

Altered Calcium-Induced Exocytosis in Neutrophils from Allergic Patients

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Key Words

Calcium · Exocytosis · GTPγS · Membrane capacitance · Neutrophils

Abstract

We have investigated the exocytotic characteristics of neutrophils from allergic patients and healthy volunteers employing the whole cell membrane capacitance (Cm) measurement. The mean serum IgE level from allergic patients (423.75 ± 12.75 IU/ml) determined by chemiluminescence immunoassay was much higher than that of healthy volunteers (28.47 ± 16.68 IU/ml). Intracellular dialysis of buffered Ca^{2+} and GTPγS triggered biphasic exocytosis. The total capacitance increment displayed a steep dependence on pipette free Ca^{2+} concentration ($[\text{Ca}^{2+}]_p$), with maximal stimulation achieved at $10 \mu\text{M}$. A significant decrease in the total capacitance increment was observed in the allergic group at $[\text{Ca}^{2+}]_p > 10 \mu\text{M}$. Moreover, at submaximal stimulatory $[\text{Ca}^{2+}]_p$ of $1 \mu\text{M}$, the maximal rate of exocytosis in allergic patients ($V_{\max} = 20.75 \pm 6.19$ fF/s) was much faster than that of the healthy control group ($V_{\max} = 7.97 \pm 2.49$ fF/s). On the other hand, the Ca^{2+} -independent exocytosis stimulated by GTPγS displayed no significant difference in either the total membrane capacitance increments or the maximal

rate of exocytosis. The results suggest that hypersecretion of neutrophils in allergic diseases may involve the development of abnormal Ca^{2+} -dependent exocytosis.

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Introduction

Neutrophils (polymorphonuclear leukocytes) play an important role in the innate immune response. They have the powerful ability of phagocytosis and evacuate microbial infection through various proteases such as peroxidase and gelatinase. The proteases are sorted into four types of granules: the primary, secondary, tertiary granules and the secretory vesicles. Different granules are believed to have distinct physiological functions and their activation may be under differential control [1]. Recently, several investigations have found that neutrophils not only play a major role in host defense against infection and inflammation, they are also involved in the pathophysiology of allergic diseases such as allergic asthma [2]. Evidence is derived from the following facts: (1) In most allergic diseases, especially chronic severe asthma, neutrophil infiltration can be found in the tissue and organ. During the development of irreversible airway obstruction in chronic asthma, neutrophils may participate in the patho-

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physiology of this airway obstruction [3]. (2) Neutrophils can hypersecrete neutrophil chemokines, such as IL-17, to induce airway inflammation [4]. (3) Neutrophils express IgE receptors and different chemokine receptors. Binding to their ligands induces exocytosis and results in allergic inflammation [5]. (4) Airway epithelial cells are chronically activated and secrete neutrophil chemoattractants, such as IL-8, and hence heighten the sensitivity to environmental pollutants, such as diesel exhaust particles [6]. Phagocytosis and exocytosis are two important functions accomplished by neutrophils. In the regulatory mechanism of neutrophil exocytosis, Ca^{2+} and $\text{GTP}\gamma\text{S}$ are the most important stimuli. Ca^{2+} as the primary regulator was widely accepted. It can interact with specific high-affinity Ca^{2+} -binding proteins, such as calmodulin, synaptotagmin, syncolin and SNAP-23, to regulate neutrophil degranulation [7]. $\text{GTP}\gamma\text{S}$ can activate GTPase, such as Rho, Rac and Cdc42, to control the assembly and disassembly of the cytoskeleton, which regulates neutrophil exocytosis [8, 9].

To investigate the mechanism underlying the hypersecretion of neutrophils in allergic diseases, we have employed membrane capacitance measurement to study the characteristics of exocytosis in neutrophils and their Ca^{2+} dependence. Ca^{2+} - and $\text{GTP}\gamma\text{S}$ -stimulated exocytosis was compared between allergic patients and healthy volunteers. Our data imply that allergic disease might involve the development of the abnormal Ca^{2+} -dependent degranulation from neutrophils.

