *et al.*, 1993; Ferro-Novick and Jahn, 1994)]. In human neutrophils, only some of these conserved molecular elements have been detected so far: VAMP-2, syntaxin 4 (Brumell *et al.*, 1995) and the mRNA for SNAP-23, a homologue of SNAP-25 (Mollinedo and Lazo, 1997).

A common feature of exocytosis in many cellular systems is its activation by cytosolic free Ca²⁺ ([Ca²⁺]_c) elevations (Burgoyne and Morgan, 1995). However, the

Ca²⁺ affinities of exocytosis obtained in different cellular systems are strikingly different. For example, studies in retinal bipolar neurons found an astonishingly low Ca²⁺ affinity of exocytosis [~190 μM Ca²⁺ (Heidelberger *et al.*, 1994)]. In contrast, a much higher Ca²⁺ affinity of exocytosis [1.6 μM Ca²⁺ (Proks *et al.*, 1996)] was found in pancreatic β-cells. The difference in affinity of Ca²⁺-dependent exocytosis in different cellular systems may reflect a cell-specific property of the exocytotic machinery. Alternatively, the Ca²⁺ affinity might be a property of each granule population. However, high- and low-affinity secretion have not been documented to co-exist together in one cell.

The neutrophil granulocyte is a particularly interesting cell type for the study of exocytosis, as it contains several subcellular compartments that undergo regulated exocytosis [referred to as primary, secondary and tertiary granules, and secretory vesicles (Borregaard *et al.*, 1993)]. These exocytotic compartments are released at different stages of neutrophil activation and therefore are in need of mechanisms that allow distinct responses to a given signal. Indeed, several studies reported different Ca²⁺ requirements for exocytosis of distinct granule populations in neutrophils. Studies using ionophore clamp or Sendai virus permeabilization suggested relatively high Ca²⁺ affinities between 0.3 and 3 μM (Lew *et al.*, 1986; Barrowman *et al.*, 1987; Borregaard *et al.*, 1993). However, in some experiments with digitonin-permeabilized cells, Ca²⁺ concentrations of 10–40 μM were necessary to achieve primary granule release (Smolen *et al.*, 1987; Smolen and Sandborg, 1990) and in previous patch-clamp studies, pipette Ca²⁺ concentrations ([Ca²⁺]_{pip}) up to 4 μM only had minor effects on exocytosis (Nüsse and Lindau, 1988).

In the light of recent reports of low affinity Ca²⁺-dependent exocytosis (Heidelberger *et al.*, 1994; von Gersdorff and Matthews, 1994) and the continuous interest in the mechanism of different signalling requirements for the release of different granules, the Ca²⁺ affinity of neutrophil exocytosis needed to be re-evaluated. To this end, we measured the increase in plasma membrane surface during exocytosis with the patch-clamp capacitance technique.

Results

To study the activation of neutrophil exocytosis by [Ca²⁺]_c, we have microperfused patch-clamped human neutrophils with highly Ca²⁺-buffered solutions (<30 nM to 5 mM free Ca²⁺). Membrane capacitance changes were recorded, until a plateau was reached, usually within 5–10 min.

Two phases of Ca²⁺-dependent capacitance increase in neutrophils

Figure 1A shows typical recordings from five different cells microperfused with different [Ca²⁺]_{pip}. With very

Fig. 1. Exocytosis and elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) by Ca^{2+} buffer perfusion in patch-clamped neutrophils. (A) Capacitance recordings of Ca^{2+} -induced exocytosis in patched neutrophils. The increase in membrane capacitance (initial capacitance, ~ 3 pF, subtracted for clarity) from five cells patched with <30 nM, 1, 10, 100 or 300 μ M free Ca^{2+} in the pipette solution ($[Ca^{2+}]_{pip}$), respectively, is plotted against the time after break-in. (B) Examples of $[Ca^{2+}]_c$ in Ca^{2+} buffer-perfused neutrophils. $[Ca^{2+}]_c$ measurements with indo-1FF from three cells with 10, 100 or 300 μ M free Ca^{2+} in the pipette solution, respectively.

low $[Ca^{2+}]_{pip}$ (<30 nM), cell capacitance did not increase during the recording. Moderately elevated $[Ca^{2+}]_{pip}$ (1 and 10 μ M) induced a monophasic capacitance increase. The plateau amplitude was higher for 10 than for 1 μ M $[Ca^{2+}]_{pip}$. Higher $[Ca^{2+}]_{pip}$ (100 and 300 μ M) stimulated a biphasic capacitance increase. The final amplitude of the second phase of the capacitance increase was a function of $[Ca^{2+}]_{pip}$, while the amplitude of the first phase was constant (~ 2 pF) within the high range of $[Ca^{2+}]_{pip}$. With all $[Ca^{2+}]_{pip}$ conditions, the capacitance recordings reached a stable plateau and were not followed by a capacitance decrease within the time scale of the recording.

