

# Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells

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Mediator release from mast cells (MC) is a crucial step in allergic and non-allergic inflammatory disorders. However, the final events in response to activation leading to membrane fusion and thereby facilitating degranulation have hitherto not been analyzed in human MC. Soluble N-ethyl-maleimide-sensitive factor attachment protein receptors (SNARE) represent a highly conserved family of proteins that have been shown to mediate intracellular membrane fusion events. Here, we show that mature MC isolated from human intestinal tissue express soluble N-ethylmaleimide sensitive factor attachment protein (SNAP)-23, Syntaxin (STX)-1B, STX-2, STX-3, STX-4, and STX-6 but not SNAP-25. Furthermore, we found that primary human MC express substantial amounts of vesicle associated membrane protein (VAMP)-3, VAMP-7 and VAMP-8 and, in contrast to previous reports about rodent MC, only low levels of VAMP-2. Furthermore, VAMP-7 and VAMP-8 were found to translocate to the plasma membrane and interact with SNAP-23 and STX-4 upon activation. Inhibition of SNAP-23, STX-4, VAMP-7 or VAMP-8, but not VAMP-2 or VAMP-3, resulted in a markedly reduced high-affinity IgE receptor-mediated histamine release. In summary, our data show that mature human MC express a specific pattern of SNARE and that VAMP-7 and VAMP-8, but not VAMP-2, are required for rapid degranulation.

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**Abbreviations:** MC: mast cells · SNAP: soluble N-ethylmaleimide sensitive factor attachment protein · SNARE: soluble N-ethylmaleimide sensitive factor attachment protein receptor ·

SLO: streptolysin-O · STX: syntaxin · TeNT: tetanus toxin ·

VAMP: vesicle associated membrane protein

## Introduction

Mast cells (MC) are well known to play a key role in allergic and a number of non-allergic inflammatory conditions. They are capable of releasing a number of pre-stored or *de novo* synthesized proinflammatory

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mediators upon a variety of stimuli [1]. Furthermore it has become evident in recent years that MC display a broad range of non-allergic immunological functions [2, 3], *e.g.* through the release and response to growth factors and cytokines [4, 5]. The intracellular pathways leading to degranulation of MC upon cross-linking of the high-affinity IgE receptors (Fc $\epsilon$ RI) have been studied extensively [6–9]. However, most of the efforts have been focused on the primary steps of the signaling cascade, while the final events leading to rapid release of mast cell granule content are still poorly understood. Exocytosis of vesicle contents requires the fusion of opposing membrane layers. Soluble N-ethyl-maleimide-sensitive factor attachment protein receptors (SNARE), a highly conserved family of proteins, have been demonstrated to be crucially involved in this process [10]. Although initially discovered and studied in neuronal cells [11], recent reports have delivered evidence that exocytosis requires SNARE in cells of the immune system as well [12–14]. Roughly, SNARE are classified as so-called v-SNARE, localized on the vesicle membrane, or t-SNARE found on the target membrane, *e.g.* the plasma membrane. SNARE-proteins assemble in a core complex of bundled helices and thereby contribute to membrane fusion [15]. Accuracy of membrane trafficking in cells is thought to be achieved at least in parts by SNARE [12]. There are numerous isoforms of t-SNARE (syntaxins and SNAP) and v-SNARE (VAMP). In addition, there are a number of proteins that display regulatory functions that have been termed SNARE-masters [15].

The role of SNARE-proteins in human MC is still largely elusive. However, experiments conducted in rodent basophilic leukemia cells (RBL-2H3), a widely used MC cell line, have provided solid data, that members of the SNARE-family are indeed specifically expressed in different compartments of these cells and that they are crucially involved in mediator secretion [16–20]. These reports attribute a key role in exocytotic events in MC to the t-SNARE SNAP-23 [21]. SNAP-23 represents the ubiquitously expressed counterpart of its neuronal isoform SNAP-25 [22] and is found in tissues throughout the body [21, 23, 24]. Furthermore, SNAP-23 has been demonstrated to mediate exocytosis in other immune cells as well [12, 25, 26]. It forms a stable SNARE complex together with a syntaxin (STX), another t-SNARE. Various STX-isoforms have been identified in myeloid cells [12, 27, 28]. Finally, docking and fusion events at the plasma membrane require v-SNARE, namely vesicle-associated membrane proteins (VAMP). Different VAMP isoforms seem to be specifically involved in mediating the various intracellular trafficking events. VAMP-1 and VAMP-2, also called synaptobrevins, have been implicated in regulated exocytosis in neurons [29] and non-neuronal cells [30], while VAMP-3, or cellu-