Patients and Methods

Subjects

Experimental Group. Eighteen subjects (11 females and 7 males, aged 13–42 years; mean: 32 ± 4.6 years) from the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, volunteered for this study. All patients with allergic rhinitis or asthma included in the study fulfilled the following criteria: (1) they showed rhinitis symptoms for at least 2 weeks, nasal congestion, sneezing, rhinorrhea and itchy nose, for example, and a total symptom score >6 ; (2) a history of allergic rhinitis during the preceding 2 years; (3) at least one common airborne allergen positive during skin prick testing; (4) a family history of inherited allergic disease, and (5) total serum IgE levels >120 IU/ml. The study also included 23 healthy volunteers (13 females and 10 males). They were 13–39 years old (mean: 31 ± 2.3) and had no previous history of allergic diseases. Their total serum IgE levels were <120 IU/ml. Informed consent and ethical commission approval were obtained from all the patients and the healthy volunteers.

Cell Preparation

Human neutrophils were isolated from 5 ml heparin-anticoagulated fresh blood obtained by venipuncture [10, 11]. The suspension

was centrifuged at 200 g for 10 min (4°C) after which the platelet-rich plasma was discarded. The leukocyte-rich layer was suspended in 5-ml aliquots of Ca^{2+} / Mg^{2+} -free Hanks' balanced salt solution (HBSS) and then carefully layered onto 5-ml aliquots of Ficoll-Hypaque (lymphocyte separation medium) with a density of 1.077 g/ml and centrifuged at 400 g (20 min, 4°C). The granulocyte pellet was resuspended in Ca^{2+} - and Mg^{2+} -free HBSS and washed by centrifugation at 200 g (5 min, 4°C). The residual erythrocytes were lysed by resuspending the cell pellet in 0.2% (w/v) sodium chloride, shaking for approximately 10 s and then adding an equal volume of 1.6% (w/v) sodium chloride. After centrifugation at 200 g (5 min, 4°C), the granulocytes were washed twice in Ca^{2+} - and Mg^{2+} -free HBSS. Finally, the purity of the obtained neutrophils was above 95%. The cell suspension was stored at 4°C until use, usually within 6 h. Cells were settled onto a glass coverslip precoated with polylysine (Gibco) for 25 min at room temperature. Subsequently, adherent cells were kept in Medium 199 (Gibco) and thoroughly rinsed with standard physiological external solution before electrical recordings.

Solution

The extracellular solution (ES) contained (in mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 Hepes and 5 glucose (pH 7.2, 318 mosm). In some experiments, 5 $\mu\text{g}/\text{ml}$ cytochalasin B was added to the ES to pretreat the cells. The standard pipette solution, internal solution (IS), contained (in mM): 125 K-glutamate, 10 NaCl, 2 MgATP and 10 Hepes (pH 7.2, 310 mosm). Pipette solutions with different Ca^{2+} concentrations were made from the standard IS by adding 10 mM HEDTA ($K_d = 3.96 \mu\text{M}$ for Ca^{2+} at pH 7.2) and the proper concentration of calcium chloride calculated by the Winmax program. To measure $[\text{Ca}^{2+}]_p$ in the micromolar range, 10 μM of the low-affinity Ca^{2+} indicator fura-2 were added to the pipette solution. $[\text{Ca}^{2+}]_p$ was determined from the ratio (R) of the fluorescence signals excited at two wavelengths (340 and 380 nm), following the equation: $[\text{Ca}^{2+}]_i = K_{\text{eff}} \cdot (R - R_{\text{min}}) / (R_{\text{max}} - R)$, where K_{eff} , R_{min} , and R_{max} are constants obtained from *in vitro* calibration. The IS with $\text{GTP}\gamma\text{S}$ was prepared from the standard IS by adding 1 mM EGTA together with 20 or 100 μM $\text{GTP}\gamma\text{S}$. All the above-mentioned reagents were purchased from Sigma.

