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Combinatorial SNARE Complexes Modulate the Secretion of Cytoplasmic Granules in Human Neutrophils

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Mobilization of human neutrophil granules is critical for the innate immune response against infection and for the outburst of inflammation. Human neutrophil-specific and tertiary granules are readily exocytosed upon cell activation, whereas azurophilic granules are mainly mobilized to the phagosome. These cytoplasmic granules appear to be under differential secretory control. In this study, we show that combinatorial soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes with vesicle-associated membrane proteins (VAMPs), 23-kDa synaptosome-associated protein (SNAP-23), and syntaxin 4 underlie the differential mobilization of granules in human neutrophils. Specific and tertiary granules contained VAMP-1, VAMP-2, and SNAP-23, whereas the azurophilic granule membranes were enriched in VAMP-1 and VAMP-7. Ultrastructural, coimmunoprecipitation, and functional assays showed that SNARE complexes containing VAMP-1, VAMP-2, and SNAP-23 mediated the rapid exocytosis of specific/tertiary granules, whereas VAMP-1 and VAMP-7 mainly regulated the secretion of azurophilic granules. Plasma membrane syntaxin 4 acted as a general target SNARE for the secretion of the distinct granule populations. These data indicate that at least two SNARE complexes, made up of syntaxin 4/SNAP-23/VAMP-1 and syntaxin 4/SNAP-23/VAMP-2, are involved in the exocytosis of specific and tertiary granules, whereas interactions between syntaxin 4 and VAMP-1/VAMP-7 are involved in the exocytosis of azurophilic granules. Our data indicate that quantitative and qualitative differences in SNARE complex formation lead to the differential mobilization of the distinct cytoplasmic granule populations in human neutrophils, and a higher capability to form diverse SNARE complexes renders specific/tertiary granules prone to exocytosis.

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The ability of intracellular secretory organelles to specifically recognize appropriate acceptor membrane targets underlies the organization of the exocytic pathway. Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are key mediators of membrane fusion (15). The neuronal SNARE proteins synaptobrevin/vesicle-associated membrane protein (VAMP)-2, syntaxin 1A, and 25-kDa synaptosome-associated protein (SNAP-25) share a homologous SNARE motif of ~60 aa (16–18), which mediates the association of SNARE proteins into a core complex composed of a tightly packed parallel four-helical bundle. VAMP-2 and syntaxin 1A contribute one α-helix each and SNAP-25 contributes two α-helices. All known SNARE motifs fall into two major subfamilies that contain either a conserved glutamine (Q) or arginine (R) at the ionic “0” layer in the middle of the bundle, leading to the classification of SNARE proteins into Q-SNARE and R-SNARE. The hydrophobic 0 layer of the neuronal SNARE complex is made of three glutamine residues from the Q-SNARE motifs (one contributed by syntaxin 1A and two by SNAP-25) and one arginine residue from the R-SNARE motif (contributed by VAMP-2) (18). The crystal structure of the endosomal SNARE complex has revealed that it is also formed by a four-helix bundle containing three Q-SNARE contributors (syntaxin 7, syntaxin 8, vti1b) and one R-SNARE (endobrevin/VAMP-8) contribution (19). This finding reveals common structural principles of SNARE core complexes consisting of four-helical bundles of the three (Q-SNARE)/one (R-SNARE) type.

A number of SNARE proteins have been identified in human neutrophils (20–26), and some of them have been recently involved in the secretion of specific and tertiary granules (24, 26). However, our knowledge on neutrophil exocytosis is far from complete. Regulation of azurophilic granule secretion is largely unknown, and a long-standing question lies on how distinct neutrophil granules are differentially mobilized upon cell activation. In this study, we show the involvement of different SNARE proteins as well as the formation of distinct SNARE complexes in the exocytosis of the major neutrophil cytoplasmic granules.

Materials and Methods

Antibodies

Specific rabbit anti-VAMP-1 Ab (Synaptic Systems) was raised against a synthetic peptide corresponding to residues 2–14 of human VAMP-1, a key mediator of membrane fusion (15). The generated cDNA was amplified by using TaqDNA polymerase (ECOGEN) and primers for human vamp-1 (5’-CCCTCTCTTAACTAGCACA-3’ and 5’-CTACACGATGATGGCAGAC-3’); vamp-2 (5’-ATGTCGCTTACCGCGTCCAC-3’ and 5’-TAAAGACTAAGTAAACTA-3’); vamp-3 (5’-ATGTCAGACCCTAAGTAAACTA-3’ and 5’-TCATGAGCTGATGGCAGAC-3’); vamp-5 (5’-ATGTCAGACCCTAAGTAAACTA-3’); vamp-7 (5’-ATGTCAGACCCTAAGTAAACTA-3’); and vamp-8 (5’-ATGTCAGACCCTAAGTAAACTA-3’). The expression vector containing the inserts was sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The detected antibodies were VAMP-1, -2, -3, -5, -7, and -8. The specificity of the antibodies was confirmed by Western blot analysis.