brevin, was attributed a role in constitutive exocytosis and vesicle recycling [31]. VAMP-7 and VAMP-8, unlike synapto- and cellubrevin insensitive to cleavage by tetanus toxin (TeNT), have so far rather been associated with endocytotic or intracellular vesicle-trafficking processes [32, 33]; however, recent reports have also delivered evidence for exocytotic functions [16, 34, 35]. With respect to leukocytes, most focus has been set on VAMP-2, which facilitates mediator release from neutrophils and eosinophils [26, 27, 36–38]. Concerning the situation in RBL-2H3 cells, VAMP-7 or VAMP-8 was found in membranes of secretory granules [16, 28]. However, the functional significance is not fully clear and mature human MC, which differ significantly from rodent MC [39], have to date not been studied with regards to SNARE-protein expression and function. Thus, we sought to determine which SNARE-isoforms are present in primary human MC. We isolated MC from human intestinal mucosa and analyzed SNARE-expression by means of RT-PCR, immunoblotting and immunofluorescence. Immunoprecipitation was utilized to demonstrate complex formation between SNAP-23, VAMP-7, VAMP-8 and STX-4. Immunofluorescence microscopy revealed translocation of both VAMP-7 and VAMP-8 to the plasma membrane and co-localization with SNAP-23 and STX-4, respectively, upon stimulation. Furthermore, targeted interference with VAMP-7 and VAMP-8, but not VAMP-2 or VAMP-3, significantly decreased histamine release, demonstrating the requirement of these VAMP for MC degranulation.

## Results

### Expression of SNARE-proteins in mature human MC

Although anticipated, the expression of SNARE in human MC has so far not been characterized. Therefore, we assessed SNARE expression on mRNA-level by RT-PCR. The results are shown in Fig. 1 and the Supporting Information. We found strong signals for SNAP-23 while SNAP-25 was barely detectable. Out of ten different cultures (from ten different individuals), it was only detected in small amounts in two samples. These results were further confirmed on the protein level by immunoblot. SNAP-25 was not detectable whereas MC showed strong SNAP-23 expression (Fig. 1B). Interestingly, VAMP-2, which has been repeatedly implicated in regulated exocytosis in granulocytes, was found to be expressed on a rather low level. In contrast, strong signals were detected for VAMP-3, VAMP-7 and VAMP-8. STX-2, STX-3, STX-4, and STX-6 were detected in considerable amounts while we found a

weaker expression of STX-1B and absence of STX-1A in MC. Notably, STX-6 mRNA expression level in relation to GAPDH was tenfold lower in MC compared to eosinophils and neutrophils (unpublished data). Neuronal tissue, namely rat brains were used as positive controls.

### SNAP-23, STX-4, VAMP-7 and VAMP-8 co-localize and form stable complexes upon activation

To further identify SNARE isoforms that participate in complex formation at the plasma membrane and thereby facilitate exocytosis of mediators in human MC, we performed fluorescence microscopy and immunoprecipitation. For this purpose, resting or iono/PMA-activated MC were fixed, permeabilized with 0.3% saponin and subsequently stained with specific antibodies as indicated. Successful degranulation of the cells was monitored by histamine release. The data presented in Fig. 2 reveal that SNAP-23 and STX-4 localized to the plasma membrane in resting as well as activated MC (Fig. 2A and B). Double staining revealed a high degree of co-localization of these two t-SNARE following activation (Fig. 2B). In contrast, VAMP-3 was preferentially found on intracellular membrane compartments; and this distribution remained unaltered upon activation (Fig. 2C and D). VAMP-7 and VAMP-8, however, which were also detected throughout the cytoplasm in resting cells (Fig. 2E and G), translocated to the plasma membrane and co-localized with SNAP-23 or STX-4, respectively, shortly after stimulation (Fig. 2F and H). In a next step, these findings were crosschecked by immunoprecipitation. Lysates from resting or acti-

vated MC that were precipitated with antibodies directed against SNAP-23 contained VAMP-7, VAMP-8 and STX-4 (Fig. 3). In contrast to VAMP-2 and VAMP-3, VAMP-7 and VAMP-8 formed stable complexes with SNAP-23 upon activation of MC. These observations indicate that SNAP-23 and STX-4 on the target membrane, and VAMP-7 and/or VAMP-8 on granule membranes, are involved in forming stable complexes upon MC activation.