Capacitance Measurement

The conventional whole-cell patch-clamp technique was used to measure the membrane capacitance change induced by intracellular $\text{GTP}\gamma\text{S}$ or Ca^{2+} introduced through patch pipette dialysis. The patch pipettes were made from borosilicate glass and were pulled in two stages using a PP-83 vertical puller (Narishige, Japan). The pipettes were heat-polished and had tip resistances in the range of 4–6 M Ω when filled with IS solution and measured in standard external solution. An Ag-AgCl wire connected to the bath was used as reference electrode. In our experiment, an EPC-9 patch-clamp amplifier was used together with Pulse+Pulsefit 8.0 software (HEKA Electronics, Germany). For capacitance measurements, we employed the Lindau-Neher technique implemented under the sine + DC mode, which is controlled by the lock-in extension of the Pulse software. A 1,000-Hz and ± 20 -mV sinusoid voltage stimulation was superimposed on a holding potential of -60 mV. The capacitance traces were imported into IGOR Pro (WaveMetrics) for further analysis. Only recordings that fulfilled the following criteria were included in the analysis: (1) membrane conductance (G_m) < 1 nS and minimum series conductance (G_s) > 50 nS; (2) the recording duration was longer than 300 s when stimulated with Ca^{2+} and 500 s with $\text{GTP}\gamma\text{S}$; (3) the changes in

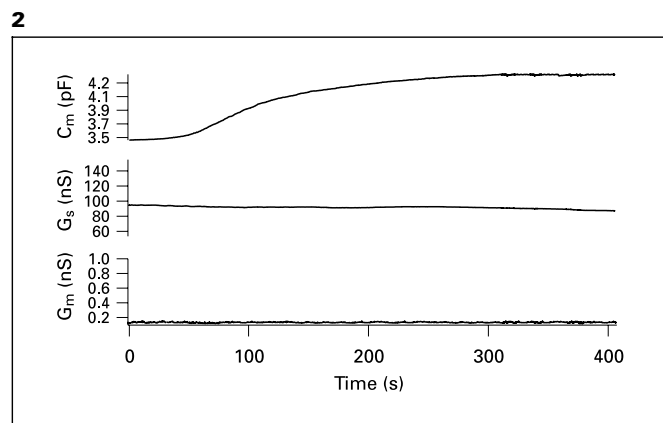
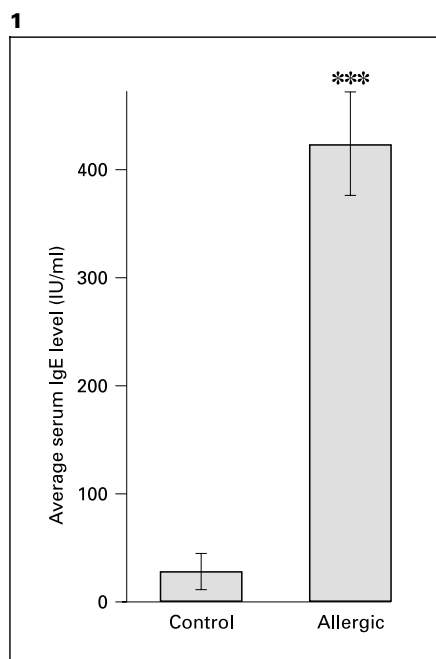


Fig. 1. Averaged serum IgE level in healthy volunteers (28.46 ± 16.68 IU/ml, $n = 23$) and allergic patients (423.75 ± 12.75 IU/ml, $n = 18$). *** $p < 0.001$.