Under our experimental conditions, the $[Ca^{2+}]_c$ elevations were due to microperfusion of highly Ca^{2+} -buffered solutions through the patch-clamp pipette. To measure kinetics and amplitude of these $[Ca^{2+}]_c$ elevations, we have used the Ca^{2+} -sensitive fluorescent dye indo-1FF (London *et al.*, 1996). This probe is a low-affinity derivative of indo-1 ($K_d = 27$ μ M) with high selectivity for Ca^{2+} over Mg^{2+} . We included this dye in its free-acid form in the patch-pipette solution. Approximately 10–20 s after break-in, the intracellular dye concentration was sufficiently high to allow ratiometric $[Ca^{2+}]_c$ measurements. Figure 1B shows examples of $[Ca^{2+}]_c$ measurements with indo-1FF and 10, 100 or 300 μ M Ca^{2+} in the pipette. Following the break-in, $[Ca^{2+}]_c$ increased over time as a function of $[Ca^{2+}]_{pip}$ and reached values close to $[Ca^{2+}]_{pip}$

within 5–10 min. Note, however, that the rise in $[Ca^{2+}]_c$ was delayed and had a sigmoidal shape. For the diffusion of Ca^{2+} and the low molecular weight Ca^{2+} chelators through the patch-pipette, an inverse exponential curve rapidly approaching $[Ca^{2+}]_{pip}$ values ($\tau \sim 30$ –60 s) would be expected (Nüße and Lindau, 1993). The deviation of the measured $[Ca^{2+}]_c$ elevations from the theoretically expected curves might hint at Ca^{2+} regulatory mechanisms of the cell, e.g. Ca^{2+} pumps or Ca^{2+} buffers, that are able temporarily to counteract the influx of Ca^{2+} from the pipette. These results underline the importance of measuring $[Ca^{2+}]_c$ during microperfusion of Ca^{2+} through the patch-pipette, in order to establish the correlation between $[Ca^{2+}]_c$ levels and exocytosis.

To obtain the dose-response curve for Ca^{2+} -induced neutrophil exocytosis, we first plotted the mean plateau capacitance values against $[Ca^{2+}]_{pip}$. As shown in Figure 2A, the observed dose-response curve was complex. Between 0.1 and 10 μ M $[Ca^{2+}]_{pip}$, the plateau capacitance values increased with rising Ca^{2+} concentrations. Raising $[Ca^{2+}]_{pip}$ from 10 to 60 μ M had little additional effect, while between 60 and 300 μ M, a very steep relationship between plateau capacitance values and $[Ca^{2+}]_{pip}$ was observed. Finally, with $[Ca^{2+}]_{pip} > 300$ μ M, plateau capacitance values declined. Thus, there was a biphasic activation of exocytosis by $[Ca^{2+}]_{pip}$, with phase 1 ranging from 0.1 to 10 μ M $[Ca^{2+}]_{pip}$ and phase 2 from 60 to 300 μ M $[Ca^{2+}]_{pip}$. In addition, there also appeared to be an inhibitory effect of very high $[Ca^{2+}]_{pip}$. In this study, we will not analyse the inhibitory effect of very high $[Ca^{2+}]_{pip}$ further, but concentrate on the activation observed with $[Ca^{2+}]_{pip}$ values up to 300 μ M. The solid line in Figure 2A is a best fit with a double logistic equation, excluding $[Ca^{2+}]_{pip}$ values > 300 μ M.

In a series of control experiments (not shown), we measured capacitance increases with different Ca^{2+} chelators in the pipette solution (HEDTA versus citrate), and with Ca^{2+} -containing or Ca^{2+} -free bath solutions. Neither of those factors influenced the $[Ca^{2+}]_{pip}$ exocytosis relationship shown in Figure 2A.

If the biphasic $[Ca^{2+}]_{pip}$ exocytosis relationship reflects the presence of two granule populations that are secreted with different Ca^{2+} affinities, it should also be possible to observe such a behaviour by plotting $[Ca^{2+}]_c$ (as measured by indo-1FF in an individual cell) against the capacitance increase in the same cell. The dose-response curve of individual cells was indeed very similar to the $[Ca^{2+}]_{pip}$ dependence of the mean amplitude of exocytosis (compare Figure 2A with B and C). Note, however, that with high $[Ca^{2+}]_{pip}$ (100 μ M, Figure 2B), phase 1 of secretion was almost completed before $[Ca^{2+}]_c$ could be monitored reliably (see technical considerations above). With lower $[Ca^{2+}]_{pip}$ (30 μ M), phase 1 could be clearly resolved (Figure 2C), and, as expected, phase 2 did not occur.

The quantitative results of the analysis of the Ca^{2+} dependency of neutrophil exocytosis based on $[Ca^{2+}]_{pip}$ or $[Ca^{2+}]_c$, are compared in Figure 3. EC_{50} and slope factor [Hill coefficient (Barlow and Blake, 1989)] for the analysis based on $[Ca^{2+}]_{pip}$ were derived from a double logistic fit of the mean capacitance increase over $[Ca^{2+}]_{pip}$, as shown in Figure 2A. For the analysis based on $[Ca^{2+}]_c$, we proceeded as follows. Phase 2 was analysed by fitting