### Inhibition of SNAP-23, STX-4, VAMP-7 and VAMP-8 significantly decreases histamine release from human MC

It has been reported previously that cleavage of VAMP-2 by TeNT significantly decreased mediator release from human eosinophils [38]. A similar approach was chosen in order to transfer these findings to MC. TeNT is a heterodimeric protein and consists of a heavy and a light chain, the latter carrying the proteolytic activity. We electroporated MC and used [<sup>125</sup>I]-tagged TeNT light chains (50 kDa) to prove the uptake of the protein by measuring radioactivity (cpm) of MC, which were washed vigorously, 1 h after electroporation. Fig. 4A and B show voltage-dependent efficacy of protein delivery and viability of MC, respectively. For efficient uptake and activity of TeNT (light chains) in human MC, cleavage of VAMP-2 was detected by Western blot in two different cultures after electroporation only or simultaneous administration of TeNT light chains (not shown). However, in spite of efficient toxin-uptake and efficient *in vitro* cleavage of target proteins no differences in activation-induced histamine release were observed as shown in Fig. 4C. This suggested a central role of one of

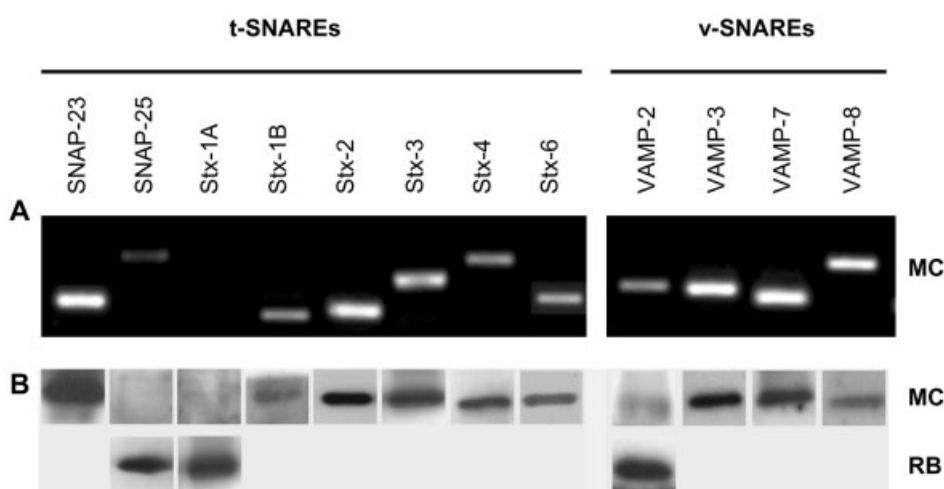


**Figure 1.** Expression of SNARE in primary human mast cells. (A) Expression of mRNA shown by RT PCR. (B) Protein expression shown by immunoblot. SNAP-23 is strongly expressed both at the mRNA and protein level, while SNAP-25 is virtually absent. SNAP-25 was only detected in two MC lysates (out of ten tested) by RT PCR at a very low level. VAMP-2 was detected in small amounts by polyclonal antibodies while it was not detectable using an mAb (not shown). Lysates of rat brain (RB) were used as controls.