Fig. 2. Time courses of C_m , G_m and G_s of a human neutrophil stimulated with pipette solution containing $1 \mu M$ Ca^{2+} . The exocytosis of the cell results in a total C_m increment ($\Delta C_{m,T}$) of 0.79 pF from its initial capacitance of 3.40 pF to its final capacitance of 4.19 pF.

C_m did not accompany concurrent changes in G_s or G_m . Statistical analysis with a probability value < 0.05 was considered statistically significant. Data were presented as means $\pm$ SEM.

Results

Comparison of Serum IgE Levels

Neutrophils have been suggested to be involved in the pathophysiology of allergic diseases such as allergic asthma [2]. It has been suggested that neutrophils can hypersecrete neutrophil chemokines, such as IL-17, to induce airway inflammation [4]. We have chosen patients with allergic rhinitis or asthma for this study to understand the mechanism of neutrophil hypersecretion. Type I hypersensitivity is primarily conducted by specific IgE. Neutrophils express IgE receptors and different chemokine receptors. Binding of IgE to its high-affinity surface receptor results in exocytosis and allergic inflammation. Because of the important role of IgE in type I hypersensitivity, we compared the serum IgE levels between allergic patients and healthy volunteers. The total serum IgE levels were detected by ACS180 (USA) and summarized in figure 1. The mean serum IgE levels were 423.75 ± 12.75 ($n = 18$) and 28.47 ± 16.68 IU/ml ($n = 23$) for the patient and control groups, respectively ($p < 0.001$).

Ca^{2+} -Stimulated Exocytosis in Neutrophils

Previous studies of exocytosis in neutrophils indicate that the final trigger to exocytosis involves Ca^{2+} and Ca^{2+} -binding proteins. It has been suggested that specific elements of secretion in individual neutrophils might involve different types of granules with different affinity for Ca^{2+} . The marked differences in Ca^{2+} sensitivity between granule populations 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 [12]. However, the difference in Ca^{2+} -induced exocytosis in neutrophils between allergic patients and healthy individuals remains unexplored. In this study, we have employed whole-cell C_m measurements to assess exocytosis. Figure 2 displays the time course of C_m , G_s and G_m recorded from a single cell that was dialyzed with a micro-pipette solution containing $1 \mu M$ $[Ca^{2+}]_p$. The cells in the recording chamber were perfused with standard ES solution. We could observe that C_m started to increase after a short lag phase usually of 50–100 s. After the delay, C_m increased from an initial value of 3.40 pF to a final value of 4.19 pF ($\Delta C_m = 0.79$) within 300 s. The C_m increase usually started with a faster phase followed by a slower phase, suggesting the existence of different granule state or different types of granules. Next we checked the Ca^{2+} dependence of exocytosis by dialyzing different levels of $[Ca^{2+}]_p$. The total capacitance increment displayed a steep