Cell culture

Human leukemia HL-60 cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, l-glutamine, and antibiotics. Neutrophil differentiation of HL-60 cells was induced with 1.3% (v/v) DMSO as described previously (26).

Neutrophil isolation and activation

Neutrophils were obtained from human peripheral blood by dextran sedimentation and Ficoll-Hypaque centrifugation as described previously (23). Freshly isolated human neutrophils were resuspended at 3–5 × 10^6 cells/ml in HEPES/glucose buffer (150 mM NaCl, 10 mM HEPES, 5 mM KCl, 1.2 mM MgCl₂, 1.3 mM CaCl₂, 5.5 mM glucose, (pH 7.5)). For cell activation assays, neutrophils were incubated with 100 ng/ml PMA for 10 min at 37°C or preincubated with 5 µg/ml cytochalasin B for 5 min at 37°C, and then stimulated with 10^{-7} M FMLP for 10 min at 37°C. Release of gelatinase, lactoferrin, β-glucuronidase, and peroxidase following neutrophil activation was determined as described elsewhere (6, 11, 31).

Subcellular fractionation

Resting neutrophils were resuspended in 50 mM Tris-HCl (pH 7.5), containing 2 mM PMSF, disrupted by repeated freeze-thaw, and soluble and membrane fractions from postnuclear extracts were prepared as described previously (24).

To prepare the distinct subcellular fractions, freshly isolated resting and PMA-activated neutrophils (~3–5 × 10^6) were gently disrupted and the postnuclear fraction was layered onto a 27-ml, 15–40% (w/w) continuous sucrose gradient, with a 1-ml cushion of 60% (w/w) sucrose, and centrifuged at 25,000 rpm in a Beckman L8-70B ultracentrifuge using a SW27 rotor (24). Subcellular fractions were assayed for marker proteins, namely lactate dehydrogenase (cytosol), HLA (plasma membrane), gelatinase (tertiary granules), lactoferrin (specific granules), and peroxidase (azurophilic granules) as described (26). The membranes from each fraction were obtained as described (26).

RT-PCR

Total RNA (10 µg) was reverse-transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega). The generated cDNA was amplified by using TaqDNA polymerase (ECOGEN) and primers for human vamp-1 (5’-CCCTCTCTTAACTAGCACA-3’ and 5’-CTACACGATGATGGCAGAC-3’); vamp-2 (5’-ATGTCGCTTACCGCGTCCAC-3’ and 5’-TAAAGACTAAGTAAACTA-3’); vamp-3 (5’-ATGTCAGACCCTAAGTAAACTA-3’ and 5’-TCATGAGCTGATGGCAGAC-3’); vamp-5 (5’-ATGTCAGACCCTAAGTAAACTA-3’); vamp-7 (5’-ATGTCAGACCCTAAGTAAACTA-3’); and vamp-8 (5’-ATGTCAGACCCTAAGTAAACTA-3’). The PCR products were 263 for vamp-1, 426 for vamp-2, 304 for vamp-3, 27 cycles for vamp-4, 25 cycles for vamp-5, 27 cycles for vamp-6, 30 cycles for vamp-7, and 30 cycles for vamp-8. The PCR products were visualized by agarose gel electrophoresis.
\textbf{Coimmunoprecipitation}

A total of $5 \times 10^6$ cells was lysed with 200 \mu l of lysis buffer (20 mM Tris-HCl, 100 mM KCl, 0.9\% Triton X-100, 10\% glycerol, 2 mM orthovanadate, and 2 mM PMSEF). Lysates were precleared with 500 \mu l of protein A-Sepharose at $4^\circ C$ for 2 h and immunoprecipitated by incubation with Abs against the indicated SNARE proteins precoupled to protein A-Sepharose for 2 h at $4^\circ C$. After extensive washing with lysis buffer, the precipitates were subjected to SDS-PAGE and Western blot analysis. P3X63 was used as a negative control for immunoprecipitation, and no signal was detected.

\textbf{Tetanus toxin (TeTx) treatment}

Recombinant toxigenic L chain TeTx, provided by Dr. J. Blasi (Universidad de Barcelona, L’Hospital de Llobregat, Barcelona, Spain), was preactivated by incubation with 10 mM DTT at $37^\circ C$ for 1 h. Neutrophil extracts or membranes (90 \mu g protein) were incubated in the presence of 0.5\% Triton X-100 for 1 h at $37^\circ C$ with or without 400 nM preactivated TeTx.