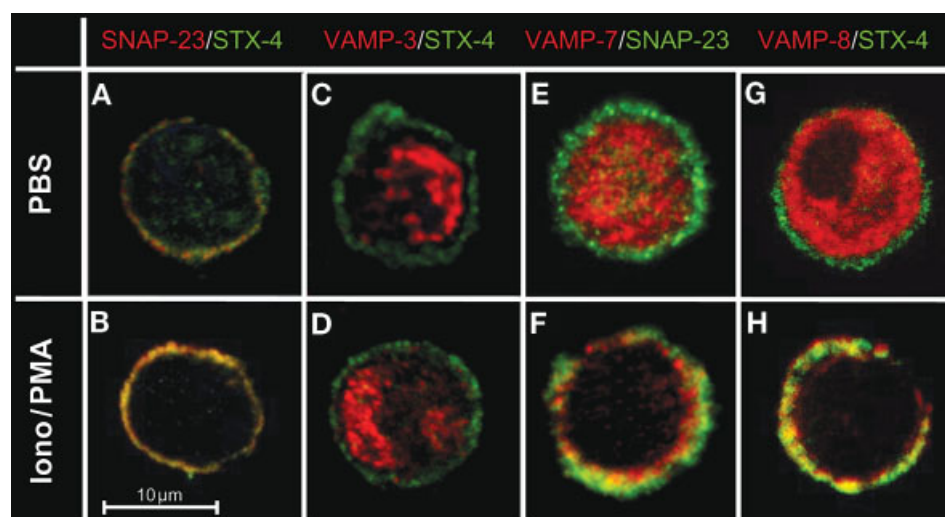


**Figure 2.** Expression of SNARE isoforms in fixed and permeabilized human MC. Representative images of five different MC cultures ( $n = 5$ ) shown. (A and B) Expression of STX-4 (green) and SNAP-23 (red) in resting (+PBS, A) and activated (+iono/PMA, B) MC. (C and D) VAMP-3 (red) and STX-4 (green) co-staining. No translocation or co-localization was observed. (E and F) In resting MC, VAMP-7 (red) is located in the cytoplasmic compartment (E), and translocates to the plasma membrane and co-localizes with SNAP-23 (green) in activated MC (F). (G and H) VAMP-8 (red) is found in the cytoplasm in resting cells (G) and co-localizes with STX-4 (green) upon activation of MC (H). Staining of VAMP-7 and VAMP-8 by immunofluorescence after stimulation of the cells with IgE and anti-IgE was not suitable because of the interference/reactivity of the secondary antibodies with the anti-IgE antibodies (not shown).

the neurotoxin insensitive VAMP, VAMP-7 and VAMP-8 that we had already identified as binding partners of SNAP-23/STX-4 (Fig. 2).

No specific inhibitors of VAMP-7 or VAMP-8 are available to date, as these isoforms, unlike VAMP-1, VAMP-2 and VAMP-3, are insensitive to cleavage by both TeNT and botulinum neurotoxin. We therefore chose to block SNARE interaction by administration of specific

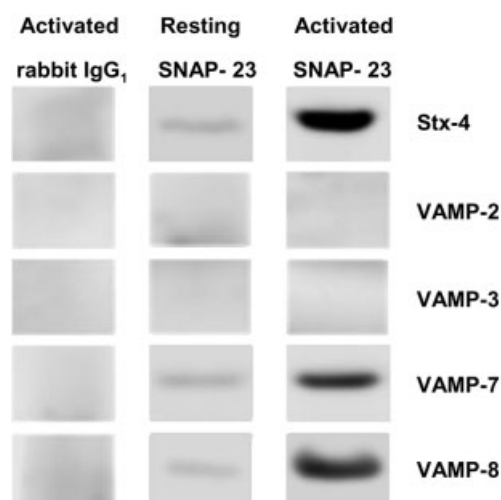


**Figure 3.** Immuno-co-precipitation of SNARE in MC. Lysates from resting and activated MC were precipitated with anti-SNAP-23 antibodies or lysates from activated MC were precipitated with an isotype antibody (rabbit IgG<sub>1</sub>) as a control, respectively, subsequently subjected to Western blotting and stained with the antibodies indicated. STX-4, VAMP-7, and VAMP-8 were found to form stable complexes with SNAP-23.

neutralizing antibodies directed against different SNARE isoforms. Antibodies recognize cytoplasmic NH<sub>2</sub>-terminal region required for SNARE binding. Since electroporation did not allow the delivery of whole antibodies (150 kDa) at the given electroporation conditions to viable MC, permeabilization was alternatively achieved by incubation with streptolysin-O (SLO). This bacterial-derived toxin allows a temporary permeabilization of vital cells in a dose dependent manner (Fig. 4D). After permeabilization, pores were re-sealed by addition of medium containing 1–2 mM Ca<sup>2+</sup>. Degranulation was induced after 2 h of incubation in normal culture medium for reconstitution. Sufficient uptake of antibody was demonstrated by immunofluorescence using fluorescence-conjugated mouse IgG (Fig. 4F). We found that administration of anti-SNAP-23 antibodies totally abolished both Iono/PMA and FcεRI mediated degranulation, while inhibition of VAMP-7 resulted in a reduction of histamine release by 50% compared to control MC that were treated with SLO and isotype Ab (Fig. 4G and H). Delivery of anti VAMP-8 mAb decreased histamine release by 60%. In contrast, pretreatment with anti-VAMP-2 or VAMP-3 antibodies did not affect the capacity of degranulation of cultured MC, supporting the data obtained with TeNT (Fig. 4C, G and H). Inhibition of STX-4, but not STX-6, caused a significant reduction of IgE and Iono/PMA mediated histamine release (Fig. 4G and H). We therefore conclude that SNAP-23, STX-4, VAMP-7 and VAMP-8 represent essential elements of the secretory apparatus in human MC.