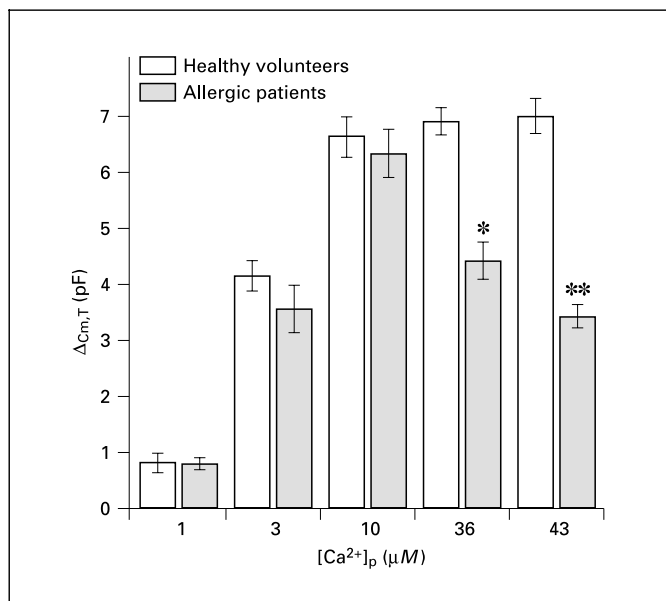


Fig. 3. Ca^{2+} dependence of the total capacitance increments from neutrophils. Averaged total capacitance increments ($\Delta C_{m,T}$) exhibit steep dependence on the pipette Ca^{2+} concentration ($[Ca^{2+}]_p$) in the allergic patients. A significant decrease in $\Delta C_{m,T}$ was evident in the allergic group at $[Ca^{2+}]_p > 10 \mu M$. At 1, 3, 10, 36 and 43 μM $[Ca^{2+}]_p$, the averaged $\Delta C_{m,T}$ are 0.81 ± 0.17 ($n = 23$), 4.16 ± 0.25 ($n = 17$), 6.63 ± 0.36 ($n = 23$), 6.92 ± 0.24 ($n = 11$) and 7.01 ± 0.31 pF ($n = 14$) for the healthy group, and 0.79 ± 0.11 ($n = 13$), 3.57 ± 0.37 ($n = 21$), 6.34 ± 0.42 ($n = 12$), 4.43 ± 0.33 ($n = 17$) and 3.42 ± 0.21 pF ($n = 11$) for the allergic group. * $p < 0.05$, ** $p < 0.01$, respectively.

dependence on $[Ca^{2+}]_p$, with maximal stimulation achieved at 10 μM (fig. 3). When we compared the total capacitance increment between allergic patients and healthy volunteers at various levels of $[Ca^{2+}]_p$, we found that while no obvious difference was found at lower $[Ca^{2+}]_p$ ($\leq 10 \mu M$), a significant decrease in the total capacitance increment was evident in the allergic group (fig. 3). Next, we compared the maximal rate of exocytosis at various $[Ca^{2+}]_p$ (fig. 4). Although no difference was found at higher $[Ca^{2+}]_p$ levels, the maximal rate of exocytosis in the allergic group ($V_{max} = 20.75 \pm 6.19$ fF/s) was much faster than that of the healthy control group ($V_{max} = 7.97 \pm 2.49$ fF/s) when stimulated at 1 μM $[Ca^{2+}]_p$.

Cytochalasin B Enhanced Exocytosis in Neutrophils in Both Groups

Several investigations have reported that cytochalasin B can markedly accelerate neutrophil exocytosis from healthy individuals [13]. We tried to determine whether the already accelerated exocytosis in neutrophils from

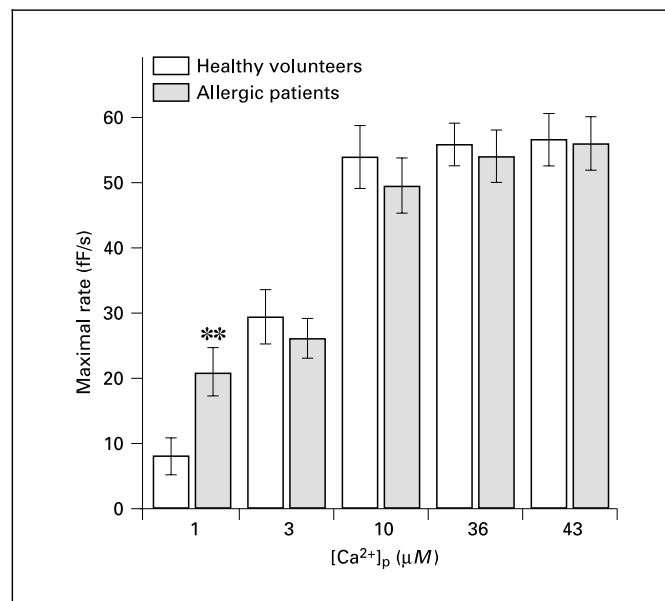


Fig. 4. Allergic patients have a higher rate of exocytosis at low Ca^{2+} concentration. The C_m trace was derived to obtain the maximal rate of exocytosis. The maximal rates of exocytosis at 1 μM $[Ca^{2+}]_p$ are faster in the allergic patients than in the healthy control group ($p < 0.01$). At higher $[Ca^{2+}]_p$, however, no significant difference in the maximal rates of exocytosis was found. The numbers of cells used for statistical analysis under each condition are from 12 to 23.