\textbf{Electropheromobilization and immunofluorescence flow cytometry}

Neutrophils were electropheromobilized by two discharges of 5 kV/cm, 25 \mu F, and 72 Ohm using a BTX electroporator (Biotechnologies & Experimental Research) as described previously (24, 32, 33). Electropheromobilized neutrophils were incubated for 5 min with different concentrations of Abs or with 400 nM TeTx for 30 min, and then incubated for 5 min with 5 \mu g/ml cytochalasin B at $37^\circ C$, followed by stimulation with 1 \mu M CaCl$_2$ (0.1 mM CaCl$_2$, 5.37 mM MgCl$_2$, 5 mM hydroethyl EDTA, 10 mM glucose) and 50 \mu M GTP-y-S for 10 min at $37^\circ C$ as described elsewhere (26). In some cases, cells were stimulated by incubation with 100 ng/ml PMA for 10 min at $37^\circ C$ without cytochalasin B pretreatment, or pretreated with cytochalasin B and then activated with 10-7 M FMLP for 10 min at $37^\circ C$. Cells were then fixed with 1\% paraformaldehyde and processed for immunofluorescence flow cytometry. Control untreated electropheromobilized cells were run in parallel. Ag cell surface expression was measured in paraformaldehyde-fixed neutrophils as described (24) using a BD Biosciences FACSCalibur flow cytometer.

\textbf{Immunoelectron microscopy}

Resting human neutrophils and exudate neutrophils from skin window chambers after phagocytosis of latex beads (34), provided by Dr. N. Borregaard (National University Hospital), were fixed and processed for ultrathin cryosectioning as described previously (26, 35). For double immunolabeling, ultrathin frozen sections were incubated with the indicated Abs followed with 10- and 15-nm protein A-conjugated colloidal gold probes (Electron Microscopy Laboratory, Utrecht University, The Netherlands) (36). After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope. Negative controls, prepared by replacing the primary Ab by a nonrelevant rabbit or mouse Ab, showed no staining.

\textbf{Results}

\textbf{VAMP expression in human neutrophils and neutrophil-differentiating HL-60 cells}

Because neutrophils contain a high number of granules and VAMPs constitute the major SNARE proteins located in vesicles (37), we first analyzed the expression of VAMP genes in human mature peripheral blood neutrophils and neutrophil-differentiating HL-60 cells by semiquantitative RT-PCR. The human HL-60 cell line can be induced to differentiate toward the neutrophil lineage, and it has been widely used as a cell culture model to study neutrophil gene expression (30). VAMP-1, VAMP-2, VAMP-3 (cellobrevin), VAMP-4, VAMP-5 (myobrevin), VAMP-7 (or tetanus neurotoxin-insensitive vesicle-associated membrane protein/TIVAMP), and VAMP-8 were expressed in both HL-60 cells and human neutrophils (Fig. 1). VAMP-1, VAMP-2, VAMP-4, and VAMP-5 mRNA levels were up-regulated following HL-60 differentiation (Fig. 1), and thus their expression was under regulatory control during neutrophil maturation. These VAMP genes were up-regulated after 1-day incubation of HL-60 cells with DMSO (Fig. 1), the time required to commit HL-60 cells toward the neutrophil lineage (38). The VAMP gene expression pattern shown in mature human neutrophils was similar to that found in DMSO-differentiated HL-60 cells (after a 5-day DMSO treatment), except for a lower expression of VAMP-8 in neutrophils (Fig. 1). Each VAMP was identified by the expected amplicon size, and by subsequent cloning and sequencing of each amplified fragment.

HL-60 cells do not form specific and tertiary granules during neutrophil differentiation (3, 39, 40), and we have previously found that VAMP-2 is mainly present in these granules in mature resting human neutrophils (26). Yet, HL-60 cells retain the ability to express some proteins normally localized in the membranes of specific and tertiary granules when driven into neutrophil maturation by retinoic acid or DMSO (3). In this regard, the expression of VAMP-2 during DMSO-induced neutrophil differentiation of HL-60 cells resembles the induced expression of CD11b Ag during HL-60 differentiation (30), even though most of this protein is located in specific and tertiary granules in mature resting human neutrophils (12). This observation suggests a different location of CD11b in differentiated HL-60 cells, namely, cell surface (30).