Fig. 2. The Ca²⁺ dependency of neutrophil exocytosis. (A) The mean ($\pm$ SEM) amplitude of the capacitance increases (plateau values) from 3–22 cells per [Ca²⁺]_{pip} condition is plotted against the pipette Ca²⁺ concentration. The line represents a double logistic fit. The capacitance rise from 0.1 to 10 μ M Ca²⁺ is termed phase 1 of Ca²⁺-induced exocytosis, the rise from 30 to 300 μ M is termed phase 2. (B) Capacitance increase of a single cell, stimulated with [Ca²⁺]_{pip} = 100 μ M, plotted against [Ca²⁺]_c, as measured in parallel in the same cell. (C) Capacitance increase of a single cell, stimulated with [Ca²⁺]_{pip} = 30 μ M, plotted against [Ca²⁺]_c. The recordings shown in (B) and (C) lasted 690 and 490 s, respectively.

the plots of Δ Cm over [Ca²⁺]_c with 100 or 300 μ M [Ca²⁺]_{pip} (as shown in Figure 2B) with a double logistic equation, which directly yielded the EC₅₀ and slope factor of phase 2 (see Materials and methods). For phase 1, experiments with 10 or 30 μ M [Ca²⁺]_{pip} were fitted by a single logistic equation. Importantly, the analysis of the Ca²⁺ dependence of the two phases of neutrophil exocytosis yielded very similar results, independently of whether it was based on [Ca²⁺]_{pip} or [Ca²⁺]_c. Both types of analysis revealed a relatively high Ca²⁺ affinity of phase 1 (EC₅₀ = 1.5–5 μ M) (Figure 3A), but a low Ca²⁺ affinity of phase 2 (EC₅₀ = 100 μ M). The steepness of the dose–response curve was relatively low in phase 1 (slope factor 1.5–1.9), but very high for phase 2 (slope factor 6–15, Figure 3B). Thus, two different approaches towards the analysis of Ca²⁺-induced exocytosis in neutro-

Fig. 3. Quantitative analysis of the two phases of neutrophil exocytosis; comparison of the analysis based on [Ca²⁺]_{pip} (see Figure 2A) and [Ca²⁺]_c (see Figure 2B and C). (A) The EC₅₀ for Ca²⁺ of phase 1 and 2, mean $\pm$ SEM. (B) The slope factor (Hill coefficient; Barlow and Blake, 1989) of the Ca²⁺ dependency of phase 1 and 2, mean $\pm$ SEM. Note: there was no statistically significant difference between the EC₅₀ and the slope factors obtained with 10 versus 30 μ M [Ca²⁺]_{pip} and 100 versus 300 μ M [Ca²⁺]_{pip} (Student's *t*-test).

phils distinguished a high-affinity phase with a relatively shallow dose–response curve, and a low-affinity phase with a steep dose–response curve.

Identification of the two phases of Ca²⁺-dependent exocytosis as peroxidase-negative and peroxidase-positive granule populations

Neutrophils have several distinct populations of granules that are secreted under different physiological conditions (Borregaard *et al.*, 1993). We therefore investigated the hypothesis that the two phases of Ca²⁺-induced exocytosis correspond to the secretion of morphologically distinct populations of neutrophil granules. We first compared the amplitude of the two phases of exocytosis with the total membrane area of all granules (Nüße and Lindau, 1988) (Figure 4A). The amplitude of phase 1 is very close to the morphology-based estimate of the size of secondary and tertiary granules. The amplitude of phase 2 is similar to the estimated size of all primary granules. Thus, phase 1 of Ca²⁺-induced exocytosis may correspond to the release of secondary and tertiary granules, whereas phase 2 may correspond to the release of primary granules.

A major constituent of primary granules is myeloperoxidase (MPO), which is absent from the other granule populations. We therefore used a light microscopic MPO stain to assess the MPO content of neutrophils after the completed patch–clamp experiment. If the above suggested correlation between granule types and phases of capacitance change is correct, stimulation with [Ca²⁺]_{pip} $\leq$ 30 μ M should not affect MPO staining, while stimulation with [Ca²⁺]_{pip} $\geq$ 100 μ M should markedly diminish MPO

Fig. 4. Identification of released granules by myeloperoxidase staining. (A) Comparison of calculated membrane capacitance based on electron microscopic data and capacitance measurements. The amplitudes of phase 1 and 2 of Ca^{2+} -induced exocytosis (Figure 2A) are compared with estimates of the total granule surface of primary granules versus secondary and tertiary granules (published in Nuße and Lindau, 1988). The size of the average granule of each population and the total number of granules per cell were estimated from electron microscopy data (Bainton *et al.*, 1971; Spitznagel *et al.*, 1974). The total membrane surface of the granules was calculated and transformed into membrane capacitance assuming a specific membrane capacitance of 10 fF/ μm^2 . (B–D) Examples of myeloperoxidase-stained granulocytes with one patched cell in front of the pipette. The composition of the internal solution and the capacitance increase before staining was: (B) 10 mM EGTA (= 0 Ca^{2+}), 0 pF; (C) 10 μM Ca^{2+} , 2.5 pF; (D) 300 μM Ca^{2+} , 6.9 pF. (E) The intensity of myeloperoxidase staining was quantitated by laser densitometry. The absorbance over each cell was integrated. Mean integrated absorbance $\pm$ SEM of three conditions are compared: 0 Ca^{2+} , $n = 8$; 10 μM Ca^{2+} , $n = 6$; 300 μM Ca^{2+} , $n = 6$.