allergic patients can still be potentiated by cytochalasin B. Figure 5a compared two exemplary C_m traces from allergic patients in the absence and presence of 5 $\mu g/ml$ cytochalasin B. As shown in figure 5a, exocytosis in neutrophils from allergic patients stimulated at 3 μM $[Ca^{2+}]_p$ is markedly enhanced by pretreatment of cytochalasin B. Further analysis suggests that cytochalasin B not only accelerates the total C_m increment, but also quickens the maximal rate of exocytosis (fig. 5b, c). In addition, cytochalasin B affected neutrophil secretion from the healthy individuals to a similar extent (data not shown). These results indicate that cytochalasin B induced a similar mechanism to regulate neutrophil exocytosis in the allergic patients and healthy individuals.

GTP γ S-Stimulated Exocytosis in Neutrophils

Finally, we compared GTP γ S-dependent exocytosis between allergic patients and healthy individuals. GTP γ S can continuously activate the members of the mammalian Rho GTPase family that regulate the organization of the actin cytoskeleton. Figure 6a shows the time course of C_m , G_s and G_m recorded from a control neutrophil dialyzed

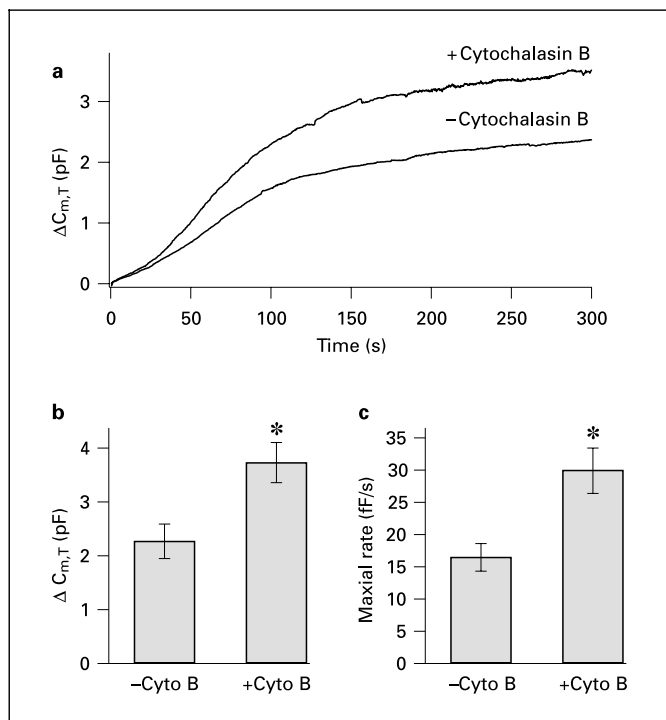


Fig. 5. Cytochalasin B enhanced exocytosis in neutrophils from the allergic patients. **a** Time course of the mean C_m with and without 5 $\mu\text{g/ml}$ cytochalasin B at 3 μM $[\text{Ca}^{2+}]_p$. **b** Comparison of the mean $C_{m,T}$ increase in the absence (2.27 ± 0.32 pF, $n = 11$) and presence of 5 $\mu\text{g/ml}$ cytochalasin B (3.73 ± 0.36 pF, $n = 9$) at 3 μM $[\text{Ca}^{2+}]_p$ ($p < 0.05$). **c** Comparison of the maximal rate of exocytosis in the absence (16.50 ± 2.13 fF/s, $n = 11$) and presence of 5 $\mu\text{g/ml}$ cytochalasin B (29.97 ± 3.32 fF/s, $n = 9$) at 3 μM $[\text{Ca}^{2+}]_p$ ($p < 0.05$).