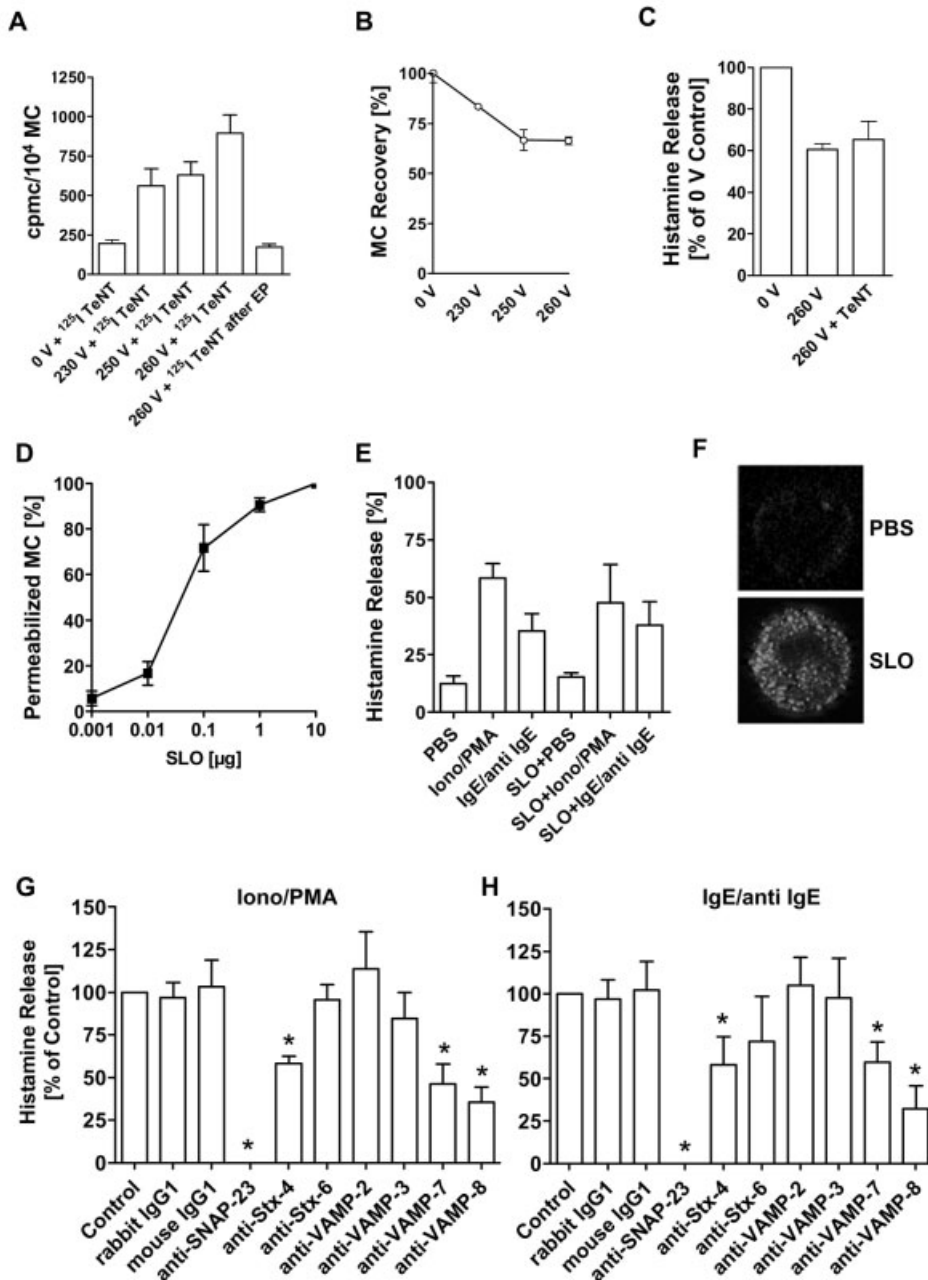


**Figure 4.** (A–C) MC were transiently permeabilized by electroporation. (A) Voltage dependency of protein uptake was demonstrated by co-incubation with [<sup>125</sup>I]TeNT and subsequent measurement of cpm. Administration of [<sup>125</sup>I]TeNT after electroporation (EP) ruled out non-specific binding of the proteins to the cell surface, as shown in the column on the far right. (B) MC viability was reduced in a voltage dependent fashion. (C) Histamine release was reduced after electroporation. Administration of TeNT light chains did not further affect MC histamine release (*n* = 3). (D–H) MC analysis after permeabilization by SLO treatment. (D) Dose-dependency of SLO-permeabilization was checked by trypan blue staining (*n* = 3). (E) Comparison of histamine release from MC after treatment with SLO and stimulated by either Iono/PMA or IgE + anti-IgE. (F) Uptake of mouse IgG after SLO-permeabilization of MC, detected by immunofluorescence using Alexa<sup>®</sup>594-conjugated mouse IgG. (G and H) Administration of different neutralizing antibodies or isotype control IgG to permeabilized MC. SNAP-23 (10 μg/mL), STX-4 (5 μg/mL), VAMP-7 (5 μg/mL) and VAMP-8 (5 μg/mL) Ab, but not STX-6 (10 μg/mL), VAMP-2 (10 μg/mL), or VAMP-3 (20 μg/mL) Ab, significantly decreased the capacity of MC to release histamine upon stimulation as indicated by \*. Histamine release is expressed as percentage of release from MC treated with SLO alone (100%) (*n* = 4). (\* *p* < 0.05).