\textbf{TeTx-sensitive and insensitive mechanisms in neutrophil exocytosis}

TeTx specifically cleaves some VAMPs, and thereby constitutes an excellent tool to study functional aspects of these proteins (41, 42). The selective action of TeTx relies on the presence of a highly conserved QF motif in the amino acid sequence of both human VAMP-1 (GASQFESS) and human VAMP-2 (GASQFETS), but not in other VAMPs, such as VAMP-7 (TF) and rat VAMP-1 (VF) (41, 42). We have recently shown that TeTx degraded VAMP-2

\begin{figure}
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\caption{Expression of VAMP genes in neutrophil-differentiating HL-60 cells and human peripheral blood neutrophils. Total RNA was purified from human peripheral blood mature neutrophils (PMN), untreated HL-60 cells, and HL-60 cells treated with 1.3\% (v/v) DMSO for the indicated times, and subjected to semiquantitative RT-PCR analysis using specific oligonucleotide primers for each gene, under conditions shown to be at the linear phase of amplification. PCR amplification of \beta-actin was used as an internal loading control. The PCR products were electrophoresed onto a 2\% agarose gel and stained with ethidium bromide.}
\end{figure}
and inhibited secretion of specific and tertiary granules in human neutrophils (26). Now, we asked whether azurophilic granules were also regulated by TeTx-sensitive proteins. To access the neutrophil cytoplasm in whole functional neutrophils with TeTx, we prepared electropermeabilized human neutrophils and treated them with 400 nM TeTx. The viability of such permeabilized cells determined by latency of lactate dehydrogenase averaged 82.3 ± 4.6% (n = 5) for at least 50 min, which is in agreement with previous estimates (32, 43). The granules and the plasma membrane of electropermeabilized human neutrophils did not show any significant difference with intact neutrophils as assessed by electron microscopy (44), and electropermeabilized neutrophils have been shown to remain functional, retaining their responsiveness to distinct stimuli (24, 26, 32, 45). The effect of TeTx on neutrophil exocytosis in electropermeabilized neutrophils was analyzed following the up-regulation of the two granule membrane markers CD63 (azurophilic membrane marker) (46) and CD66b (specific/tertiary granule membrane marker) (26, 47) at the cell surface, which constitutes a reliable method to monitor neutrophil degranulation in electropermeabilized neutrophils (24, 33). We have previously shown that most of the intracellular CD63 and CD66b pools (>80%) were shown incorporated into the cell surface of electropermeabilized neutrophils after cell activation with Ca²⁺ and GTP-γ-S (24). TeTx practically prevented CD66b up-regulation, but only partially inhibited the CD63 up-regulation following cell activation with Ca²⁺ and GTP-γ-S (Fig. 2A). This finding suggests that exocytosis of specific/tertiary and azurophilic granules is mainly mediated by TeTx-sensitive and insensitive proteins, respectively. Azurophilic granules are lysosome-like organelles, and TeTx-insensitive VAMP-7 has been involved in

![FIGURE 2. Effect of TeTx on neutrophil exocytosis and identification, subcellular distribution and TeTx sensitivity of VAMPs in human neutrophils.](http://www.jimmunol.org/)
Thus, we next asked for the putative involvement of VAMP-7 in neutrophil exocytosis. In addition, because VAMP-1 and VAMP-2 expression was up-regulated during neutrophil differentiation of HL-60 cells, and both proteins are cleaved by TeTx (42), we further analyzed their respective roles in neutrophil exocytosis.

Previously, we and others showed the expression of TeTx-sensitive VAMP-2 in human neutrophils (20, 25, 26). Here we found that specific anti-VAMP-1 and anti-VAMP-7 mAbs recognized bands of 18 and 25 kDa, respectively, in both neutrophil extract and brain homogenate, although greater amounts of neutrophil protein were loaded to allow protein detection (Fig. 2B). These immunoreactive bands were detected in the membrane fraction of human neutrophils, but not in the soluble fraction containing the cytosol (Fig. 2C), indicating that both proteins were membrane bound. The immunoreactive 18-kDa band was extensively degraded by treatment with TeTx, whereas the immunoreactive 25-kDa band was resistant (Fig. 2D), further confirming their identity as VAMP-1 and TeTx-insensitive VAMP-7, respectively. TeTx also degraded VAMP-1 in electroporemeabilized cells (Fig. 2D), suggesting a role for this protein in TeTx-sensitive neutrophil exocytosis.

To determine the subcellular localization of VAMPs in resting human neutrophils we performed subcellular fractionation assays that resolved cytosol, plasma membrane, as well as tertiary, specific, and azurophilic granules (Fig. 2E). We found that VAMP-1 was located in the membranes prepared from subcellular fractions the mobilization of lysosomes in different systems (48–51). Thus, we next asked for the putative involvement of VAMP-7 in neutrophil exocytosis. In addition, because VAMP-1 and VAMP-2 expression was up-regulated during neutrophil differentiation of HL-60 cells, and both proteins are cleaved by TeTx (42), we further analyzed their respective roles in neutrophil exocytosis.