with the pipette solution containing 20 μM GTP γ S and 1 mM EGTA. The cell was pretreated with cytochalasin B (5 $\mu\text{g/ml}$) to shorten the onset and to accelerate the degranulation process. Neutrophils from healthy individuals and allergic patients were stimulated with 20 μM GTP γ S and compared for their total C_m increment and maximal rate of exocytosis. As summarized in figure 6b and 6c, no statistical significance was observed in the total C_m increment and the maximal rate of exocytosis between the allergic and control groups.

Discussion

This study covers Ca^{2+} - and GTP γ S-induced exocytosis in neutrophils from allergic patients and healthy volunteers. Our results demonstrate that the Ca^{2+} -dependent exocytosis from neutrophils might be differentially modu-

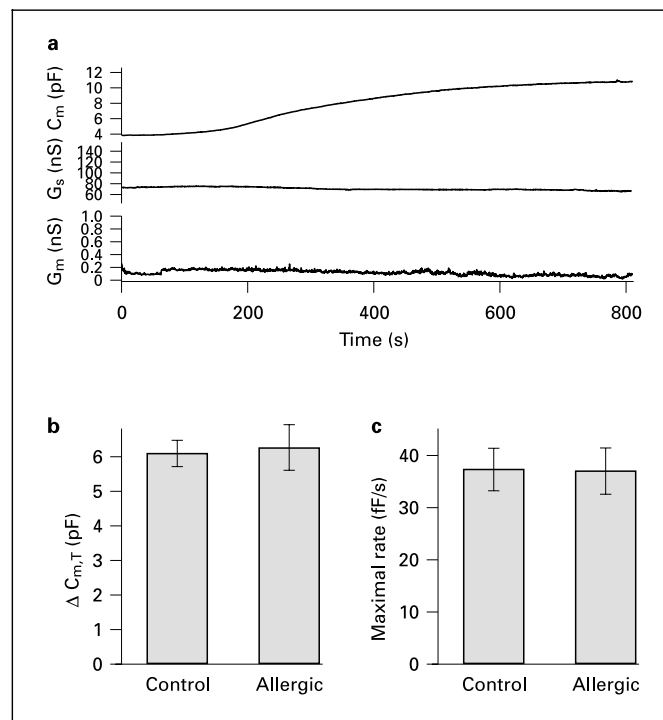


Fig. 6. No alteration in the GTP γ S-triggered exocytosis in neutrophils from allergic patients. **a** Time course of C_m , G_m and G_s of a human neutrophil from a healthy individual stimulated with 20 μM GTP γ S. The cell was pretreated with 5 $\mu\text{g/ml}$ cytochalasin B to accelerate the exocytotic processes. Please note that the GTP γ S-triggered C_m increase proceeds much slower compared with the Ca^{2+} -triggered exocytosis. **b**, **c** Comparison of the averaged total capacitance increments (**b**) and the maximal rate of exocytosis (**c**) induced by 20 μM GTP γ S between the control and the allergic groups. No significant difference was found. The numbers of cells used for statistical analysis are 11 and 15 for control and allergic patients, respectively.

lated at different Ca^{2+} concentrations in allergic diseases, whereas GTP γ S-stimulated exocytosis remains unaltered.