## Discussion

A number of studies performed in a rodent cell-line (RBL-2H3) address the question of SNARE protein involvement in exocytosis in these cells [16–20]. However, SNARE expression or function in primary human MC or basophils has hitherto not been analyzed. At this point, it is particularly noteworthy that mature human MC differ significantly from RBL-2H3 and primary mouse MC in a number of important biological properties like expression and response to cytokines, as well as activation of signaling pathways [39]. In order to understand molecular mechanisms of allergic and anaphylactic disorders and to identify potential novel targets of anti-allergic treatment, it seems essential to study MC isolated from human tissue.

Here, we show that human mucosal MC indeed express specific isoforms of SNARE (Fig. 1 and 2). As could be expected, SNAP-23 was strongly expressed while SNAP-25 was virtually absent, both on the protein and on the mRNA level. This is in accordance to the consensus that SNAP-23 represents the non-neuronal isoform of SNAP-25 [22]. Furthermore, this finding is in line with data demonstrating a pivotal role for SNAP-23 in secretory events in different immune cells [12, 25, 26]. Unexpectedly, expression of VAMP-2 was hardly detectable on protein level in MC in contrast to what has been reported for human eosinophils and rodent RBL-2H3 cells [38]. However, other studies report a low expression of VAMP-2 in eosinophils [37].

More recently, a central function has been attributed to SNAP-23, VAMP-7 and STX-4 in human eosinophil and neutrophil exocytosis [34]. In agreement with these data we found striking evidence for a critical role of these SNARE proteins in human MC as well. We could demonstrate interaction of SNAP-23, STX-4 and VAMP-7 in terms of translocation and complex formation upon MC activation (Fig. 2 and 3). In addition, we found that VAMP-8 also co-localizes with STX-4 upon activation. We were able to show that administration of neutralizing antibodies to VAMP-7 significantly reduced histamine secretion from permeabilized MC (Fig. 4). Moreover, we found an additional requirement of VAMP-8 for MC-degranulation. In contrast, inhibition of VAMP-2 and VAMP-3 by TeNT or anti VAMP-2 and VAMP-3 antibodies did not alter histamine release. These findings are based on neutralizing antibodies and it will be important to confirm the results in future studies using alternative approaches like RNAi. VAMP-8 has been repeatedly implicated in mediating endocytotic membrane traffic. However, a very recent report delivers evidence for the involvement of VAMP-8 in granule content release from human platelets [40, 41]. The fact that MC degranulation is facilitated by compound exocytosis, which requires fusion of vesicles prior to exocytosis [42],

might hold an explanation for the involvement of two distinct VAMP. However, it is specifically noteworthy that VAMP-8 has been shown to be not essential for mediator release from human eosinophils [34], underlining the differential functions of these two cell types.

Antiallergic treatment has so far been focused on the inhibition of MC- (or eosinophil) derived mediators, most prominently histamine [43]. An alternative approach is to interfere prior to the release of inflammatory mediators from activated MC. Sodium cromoglycate, and more recently, anti-IgE mAb (Omalizumab) have hitherto represented the only suitable compounds. Neurotoxins like botulinum or TeNT specifically cleave SNARE proteins and thereby mediate their fatal effects by inhibiting neurotransmitter release and causing paralysis or generalized muscle contraction [44]. VAMP-7 and VAMP-8 however are insensitive to the proteolytic activity of neurotoxins making these molecules a little promising tool for SNARE inhibition in MC. On the other hand, there are STX sensitive to botulinum neurotoxin serotype C and it is furthermore conceivable that other SNARE isoforms govern important membrane-fusion steps upstream to the fusion of MC granules with the plasma membrane. Moreover, the SNARE machinery is tightly controlled by regulatory molecules, often called SNARE masters [17, 45], manipulation of which could represent an alternative way of MC inhibition as anti-allergic strategy.