**FIGURE 3.** Electron microscopy characterization of VAMP-1 and VAMP-7 positive granules. Cryosections of neutrophils were double-labeled for VAMP-1 and lactoferrin (Lf) (A and B), gelatinase (Gel) (C), or myeloperoxidase (MPO) (D), respectively, using specific immunogold-labeled Abs with different gold particle sizes (10 and 15 nm) as indicated. Double-labeled granules are indicated (arrows). VAMP-1 was also seen in Golgi (G) and multivesicular bodies (mvb). E, Neutrophils were immunogold-labeled with anti-VAMP-7 and anti-myeloperoxidase specific Abs, showing colocalization of both proteins (arrows). Scale bar, 200 nm.

**FIGURE 4.** Differential subcellular localization of VAMP in neutrophil granules. Ultrathin cryosections were double immunogold-labeled for the corresponding VAMP and either myeloperoxidase (marker for azurophilic granules), lactoferrin (marker for specific granules), or gelatinase (marker for gelatinase-rich tertiary granules). Histograms indicate the percentage of VAMP-positive granules displaying colocalization with each granule marker. For each experiment, at least 200 positive granules were analyzed.

**FIGURE 5.** VAMP-1 and VAMP-7 are present in phagolysosomes. Cryosections of exudate neutrophils from skin window chambers after phagocytosis of latex beads were incubated with anti-VAMP-1 (A) and anti-VAMP-7 (B) Abs for immunogold detection. A, An area of a cell shows labeling of VAMP-1 (arrows) on the outer membrane and on vesicles in the phagoslyosomes (ph) and in the endoplasmic reticulum (er). B, Three phagolysosomes are labeled (arrows) for VAMP-7. Scale bar, 200 nm.
4–6, enriched in specific and tertiary granules, as well as from fractions 7–8, enriched in azurophilic granules (Fig. 2F). This subcellular location differed from that of VAMP-2, mainly localized in the readily mobilizable tertiary and specific granules (Fig. 2F). When human neutrophils were activated with PMA that released tertiary and specific granules, but not azurophilic granules (26), VAMP-2 and the tertiary/specific granule location of VAMP-1 were translocated to the plasma membrane, whereas the azurophilic granule-located VAMP-1 remained in the last fractions of the subcellular fractionation (Fig. 2F). Interestingly, we found that VAMP-7 was mainly localized in the membranes of the fractions enriched in azurophilic granules in resting human neutrophils (Fig. 2F), and was not mobilized upon PMA activation (Fig. 2F).

To further define the subcellular localization of VAMP-1, VAMP-2 and VAMP-7, cryosections of resting human neutrophils were double-labeled with Abs against these VAMPs and with markers for the different cytoplasmic granules, namely myeloperoxidase (azurophilic granules), lactoferrin (specific granules), or gelatinase (tertiary granules). Cryosections were analyzed by immunogold electron microscopy. VAMP-1 was localized on the membranes of specific, tertiary and azurophilic granules (Fig. 3, A–D), and on the internal vesicles of multivesicular bodies (Fig. 3B). VAMP-2-positive granules were mostly positive for specific and tertiary granule markers (data not shown), corroborating our previous findings (26). Interestingly, VAMP-7 was mainly located on the membrane of myeloperoxidase-positive granules (Figs. 3E).