staining. Figure 4B–D shows typical examples of MPO staining after a patch-clamp experiment with $[Ca^{2+}]_{pip}$ of <30 nM, 10 μM and 300 μM , respectively. To quantitate the loss of MPO during the patch-clamp experiments, photographs of the MPO-stained cells were analysed densitometrically (Figure 4E). There was no difference in the staining of cells patched with $[Ca^{2+}]_{pip}$ <30 nM or 10 μM . In contrast, with $[Ca^{2+}]_{pip} = 300 \mu M$, the MPO staining was reduced significantly. These results demonstrate that: (i) no significant secretion of MPO-containing granules occurred during the high-affinity phase of capacitance increase, suggesting that it was due predominantly to release of MPO-negative (secondary and tertiary) granules; and (ii) substantial loss of cellular MPO content occurred during the low-affinity phase of capacitance increases, suggesting that it was due predominantly to release of MPO-positive (primary) granules.

Kinetics of Ca^{2+} -dependent secretion

We next analysed the relationship between Ca^{2+} and the rate of secretion. Visual inspection of the capacitance increase with $[Ca^{2+}]_{pip} = 300 \mu M$ already suggests that the two phases of neutrophil secretion proceed at different rates (Figure 5A). The rate of capacitance increase can be obtained directly by differentiation of the capacitance

Fig. 5. The Ca^{2+} dependence of the rate of exocytosis.

(A) Capacitance recording of an experiment with 300 μM Ca^{2+} in the internal solution. (B) The rate of exocytosis was determined as the derivative of the smoothed capacitance curve shown in (A). Two peaks corresponding to phase 1 and 2 of secretion were identified. (C and D) The maximal rates of exocytosis were determined for peak 1 (C) and 2 (D) of Ca^{2+} -induced capacitance increase. The mean $\pm$ SEM values ($n = 3$ –22) are plotted for each phase separately against $[Ca^{2+}]_{pip}$ and fitted using a logistic equation.

versus time plot (Figure 5B). This analysis revealed two peaks, corresponding to the maximal rates of exocytosis in phase 1 and phase 2. We determined the maximal rates of the two phases as a function of $[Ca^{2+}]_{pip}$. The results are summarized in Figure 5C and D. Peak 1, the maximal rate of capacitance increase of phase 1, reached a plateau of 32 fF/s at $\sim 10 \mu M$ $[Ca^{2+}]_{pip}$ and did not increase further with higher $[Ca^{2+}]_{pip}$. Peak 2, the maximal rate of capacitance increase of phase 2, became detectable with $[Ca^{2+}]_{pip}$ values of 100 μM , and reached a plateau of 140 fF/s at $\sim 300 \mu M$ (Figure 5D). These data indicate that the rate of exocytosis of both phases depended on the Ca^{2+} concentration in the internal solution. However, both phases reached a maximal rate of exocytosis that could not be augmented by higher $[Ca^{2+}]_{pip}$. The maximal rate of capacitance increase is not only determined by the rate of fusion (i.e. number of granules/s), but also by the membrane surface of a granule (fF/granule). To compare fusion of different granules in different cell types, the rate of fusion was normalized to the available plasma membrane surface to yield the 'specific rate of fusion' (Huang and Neher, 1996). When such a normalization was applied to the analysis of exocytosis of the two

Table I. Ca²⁺ affinity and secretion rates of different neutrophil granule population in comparison with endocrine cells and nerve terminals

Cell type	EC ₅₀ for Ca ²⁺ (μM)	Slope factor	Max rate (fF/s)	Cell surface (μm ²)	Granule capacitance (fF)	Rate of fusion (1/s)	Specific rate of fusion [1/(μm ² ×s)]
β-Cell ^a	1.6	2.5	59	400	2	30	0.075
Chromaffin cell ^b	1.6	1.85	45	600	2	23	0.038
Neutrophil ^c							
phase 1	1.5–5	1.5–1.9	32	300	1.1 ^d	29	0.097
phase 2	100	6–15	140	500 ^e	2.4 ^f	58	0.116
Nerve terminal ^g	194	≥4	730	300	0.077	9500	32

Adapted from Huang and Neher (1996).

The average cell surface is derived from the initial capacitance of unstimulated cells (C_i). However, phase 2 of neutrophil secretion begins after phase 1 at C_i + ΔC_m (phase 1). The specific rate of granule fusion is the rate of secretion normalized for the membrane surface.

^{a,b}Sustained secretion ^afrom mouse pancreas (Proks *et al.*, 1996) and ^bfrom bovine adrenal medulla (Augustine and Neher, 1992; Burgoyne and Handel, 1994).

^cThis study.

^dMean of secondary and tertiary granules (Lollike *et al.*, 1995).

^eAfter completion of phase 1.

^fLollike *et al.*, 1995; Nüße and Lindau, 1988.

^gRapid secretion of a limited vesicle pool from goldfish retinal bipolar neurons (Heidelberger *et al.*, 1994; von Gersdorff and Matthews, 1994).

neutrophil granule populations, similar specific rates of fusion were obtained for both granule populations (Table I). Comparable results were reported for the sustained secretory responses in endocrine cells which were induced by microperfusion of Ca²⁺ buffers through the patch-pipette (Burgoyne and Handel, 1994; Proks *et al.*, 1996) (Table I).