Altered Ca^{2+} -Dependent Exocytosis in Neutrophils from Allergic Patients

Neutrophil granulocytes contain primary, secondary and tertiary granules and secretory vesicles. There is a total number of $\sim 1,300$ primary granules and $\sim 4,600$ secondary and tertiary granules per cell, corresponding to a total membrane capacitance of ~ 3.6 pF for primary granules and about 2.4 pF for secondary and tertiary granules [12, 14]. In the whole cell configuration, the composition of the cytoplasm is tightly controlled by the pipette solution. We have observed a maximum C_m increment of ~ 6.5 pF, which correlates well with the total granule pop-

ulations in neutrophils. The maximum C_m increment was achieved at $10 \mu M [Ca^{2+}]_p$, a Ca^{2+} level similar to that reported in a previous study [12]. However, the increment in C_m was much larger at $10 \mu M [Ca^{2+}]_p$ compared to the previous report [12]. The reason for this discrepancy is unclear. It may be due to different subjects studied and the difference in the preparation of neutrophils. At $[Ca^{2+}]_p < 10 \mu M$, we have found that both the total C_m increment and the exocytotic rate increased with the pipette calcium concentration. Stimulation at $1 \mu M [Ca^{2+}]_p$ caused a total ΔC_m of only 0.7–0.8 pF. It remains puzzling that sustained exposure to a submaximal Ca^{2+} concentration stimulated submaximal secretion in neutrophils, as in a simple process of Ca^{2+} -dependent exocytosis, no incomplete exocytosis should occur. This graded response might reflect a mechanism of adaptation to various stimulations, as suggested previously [12, 15]. A comparison of the total C_m increment in neutrophils under different Ca^{2+} concentrations between healthy volunteers and allergic patients revealed no obvious difference at $[Ca^{2+}]_p < 10 \mu M$. However, a significantly decreased total C_m increment was noted at higher $[Ca^{2+}]_p (> 10 \mu M)$ in the allergic group (fig. 3). It has been suggested that low Ca^{2+} concentrations might specifically induce the release of the secondary and tertiary granules, whereas high Ca^{2+} concentrations release the primary granules. Thus, our results might suggest that while the total number of the secondary and tertiary granules remains unaltered, the number of the primary granules is decreased in allergic patients, perhaps due to a depletion of primary granules in hypersecreted neutrophils from allergic patients. Moreover, when we compared the maximal rate of exocytosis between both groups, a more than twofold increase in the maximal rate of exocytosis was found at $1 \mu M$ but not at higher $[Ca^{2+}]_p$. Hence, in contrast to the primary granules, the Ca^{2+} sensitivity of the secondary and tertiary granules might be sensitized in allergic patients. However, it could also be possible that the effect of sensitization of the secretory granules in neutrophils can only be observed at lower Ca^{2+} concentrations, as higher Ca^{2+} tends to maximize the stimulation. The Ca^{2+} sensors responsible for exocytosis in neutrophils have remained elusive. Recently, synaptotagmin II but not I has been found in human neutrophils [16], but its function in exocytosis is not clear. Indeed, there must exist multiple Ca^{2+} sensors in neutrophils to account for the different populations of granules with different affinity for Ca^{2+} . Thus, other isoforms of synaptotagmin, or other calcium-sensitive proteins, e.g. calcium-dependent activator protein for secretion and annexin [17–19], might also serve as calcium sensors involved in

the late stages of exocytosis. Further experiments to investigate the modulation of these proteins under allergic diseases might help to understand the underlying mechanism of hypersensitivity in neutrophils.

GTP γ S-Induced Exocytosis Is Not Altered in Neutrophils from Allergic Patients

It is reported that GTP γ S could induce neutrophils to degranulate completely via continuous activation of some members of the mammalian Rho GTPase family, such as Rho, Rac2 and Cdc42 [20]. Many studies showed that activated Rho, Rac2 and Cdc42 GTPase could regulate the organization of the cytoskeleton, e.g. by directly regulating the assembly and disassembly of the microtubules. The cytoskeleton has been ascribed an important role in vesicle transportation and exocytosis [21]. After pretreatment with $5 \mu g/ml$ cytochalasin B, no difference in either the total C_m increment or the maximal rate of exocytosis in neutrophils was found in allergic patients. Thus, the signaling pathways of GTP γ S seem not to be involved in the hypersecretion of neutrophils under allergic diseases.

Acknowledgments

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