**FIGURE 6.** Effect of Abs against VAMPs and syntaxin 4 on the up-regulation of CD63 and CD66b cell surface expression in activated electropor permeabilized human neutrophils. A, Electropor permeabilized neutrophils (PMN) were incubated in the absence (Control) or in the presence of P3X63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), anti-CD3 mAb (20 μg/ml), or of increasing concentrations of anti-VAMP-1, anti-VAMP-2, and anti-VAMP-7 Abs, and then activated with Ca²⁺ + GTP-γ-S, and assayed for CD63 and CD66b Ag expression by flow cytometry. In addition, electropor permeabilized cells were also incubated with anti-VAMP-1 + anti-VAMP-7 Abs at the indicated concentrations. Data are expressed as the percentage of the cell surface Ag increase upon electropor permeabilized neutrophil activation compared with the CD63 and CD66b cell surface up-regulation detected in control Ca²⁺ + GTP-γ-S-stimulated electropor permeabilized neutrophils in the absence of any Ab (Control), considered as 100% increase in cell surface Ag expression. Mean values ± SD of three independent determinations are shown. B, Electropor permeabilized neutrophils (PMN) were incubated in the absence (Control) or in the presence of P3X63 (20 μg/ml), or of increasing concentrations of anti-syntaxin 4 (STX4) Ab, and then activated with Ca²⁺ + GTP-γ-S (Ca²⁺ + GTP), and assayed for CD63 and CD66b Ag expression by flow cytometry. The results shown are representative of three separate experiments. C, Electropor permeabilized neutrophils (PMN) were incubated in the absence (Control) or in the presence of P3X63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), anti-CD3 mAb (20 μg/ml), increasing concentrations of anti-syntaxin 4 (STX4) Ab, or combinations of Abs against syntaxin 4 and different VAMPs, and then activated with Ca²⁺ + GTP-γ-S, and assayed for CD63 and CD66b Ag expression by flow cytometry as described. Data are expressed as in A. Mean values ± SD of three independent determinations are shown.
and 4). The degree of colocalization of the VAMPs with the distinct granule markers is shown in Fig. 4, after analyzing at least 200 positive granules for VAMPs. Because labeling with anti-VAMP Abs was much weaker than labeling with the corresponding granule markers and only one section was examined for each granule, we analyzed colocalization only in VAMP-positive granules to avoid that the less abundant granule constituent, i.e., VAMP could be missed in a particular section of the same granule. VAMP-1 was evenly detected in the three cytoplasmic granules, whereas VAMP-2 was predominantly present in specific and tertiary granules and VAMP-7 in azurophilic granules (Fig. 4). Because the major function of neutrophil azurophilic granules is to fuse with the phagosome during phagocytosis, we analyzed the putative presence of VAMP-1 and VAMP-7 in phagolysosomes. We used exudate skin window neutrophils stimulated to phagocytosis by Ig-coated latex beads (34), which release part of their specific granules but contain an intact azurophilic population (52). Formation of phagolysosomes has been shown to be much higher in exudate neutrophils from skin windows than in blood neutrophils (34). Fig. 5 shows that VAMP-1 (Fig. 5A) and VAMP-7 (Fig. 5B) are present in the membrane of phagolysosomes after uptake of latex beads by human neutrophils, further supporting the azurophilic granule location of these proteins. Because only one section was examined for each phagolysosome, both its apparent size and the presence of a higher or lower amount of VAMP-1 or VAMP-7 are largely dependent on each particular section.

VAMPs mediate exocytosis of distinct granules in human neutrophils

Next we analyzed whether the VAMPs, with different subcellular locations, could play a role in neutrophil exocytosis. To this aim, we prepared electropermeabilized neutrophils that allowed rapid access of Abs into the cytoplasm and were able to undergo exocytosis of cytoplasmic granules upon cell activation with Ca\(^{2+}\) and GTP-\(\gamma\)-S (24, 33). More than 95% of electropermeabilized neutrophils were permeable to either propidium iodide or FITC-conjugated anti-CD3 mAb (24, 33). Incubation of electropermeabilized neutrophils with irrelevant mouse Igs, including P3X63 myeloma culture supernatant or isotype-matched unrelated mouse mAbs anti-CD20 or anti-CD3, used as negative controls, had no effect on neutrophil degranulation (Fig. 6A). However, incubation of electropermeabilized neutrophils with specific anti-VAMP-1 Abs inhibited exocytosis of both CD63- and CD66b-rich granules although a higher inhibitory effect on the secretion of azurophilic granules was observed (Fig. 6A). Specific anti-VAMP-2 Abs inhibited CD66b up-regulation in a dose-dependent manner, but had no effect on CD63 up-regulation (Fig. 6A). In contrast, specific
anti-VAMP-7 Abs showed a potent inhibitory action on the exocytosis of azurophilic granules with little effect on CD66b-positive granule secretion (Fig. 6A). The combination of anti-VAMP-1 and anti-VAMP-7 Abs highly potentiated the inhibitory effect on azurophilic granule secretion, but no further inhibitory action was observed on CD66b-rich granule exocytosis (Fig. 6A). Ab neutralization of SNAREs has been shown to impair secretory responses in a variety of cell types (24, 26, 53, 54), likely by hampering SNARE complex formation through steric hindrance or conformational change.