Submaximal responses within granule populations

In a simplest model, Ca²⁺-induced exocytotic granule fusion may be described as a first order reaction: C_t = C_{max} * (1 - e^{-t/τ}) (C_t = cumulative capacitance increase at a given time point; C_{max} = capacitance increase due to the insertion of the total available granule population; τ = time constant of the reaction). The effect of Ca²⁺ in such a system might be either a modulation of τ, i.e. the speed of the reaction, or a modulation of C_{max}, i.e. the size of the available granule population. Figure 6A and B illustrates the effects of modulation of τ and C_{max} on a first order reaction. Clearly, modulation of τ has no effect on the final amplitude of the reaction. Figure 6C and D shows averaged capacitance traces at various low [Ca²⁺]_{pip} (phase 1) and high [Ca²⁺]_{pip} (phase 1 + 2). Note that [Ca²⁺] clearly modulated the final amplitude of the capacitance increases (compare also with Figures 1A and 2A). Thus, we observed a submaximal exocytotic response to submaximal Ca²⁺ concentrations, which excludes that Ca²⁺ simply modulated the rate of exocytosis. To investigate further to what extent substrate limitation (i.e. granule depletion) contributed to the termination of the observed capacitance increases, we plotted the rate of exocytosis as a function of the fraction of secreted granules for various [Ca²⁺]_{pip} (Figure 6F and G). This analysis showed that the maximal rate of secretion for phase 1 and 2 was reached when less than one-third of the respective granule population was released. Thus the down-stroke in the rate of exocytosis could not be explained by a lack of available granules. Instead, [Ca²⁺]_{pip} determined which fraction of granules was secreted. Thus, both peroxidase-positive and peroxidase-negative granules in neutrophils were only released in part in response to submaximal Ca²⁺ concentrations.

Discussion

In this study, we have analysed Ca²⁺-induced exocytosis in human neutrophils. The interest in investigating exocytosis in neutrophils derives in part from its importance in host defence and inflammation. An aspect of general interest for cell biology is the presence of well defined granule populations with distinct exocytotic signalling requirements within a single cell.

Ca²⁺ affinity of neutrophil exocytosis

Previous studies using ionophore clamp techniques had suggested that [Ca²⁺]_c elevations induce exocytosis of all neutrophil granule populations with a relatively high affinity [0.3 and 3 μM for MPO-negative and -positive granules, respectively (Lew *et al.*, 1986; Barrowman *et al.*, 1987; Sengelov *et al.*, 1993)]. As these numbers were in line with fluorescence measurements of average [Ca²⁺]_c, they were thought to represent the 'in vivo' Ca²⁺ sensitivity of the exocytotic machinery. The low-affinity Ca²⁺-activated exocytosis observed in permeabilized neutrophils (Smolen *et al.*, 1987; Smolen and Sandborg, 1990) was attributed to cell and membrane damage through permeabilization (Lew *et al.*, 1986). However, recent changes in concepts and novel observations require a reassessment of these arguments: (i) at least in some neuronal and endocrine cells the presence of low affinity Ca²⁺-dependent exocytosis has been clearly established (Thomas *et al.*, 1993; Heidelberger *et al.*, 1994; von Gersdorff and Matthews, 1994); (ii) Ca²⁺ influx through plasma membrane Ca²⁺ channels may generate submembraneous [Ca²⁺] in the high micromolar range (Henkel and Almers, 1996), values that were not anticipated from average [Ca²⁺]_c measurements; and (iii) Ca²⁺ ionophores may create local domains of high [Ca²⁺]_c which might shift the apparent dose-response curve of ionophore-induced secretion (Nüße *et al.*, 1997).

The patch-clamp technique avoids several of the problems inherent in other approaches to determine the Ca²⁺ affinity of exocytosis. There is no need for permeabilizing agents, and the membrane alteration due to the break-in is limited to a small fraction of the plasma membrane

Fig. 6. Submaximal secretion at suboptimal stimulation. (A and B) Illustration of the time course of a calculated capacitance increase over time (C_t) following a first order reaction: $C_t = C_{max} \cdot (1 - e^{-t/\tau})$. Complete secretion corresponds to 100%. Variations in the speed, τ (A), or in the amplitude, C_{max} (B), of the reaction are modelled. (C and D) Measured mean capacitance traces of 4–7 patched neutrophils per $[Ca^{2+}]_{pip}$ condition. (E and F) Mean rate of exocytosis over the fraction of granules secreted ($\Delta C_m/C_{total}$; total = 7.5 pF) derived from the mean traces in (C) and (D). The fraction of 0.3 (30%) of the total, as obtained with 10 μM (E) and 60 μM $[Ca^{2+}]_{pip}$ (F), corresponds to the maximal release of phase 1, 2.25 pF.

surface. $[Ca^{2+}]_c$ elevations achieved by perfusion of the cell with highly Ca^{2+} -buffered solutions through the patch-pipette are likely to generate uniform $[Ca^{2+}]_c$ elevations (Oliva *et al.*, 1988) without microdomains of $[Ca^{2+}]_c$ higher than $[Ca^{2+}]_{pip}$.

Using the patch-clamp technique, we could clearly identify two phases of secretion with high and low Ca^{2+} affinity within individual human neutrophils. The MPO-staining results show that the high-affinity component consisted of MPO-negative (secondary and tertiary) granules, while the low-affinity component consisted of MPO-positive (primary) granules.