In conclusion, we show here that a specific pattern of SNARE proteins is expressed in human MC and that they act as essential parts of the secretory machinery. It is tempting to speculate whether atopic diseases might be associated with an altered expression of SNARE and in how far that could contribute to the hyper-responsiveness of MC in these individuals. Detailed characterization of SNARE functions in human MC will add important information to our understanding of molecular mechanisms of allergy as well as MC-mediated responses in innate immunity.

## Materials and methods

### Isolation, purification and culture of human intestinal MC

Human intestinal MC were isolated from surgical tissue specimens derived from patients undergoing bowel resection due to intra-abdominal carcinomas. Permission to conduct the study was obtained from the local Ethical Committee. The methods of mechanical and enzymatic tissue dispersion yielding single-cell preparations containing  $4 \pm 2\%$  (mean  $\pm$  SD) MC have been described in detail elsewhere [46]. After overnight incubation in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM glutamine, 100  $\mu$ g/mL streptomycin, 100  $\mu$ g/mL gentamicin,

100 U/mL penicillin, and 0.5 µg/mL amphotericin; all reagents: Invitrogen Life Technologies, Paisley, UK), MC were enriched by positive selection of *c-kit*-expressing cells using magnetic cell separation (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and the mAb YB5.B8 (BD PharMingen, Hamburg, Germany) as described [5, 46]. The fraction containing the *c-kit*-positive cells (mast cell purity  $60 \pm 20\%$ ) was cultured at a density of  $2\text{--}10^5$  MC/mL in medium supplemented with 25 ng/mL of recombinant human stem-cell factor (SCF; Peprotech, Rocky Hill, NJ). Cultured MC were fed once a week by exchanging half of the culture medium supplemented with SCF. During culture for 14–28 days, the purity of MC increased to 97–100% MC. MC purity was determined by differentiation of the cells on cytopins stained with May-Grünwald/Giemsa [5, 46].

### RNA-isolation and RT-PCR

Total RNA was prepared from  $1 \times 10^5$  human intestinal cells containing 98–100% MC and RT-PCR was performed as described [4]. Briefly, total RNA was treated for 15 min at 37°C with 10 U RNase-free DNase (Promega, Madison, WI) to remove genomic DNA. After denaturation for 10 min at 70°C, cDNA was synthesized for 1 h at 49°C by adding Superscript<sup>TM</sup> reverse transcriptase III (Invitrogen) and 20 pmol oligo dT primers (Pharmacia, Uppsala, Sweden). Thirty-five PCR cycles (30 s at 94°C, 45 s at 60°C, 35 s at 72°C) were performed with Platinum Taq DNA polymerase (Invitrogen) and 20 pmol of sense and antisense primers. The specific primers are as follows: SNAP-23 (5'-GTACGAGGAGAATCCTGGGT-3'; 5'-CAGACAAAGGCCACAGCA-3'), SNAP-25 (5'-AAGCCTGGGG-CAATAATCAGG-3'; 5'-CTTTGTTGCACGTTGGTT GGC-3'), STX-1A (5'-TGCCTCTGGGATCATCATGG-3'; 5'-ACTTGACGG-CCTTCT TGGTG-3'), STX-1B (5'-TTGAGACGAGGCACAAT-GAG-3'; 5'-ATGCGGTCA ATCATCTCTCC-3'), STX-2 (5'-TGCTGTCTCGGAAGTTTGTG-3'; 5'-CTGTGG TGGTTCTCC-CAGTT-3'), STX-3 (5'-GAGCCAAAAACCAAGGATGA-3'; 5'-TTG GTCATCACCTCCACAAA-3'), STX-4 (5'-CAGAAGGAG-GAAGCTGATGA-3'; 5'-TGCGTGTCTTCAGGATATT-3'), STX-6 (5'-TCATCTGTGCAGGCATTAGC-3'; 5'-TGCTGCTCCTCAAT-GAAATG-3'), VAMP-2 (5'-GTAACAGGAGACTGCAGCAG-3'; 5'-TTTGCGCTTGAGCTTGCTG-3'), VAMP-3 (5'-GAAGCTCTCT-GAGTTAGACG-3'; 5'-GAGACAACCCACACGATGAT-3'), VAMP-7 (5'-CTTCTGGAGGTGACAGAGC-3'; 5'-CGGGAACGTT-CAAAATCATC-3'), VAMP-8 (5'-GTGCGGAACCTGCAAAGT-GA-3'; 5'-GAAGGCACCAGTGCGCAAAGA-3').