Role of syntaxin 4 in VAMP-mediated neutrophil exocytosis

The granule membrane VAMPs discussed are expected to interact with target plasma membrane proteins during exocytosis. Thus, we next analyzed the role of syntaxin 4, which is located in the plasma membrane of human neutrophils (20, 26), in the exocytosis of the distinct granule populations. Incubation of electropermeabilized neutrophils with specific anti-syntaxin 4 mAb inhibited CD66 up-regulation in a dose-response manner (Fig. 6, B and C). Higher amounts of anti-syntaxin 4 mAb were required to inhibit CD66b up-regulation (Fig. 6, B and C). Following dose-response assays, we found that exocytosis of azurophilic granules was 3.5-fold more sensitive to the effect of the anti-syntaxin 4 Ab than the corresponding exocytosis of specific/tertiary granules. Ab inhibition experiments suggested that distinct VAMP/syntaxin 4 complexes were involved in the exocytosis of neutrophil granules (Fig. 6C). Combinations of anti-syntaxin 4/anti-VAMP-1 Abs potentiated the inhibition of the secretion of azurophilic and specific/tertiary granules, whereas combinations of anti-syntaxin 4/anti-VAMP-2 Abs and anti-syntaxin 4/anti-VAMP-7 Abs further enfeebled specific/tertiary and azurophilic granule secretion, respectively (Fig. 6C). These data suggest that syntaxin 4 mediates secretion of specific/tertiary and azurophilic granules via its putative interaction with VAMP-1 or VAMP-2 and VAMP-1 or VAMP-7, respectively.

SNAP-23 is involved in VAMP-mediated exocytosis of specific and tertiary granules

The requirement for higher amounts of anti-syntaxin 4 Abs to block exocytosis of specific/tertiary granules, as compared with azurophilic granule mobilization (Fig. 6C), would be compatible with a higher number of syntaxin 4-containing SNARE complexes involved in the secretion of specific/tertiary granules. In addition, a higher diversity of SNARE proteins in specific and tertiary granules might lead to their proneness to exocytose. We have recently reported by subcellular fractionation the presence of SNAP-23 in specific and tertiary granules, with a minor location in plasma membrane (24). In this study, we have found by electron microscopy that SNAP-23 was mainly present at the membranes of specific and tertiary granules with a secondary location in the plasma membrane (Fig. 7). Labeling of SNAP-23 in docked granules in contact with the plasma membrane was also observed (Fig. 7A, inset), further supporting its role in membrane fusion processes in these cells. Incubation of electropermeabilized neutrophils with specific anti-SNAP-23 Abs blocked CD66b up-regulation following cell activation with Ca²⁺ and GTP-γ-S, without affecting CD63 up-regulation (Fig. 8). However, incubation of electropermeabilized neutrophils with anti-SNAP-23 Abs previously preincubated with SNAP-23 recombinant protein did not affect exocytosis, further demonstrating the specific effect of the anti-SNAP-23 Ab on the exocytosis of specific/tertiary granules (data not shown). In addition, a combination of Abs against SNAP-23 with either anti-VAMP-1 or anti-VAMP-2 Abs further inhibited secretion of specific and tertiary granules, with no effect on the release of azurophilic granules (Fig. 8).

SNARE proteins are involved in neutrophil exocytosis triggered by distinct stimuli

Human neutrophils electropermeabilized under the same conditions as in this work have been previously shown to respond to different stimuli, including the phorbol ester PMA and the chemotactic factor FMLP (32). PMA induces secretion of specific and tertiary granules, whereas FMLP is a general secretagogue that releases contents of tertiary, specific, and azurophilic granules (10). We found that PMA, without cytochalasin B pretreatment, induced a potent up-regulation of CD66b, similar to that induced by Ca²⁺ and GTP-γ-S electropermeabilized cells, but it did not prompt CD63 up-regulation. This observation is in agreement with the known ability of PMA to induce secretion of specific and tertiary granules (6, 55). The complete secretagogue FMLP promoted a weaker exocytic response, ~42% of that induced by Ca²⁺ and GTP-γ-S. Similarly to what we obtained in electropermeabilized neutrophils activated with Ca²⁺ and GTP-γ-S, we found that Abs against VAMP-2 and SNAP-23 were potent inhibitors of CD66b up-regulation following stimulation with either PMA (Fig. 9A) or FMLP (Fig. 9B). Abs against VAMP-1 and syntaxin 4 were also able to inhibit CD66 up-regulation, although to a lesser extent, whereas anti-VAMP-7 Abs hardly affected the exocytosis of CD66b-positive granules (Fig. 9). In contrast, Abs against VAMP-7, syntaxin 4, and VAMP-1 drastically inhibited CD63 up-regulation, whereas Abs against VAMP-2 and SNAP-23 did not affect CD63 up-regulation (Fig. 9B). These results further support
that exocytosis of specific and tertiary granules is mediated by VAMP-1, VAMP-2, SNAP-23, and syntaxin 4, whereas exocytosis of azurophilic granules is mediated by VAMP-1, VAMP-7, and syntaxin 4, irrespective of the stimulus used for secretion.