The two phases of Ca^{2+} -dependent exocytosis in neutrophils were distinct not only with respect to the absolute values of their Ca^{2+} -dependence, but also with respect to the steepness of the Ca^{2+} -exocytosis dose-response curve. The strikingly high slope factors of 6–15 for the low-affinity phase of exocytosis indicate that membrane recruitment and/or fusion of primary granules is a highly

cooperative process. The molecular basis of this apparent cooperativity might be multiple Ca^{2+} -binding sites on the $[Ca^{2+}]_c$ -sensing protein(s) of the exocytotic machinery (Thomas *et al.*, 1993; Huang and Neher, 1996) or cooperativity between multiple Ca^{2+} -binding proteins. The shallower dose-response curve of the high-affinity phase of exocytosis may suggest a different, non-cooperative process. However, as MPO-negative granules consist of several subpopulations that probably have different Ca^{2+} affinities (Sengelov *et al.*, 1993), it would also be conceivable that each subpopulation is released through a cooperative process, and the shallow Ca^{2+} -exocytosis curve is generated through an overlap of the release from different granule subpopulations.

Submaximal responses to submaximal $[Ca^{2+}]_c$ concentrations

Submaximal secretion in response to sustained exposure to submaximal Ca^{2+} concentrations has been described

previously for populations of permeabilized cells (Knight and Baker, 1982; Hide *et al.*, 1993). These observations are puzzling, as in a simple enzymatic process where Ca²⁺ regulates the rate of the reaction, no incomplete exocytosis should occur (Figure 6A and Hide *et al.*, 1993; Vogel *et al.*, 1996). Indeed, despite the apparently submaximal Ca²⁺-induced exocytosis observed in populations of mast cells, individual mast cells were shown to respond in an all-or-none fashion (Hide *et al.*, 1993). A patch-clamp study in single chromaffin cells also found that Ca²⁺ regulates the rate rather than the amplitude of exocytosis (Augustine and Neher, 1992). Instead, Ca²⁺ dependence of the amplitude and rate of secretion from individual cells was described for PC12 cells (Kasai *et al.*, 1996). A recent study in homogenized sea urchin eggs has raised the possibility that submaximal exocytosis may be a property of granules, rather than of cells (Vogel *et al.*, 1996). Our data demonstrating submaximal exocytosis in response to sustained exposure to submaximal Ca²⁺ concentrations in individual cells provides new evidence for the latter concept. Taken together, the various studies raise the possibility that the graded secretory response in populations of various cell types might be due to different underlying mechanisms. As neutrophils are individually acting cells, a graded response of the individual cell may be a mechanism of adaptation to variable physiological situations. On the other hand, for chromaffin cells which are part of a coordinated tissue, an increase in the number of responding cells rather than in the response of an individual cell might be physiologically advantageous.

At this point, the mechanism of how a single cell can respond with submaximal exocytosis is not known. The results in sea urchin eggs suggest that individual granules might show differences in Ca²⁺ affinity due to a different density of fusion complexes (Vogel *et al.*, 1996). Alternatively (or in addition), however, ongoing exocytosis might lead to a decrease in Ca²⁺ affinity of the remaining granules through negative feedback mechanisms as described for squid nerve terminals (Hsu *et al.*, 1996).

In neuronal and endocrine cells, exocytosis is often followed by endocytotic membrane retrieval to regenerate secretory vesicles (Parsons *et al.*, 1994; Matthews, 1996). Therefore, a plateau in membrane capacitance might be due to an onset of endocytosis, stimulated by preceding exocytosis or by high [Ca²⁺]_c. However, neutrophils do not appear to regenerate their granules after secretion, and several points argue against the onset of substantial endocytosis. (i) Under all [Ca²⁺]_{pip} conditions, capacitance reached a plateau that remained stable for >5 min. This is in contrast to neuronal cells, where membrane capacitance usually returns to resting levels (Matthews, 1996). It appears unlikely that endocytosis balanced exocytosis for such a long time without net changes in capacitance. (ii) Previous high-resolution measurements of Ca²⁺ ionophore-induced neutrophil secretion have shown that the capacitance increase stopped when no more granules fused, as seen by the lack of stepwise capacitance increases (Lollike *et al.*, 1995). (iii) Receptor-mediated endocytosis in neutrophils and HL60 cells was reduced rather than enhanced by elevations in [Ca²⁺]_c (Iacopetta *et al.*, 1986; Andersson *et al.*, 1987). Taken together, these observations suggest that membrane

retrieval after exocytosis is unlikely to account for the observed submaximal responses.