### Immunoprecipitation and Western blot analysis

To obtain whole-cell extracts  $0.5\text{--}1 \times 10^6$  MC were lysed and protein concentration was determined. Cell extracts (10–20 µg protein each) were denatured, separated on a 12% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Schleicher and Schuell, Einbeck, Germany). Membranes were probed with the following antibodies: anti-SNAP-25 mouse mAb (Sternberger Monoclonals, Lutherville, MD, USA), anti-SNAP-23 rabbit Ab (Synaptic Systems, Göttingen, Germany), anti-VAMP-2 rabbit Ab (Synaptic Systems), anti-VAMP-2 mouse mAb (Synaptic Systems), anti-VAMP-3, rabbit Ab (Affinity BioReagents, Golden, CO, USA), anti-VAMP-7

mouse mAb (Cl 158.2, [47]), anti-VAMP-8 rabbit Ab [16], anti-STX-1A, rabbit Ab (Synaptic Systems), anti-STX-1B, rabbit Ab (Synaptic Systems), anti-STX-2 rabbit Ab (StressGen Biotechnologies/Assay Design, Ann Arbor, MI, USA), anti-STX-3 rabbit Ab (Abcam), anti-STX-4, mouse mAb (BD Biosciences PharMingen, Franklin Lakes, NJ, USA), and anti-STX-6 mouse mAb (BD Biosciences PharMingen). Ab reactivity was monitored using an electrochemiluminescence detection system as described by the manufacturer (NEN<sup>TM</sup> Life science, Boston, MA). For immunoprecipitation, cells were stimulated with iono/PMA or PBS as control for 5 min, harvested, washed twice and lysed. Lysates were then precleared by incubation with Protein G slurry and lysis buffer (containing proteinase-inhibitor) on ice for 30–60 min and subsequent washing. Ab against SNAP-23 was added to the precleared lysates and allowed to bind for 1 h at 4°C. Ab-Ag complexes were precipitated by addition of Protein G slurry and subsequently transferred to immunoblotting.

### Immunofluorescence

For immunofluorescence, MC were harvested, washed, and then fixed in 2% PFA at 4°C for 30 min followed by repeated washing. The cells were then permeabilized in PBS containing 0.3% saponin as well as blocking reagent (containing goat-serum, swine-serum and human IgG) for 10 min. Primary Ab (for Ab sources see *Immunoprecipitation and Western blot Section*) were added and allowed to bind for 30 min at room temperature. After washing, cells were incubated with fluorescence-conjugated secondary Ab Alexa Fluor<sup>®</sup> goat anti-mouse 488 or Alexa Fluor<sup>®</sup> donkey anti-rabbit 594 (Caltag Laboratories) for another 30 min at room temperature, washed, and finally subjected to fluorescence microscopy (Axiovert 200M ApoTome microscope, Carl Zeiss AG, Oberkochen, Germany). The ApoTome microscope uses “grid projection” which allows optical sectioning through the sample. For the analysis of activated cells, MC were treated with the phorbol ester PMA (1 µM) and ionomycin (iono, 1 µM) for 15 min prior to fixation.

### Cell permeabilization and treatment with neurotoxins and neutralizing antibodies

For permeabilization of vital MC, SLO (Sigma) was used as described in detail elsewhere [34, 48]. In brief, MC were harvested, washed and resuspended in 0.5 mL  $\text{Ca}^{2+}$ -free HBSS buffer containing 30 mM HEPES and SLO at concentrations yielding approximately 60–80% trypan blue staining of the cells (20 µg/mL). Antibodies against SNARE were added at various concentrations (1–20 µg/mL). Alexa<sup>®</sup>594-conjugated mouse IgG (5 µg/mL) was added as control for antibody uptake. After incubation in the presence of antibodies for 15 min at 37°C, the reaction was stopped and the pores were sealed by addition of 1.5 mL of ice-cold medium containing 1–2 mM  $\text{Ca}^{2+}$ . After about 1–2 h of reconstitution MC were subjected to degranulation experiments. Alternatively, MC were permeabilized by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) in the presence or absence of purified TeNT light chains, carrying the enzymatic activity or <sup>125</sup>I-tagged TeNT light chains to determine the efficiency of the toxin-uptake.

## MC stimulation and measurement of histamine release

MC histamine release was investigated as described [49]. Briefly, MC were stimulated with 1  $\mu$ M iono/PMA or by IgE receptor-cross linking through sensitization with myeloma IgE for 90 min and subsequently stimulation with 1  $\mu$ g/mL anti-human IgE Ab for 30 min. Histamine in the supernatants was measured by commercial RIA (Coulter-Immunotech, Krefeld, Germany).

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