In vivo interaction between SNARE proteins in activated neutrophils

We next sought whether VAMPS and SNAP-23, located in granules, and syntaxin 4, located in the plasma membrane, could interact each other during neutrophil exocytosis resulting in the formation of SNARE complexes. Using similar activation conditions as those used in Fig. 9, we found that SNAP-23 immunoprecipitated with VAMP-1, VAMP-2, and syntaxin 4 after neutrophil activation with PMA (Fig. 10A), which induced selective secretion of specific and tertiary granules (>67% secretion of gelatinase and lactoferrin, with <5% secretion of the azurophilic granule marker β-glucuronidase, following incubation with 100 ng/ml PMA for 10 min). Interestingly, VAMP-2 immunoprecipitated with SNAP-23 and syntaxin 4, but not with VAMP-1, after PMA stimulation (Fig. 10A), suggesting that VAMP-1 or VAMP-2 are mutually exclusive of each other in the formation of SNARE complexes. These data suggest that VAMP-1, VAMP-2, and SNAP-23, mainly found in granules, and syntaxin 4, mainly found in plasma membrane, are brought together during neutrophil activation forming two combinatorial SNARE complexes, VAMP-2/SNAP-23/syntaxin 4 and VAMP-1/SNAP-23/syntaxin 4, which eventually lead to secretion of specific and tertiary granules. VAMP-1 and syntaxin 4 immunoprecipitated after cell activation with the specific inducer of specific/tertiary granule exocytosis PMA (Fig. 10B). Immunoprecipitation of VAMP-1 and syntaxin 4 was further enhanced following cell stimulation with the complete secretagogue FMLP (Fig. 10B), which induced the discharge of specific/tertiary granules and azurophilic granules (release of 55% lactoferrin, 63% gelatinase, and 36% β-glucuronidase, following stimulation with 10^-7 M FMLP for 10 min). This result suggests the involvement of VAMP-1/syntaxin 4 complexes in the secretion of specific/tertiary and azurophilic granules. We found coimmunoprecipitation of syntaxin 4 and VAMP-7 only under conditions that promoted azurophilic granule exocytosis, i.e., in FMLP-treated neutrophils, but not in untreated or PMA-treated neutrophils (Fig. 10B), suggesting that VAMP-7/syntaxin 4 complexes were specifically formed during the azurophilic granule secretion. These results further support a role for syntaxin 4 as a major target Q-SNARE in the plasma membrane of human neutrophils, acting as an anchor for the distinct granule-located SNAREs.

Discussion

The release of neutrophil granule contents play a critical role in inflammation and host defense against infection. However, the molecular mechanisms controlling the differential exocytosis of neutrophil granules are ill-defined. Our present findings square with a model in which qualitative and quantitative differences in the formation of combinatorial SNARE complexes underlie the differential exocytosis of the distinct granule subpopulations of human neutrophils (Fig. 11). Our data indicate that at least two SNAP-23 complexes of the 3 (Q-SNARE)/1 (R-SNARE) type, namely syntaxin 4/SNAP-23/VAMP-1 and syntaxin 4/SNAP-23/VAMP-2, are involved in the exocytosis of specific and tertiary granules, bringing together plasma membrane syntaxin 4 and granule membrane VAMP-1, VAMP-2, and SNAP-23. The Q-SNARE SNAP-23 would contribute with two Q-SNARE motifs in the four-helix bundle of the SNARE core complex, whereas the Q-SNARE syntaxin 4 as well as the R-SNAREs VAMP-1 and VAMP-2 would contribute with one SNAP-23 motif each. In contrast, one Q-SNARE, syntaxin 4, and two R-SNAREs, VAMP-1 and VAMP-7, are involved in the exocytosis of azurophilic granules. Thus, if a 3 (Q-SNARE)/1 (R-SNARE) type complex were formed during azurophilic granule mobilization, the R-SNARE of these granules should interact with additional Q-SNARE motifs that remain to be identified. However, it cannot be ruled out that release of azurophilic granules could involve the formation of weaker dimeric combinations of SNARE proteins or the participation of additional non-SNARE proteins. The high number of SNAP-23 proteins in specific and tertiary granules bestows an additional advantage to form SNARE complexes during secretion of these organelles, becoming exocytosis-prone granules. The present data also indicate that qualitative differences, in addition to quantitative differences, are important in the differential regulation of the exocytosis of specific/tertiary granules and azurophilic granules. SNAP-23 and VAMP-2 are mainly involved in the release of specific and tertiary granules, whereas VAMP-7 is mainly involved in...
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References