Comparison of the characteristics of the two phases of neutrophil exocytosis with Ca²⁺-dependent exocytosis in other cellular systems

To our knowledge, this study is the first patch-clamp analysis of Ca²⁺-dependent exocytosis that reveals—within a single cell—two granule populations with a markedly different Ca²⁺ dependence. However, marked differences in the Ca²⁺ affinity of exocytosis in different cell types have been described. For example, high-affinity exocytosis has been demonstrated in insulin-secreting β -cells and in chromaffin cells (Augustine and Neher, 1992; Burgoyne and Handel, 1994; Proks *et al.*, 1996). In contrast, Ca²⁺ stimulated exocytosis with low affinity in nerve terminals from retinal bipolar neurons (Heidelberger *et al.*, 1994). The comparison of endocrine and synaptic secretion with Ca²⁺-induced secretion in human neutrophils is summarized in Table I. In terms of EC₅₀ and slope factor, we found striking similarities between β -cells, chromaffin cells and MPO-negative granules on one hand, and between nerve terminals and MPO-positive granules on the other hand. The presence of these two ‘modes’ of secretion in a single neutrophil provides first evidence that granule-specific rather than cell-specific factors regulate the Ca²⁺ sensitivity of exocytosis.

Kinetics of Ca²⁺-dependent exocytosis

Similarly to what has been described for endocrine secretion (Augustine and Neher, 1992; Proks *et al.*, 1996), we found for both phases of neutrophil exocytosis a maximal rate which could not be increased by further Ca²⁺ elevations. When normalized for the number of granules and the available plasma membrane surface, both phases of neutrophil exocytosis have similar kinetics to the sustained exocytosis described in β -cells and chromaffin cells (Table I). It appears that the rate-limiting factors for sustained release of different granules in different cells are similar, and they cannot be overcome by high Ca²⁺. Values for the specific rate of secretion reported for nerve terminals are >100-fold higher (von Gersdorff and Matthews, 1994). These values, however, refer to the release of a limited pool of pre-docked vesicles. It is interesting to note that some endocrine cells also contain a small pool of docked vesicles that can be released very quickly (40 000 vesicles/s; Heinemann *et al.*, 1994). Taken together, one might speculate that the rate-limiting steps for the relatively slow rate of secretion in neutrophils are the transport and docking of granules to the plasma membrane. The latter notion would also be compatible with previous electron micrographs of neutrophils which did not reveal plasma membrane-associated, docked granules (Borregaard *et al.*, 1993). Note, however, that a small fraction of docked granules in neutrophils might go unrecognized in electron micrographs, and would also not have been detected in our study.

Potential Ca²⁺ sensors in Ca²⁺-induced exocytosis

The identity of the proteins which act as Ca²⁺ sensors in exocytosis has not yet been established unequivocally (Burgoyne and Morgan, 1995). Synaptotagmin has not

only been suggested to be the low-affinity Ca^{2+} sensor in synaptic secretion (Geppert *et al.*, 1994), but may also play a role in high-affinity secretion in insulin-secreting cells (Lang *et al.*, 1997). High- and low-affinity Ca^{2+} -dependent interactions of synaptotagmin with phospholipids and syntaxin, respectively, might be the basis for the role of synaptotagmin in high- and low-affinity Ca^{2+} -induced secretion (Südhof and Rizo, 1996; Lang *et al.*, 1997). Thus, synaptotagmin might be an interesting candidate to mediate both phases of neutrophil secretion. However, (i) hitherto, neither synaptotagmin nor a homologue have been detected in neutrophils, and (ii) it is not clear how the same Ca^{2+} -binding protein could mediate exocytosis of different granule populations in the same cell with such substantial differences in affinity. Other candidates for Ca^{2+} sensors in exocytosis are, for example, annexins (Meers *et al.*, 1993; Hung *et al.*, 1996), S-100 proteins (Schäfer and Heizmann, 1996) and syncollin (Edwardson *et al.*, 1997). Thus, one should consider the possibility that exocytosis of the two neutrophil granule populations involves different Ca^{2+} sensors, which localize specifically to the respective granule populations.

In summary, we show the presence of high- and low-affinity Ca^{2+} -induced secretion in individual neutrophils, suggesting that the Ca^{2+} affinity of exocytosis includes granule-specific elements. We also provide evidence for submaximal exocytosis in response to submaximal Ca^{2+} concentrations in individual cells, supporting the idea of heterogeneous Ca^{2+} affinities within granule populations. Both the marked differences in Ca^{2+} sensitivity between granule populations and the existence of submaximal granule release within a granule population may be physiologically relevant, as they permit the release of different granule populations at different stages of neutrophil activation and adapt the response of a single cell to the strength of the stimulation.

Materials and methods

Material and solutions

Medium 199 was obtained from Gibco, indo-1 FF from TEFLABS (Austin, TX), GTP γ S from Boehringer Mannheim (Mannheim, D) and capillaries for patch-pipettes from Clark Instruments (Reading, UK). All other chemicals were obtained 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 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.

Cell preparation

Citrated blood was drawn from healthy volunteers, and neutrophils were prepared by one of two methods (Nüße and Lindau, 1988; Schrenzel *et al.*, 1996). In method A, most of the red blood cells were removed by dextran sedimentation. The leucocytes were then separated by centrifugation on a Ficoll layer and the remaining red blood cells were lysed by hypotonic shock. In method B, citrated blood was mixed 1:1 with Hank's balanced salt solution (HBSS) and centrifuged over a Percoll layer, and the remaining red blood cells were removed by hypotonic lysis. The cells from both types of preparations were kept in Medium 199 on ice and used within 10 h.

Capacitance measurement of secretion

For patch-clamp recordings, the neutrophils were placed on glass coverslips. After 5–10 min, non-adherent cells were washed away with ES. The adherent cells were incubated with ES + 5 $\mu\text{g}/\text{ml}$ cytochalasin B. All experiments were done at room temperature. They were performed with an inverted microscope (Nikon, Diaphot) with a 40 $\times$ 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). Patch-clamp recordings were performed with a List EPC-7 amplifier (List, Darmstadt, Germany) in voltage clamp mode. Voltage pulses were generated with the program ACQUI (SICMU, Geneva) on a Windows-operated computer (486 or pentium) and fed into the amplifier via an ACQUI D/A converter. Whole cell capacitance was measured with the time domain technique (Lindau and Neher, 1988; Nüße and Lindau, 1988). From a holding potential of 0 mV, current pulses of -20 mV and 4 ms duration were given once every second. Current was recorded at 200 kHz for 1 ms at the beginning of each pulse via a Labsys A/D-D/A converter, driven by the program DAQSYS (SICMU, Geneva). Data were stored and analysed off-line with a single exponential fit performed by the program Matlab (The MathWorks, Natick, MA). Membrane capacitance (C_m), membrane conductance (G_m) and access resistance (R_a) were calculated from the exponential fit. For further analysis and plotting, data were transferred into Origin (Microcal, Northampton, MA).

Calcium buffers

HEDTA or citrate-based calcium buffers were prepared according to calculations performed with the program MaxChelator version 6.7 (Chris Patton, Stanford, CA). Pipette solutions with variable concentrations of free Ca^{2+} were prepared with standard IS plus mixtures of 5 mM HEDTA or 10 mM citrate plus CaCl_2 .

Calcium measurements

For measurements of $[\text{Ca}^{2+}]_c$ in the micromolar range, 10 μM of the new low-affinity ($K_d = 27 \mu\text{M}$) Ca^{2+} indicator indo-1FF (TEFLABS, Austin, TX) was included in the pipette solution. Indo-1FF is a derivative of indo-1 and can be measured at the same wavelengths (excitation 360 nm). To eliminate most of the fluorescence from the pipette, the fluorescence recording was restricted to the cell by a pinhole in the emission light path. Fluorescence emission was split with a 455 nm dichroic mirror and filtered at 405 nm (F_1) and 480 nm (F_2). The intensity at each emission wavelength was recorded in parallel at 10 Hz with two photomultipliers (Nikon P100s and P101) and an ACQUI A/D converter (SICMU, Geneva). Background fluorescence of the cell and the tip of the pipette was measured in the cell-attached configuration and subtracted from the fluorescence traces before the ratio F_1/F_2 was calculated. Free Ca^{2+} concentrations were calculated from the indo-1FF fluorescence (F) according to the formula of Grynkiewicz *et al.* (1985): $[\text{Ca}^{2+}]_c = K_d \times \beta \times (R - R_{\min}) / (R_{\max} - R)$. $R_{\min}$ (0.152) and $R_{\max}$ (0.967) were determined from patch-perfusion experiments with 10 mM EGTA or 5 mM CaCl_2 in the pipette solution (Nüße and Lindau, 1990). The factor $K_d \times \beta$ (134.4) was calculated with the mean plateau values of F_1/F_2 obtained with 30, 60 and 100 μM Ca^{2+} in the pipette.

Myeloperoxidase staining and analysis

Primary neutrophil granules were stained for their contents of MPO with the method of Kaplow (1965). At the end of the patch-clamp recording, cells were fixed for 5 min *in situ* by adding an equal volume of 4% paraformaldehyde to the bath. The cells were washed with 0.9% NaCl and then stained for 90 s with the benzidine hydrochloride-based Kaplow stain. The staining solution was washed away prior to photography. Photographs (slides) were taken at high magnification (100 $\times$) before and after staining. The slides were analysed by two-dimensional laser densitometry (Model 300A, Molecular Dynamics). The background of the slide was determined in an area without cells. The absorbance of all pixels on a cell above background (usually ~ 1000 pixels) was integrated, and the average background of an unstained cell was subtracted. The method probably underestimated the MPO content of fully stained cells because the absorbance saturated. The absolute values of integrated absorbance showed cell to cell variability and have to be considered with caution. In contrast, the relative changes between the experimental conditions correlated with the capacitance recordings (not shown). Normalization of the absorbance of the patched cell to the absorbance of the surrounding unpatched cells on the same slide gave the same results as those shown in Figure 4E.

Data presentation and analysis

The moment when the patch is broken and the whole cell configuration is obtained is set as time 0 (break-in). For clarity, the initial capacitance (range 2–5 pF, mean 3.08 ± 0.09 pF, $n = 141$) of each cell has been subtracted. Capacitance data were fitted with a double logistic equation when two phases of secretion were visible:

$$\Delta C_m = A1/[1 + (\text{EC}_{50} - 1/x)^{n1}] + A2/[1 + (\text{EC}_{50} - 2/x)^{n2}]$$

where x was either $[Ca^{2+}]_c$ or $[Ca^{2+}]_{pip}$. A1 and A2 were the amplitudes of phase 1 and 2, respectively, and n_1 and n_2 were the slope factors (Hill coefficient). A simple logistic equation was used to fit data showing only one phase of secretion. Traces were smoothed by adjacent averaging. All experiments were performed at least in triplicate. Data are shown as representative traces or as mean $\pm$ SEM.

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