The regulated exocytosis of enlargeosomes is mediated by a SNARE machinery that includes VAMP4

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Accepted 11 June 2008
Journal of Cell Science 121, 2983-2991 Published by The Company of Biologists 2008
doi:10.1242/jcs.032029

Summary

The mechanisms governing the fast, regulated exocytosis of enlargeosomes have been unknown, except for the participation of annexin-2 in a pre-fusion step. We investigated whether any SNAREs are involved. In PC12-27 cells, which are enlargeosome-rich, the expressed SNAREs exhibited various distributions (trans-Golgi network, scattered puncta, plasma membrane); however, only VAMP4 was colocalized in discrete puncta with the enlargeosome marker desmoyokin. The exocytosis of the organelle, revealed by capacitance increases and by surface appearance of desmoyokin, was largely inhibited by microinjection of anti-VAMP4, anti-syntaxin-6 and anti-SNAP23 antibodies, by incubation with botulinum toxin E, and by transfection of VAMP4 and syntaxin-6 siRNAs. Microinjection of the antibodies anti-VAMP7, anti-VAMP8 and anti-syntaxin-4, and transfection with the VAMP4 siRNA were ineffective. Inhibition of enlargeosome exocytosis by VAMP4 siRNA also occurred in a cell type that was competent for neurosecretion, SH-SY5Y. Moreover, in cells expressing a VAMP4-GFP construct, enlargeosome exocytosis and surface appearance of fluorescence occurred concomitantly, and many ensuing surface patches were co-labelled by GFP and desmoyokin. VAMP4, an R-SNARE that has never been shown to participate in regulated exocytoses, therefore appears to be harboured in the membrane of enlargeosomes and to be a member of the machinery mediating their regulated exocytosis. Syntaxin-6 and SNAP23 appear also to be needed for the process to occur; however, the mechanism of their participation, whether direct or indirect, remains undefined.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/18/2983/DC1

Key words: PC12 cells, SNAP23, Syntaxin-6, Ca2+, Ahnak

Introduction

Enlargeosomes are small vesicles, first identified in a clone of rat neuroblastoma PC12 cells that were defective of neurosecretion – the PC12-27 clone (Borgonovo et al., 2002) – and then shown to exist, although at lower levels, also in a variety of other cell lines, in primary cultures and in the cells of various types of tissues. Among the enlargeosome-positive cells are: wild-type (wt) PC12 differentiated by treatment with nerve growth factor (NGF); macrophages; dendritic cells; astrocytes; and fibroblasts (Borgonovo et al., 2002; Cocucci et al., 2004; Falcone et al., 2006). All attempts made for the isolation of enlargeosomes as a pure subcellular fraction have failed. Therefore, their precise composition is unknown. So far, however, no diffusible molecules segregated within the lumen of the vesicles have been demonstrated. Therefore, the main property of enlargeosomes, their rapid exocytosis (t½ ~1 second) in response to μM rises of the cytosolic Ca2+ concentration ([Ca2+]i), seems to not induce the discharge of secretion products but only to cause the enlargement of the plasma membrane (Borgonovo et al., 2002; Cocucci et al., 2004; Cocucci et al., 2007; Kasai et al., 1999). This result, which appears to meet the needs of cells undergoing processes of rapid surface dynamics and expansion, including growth, differentiation, migration, plasma-membrane internalization and wound healing, might be of importance not only in physiological, but also in pathological, conditions.

Regulated exocytoses, as well as most other processes of specific membrane fusion, at the surface and within the cells, are triggered by the interaction of SNARE proteins harboured in the two interacting membranes, through the formation of complexes composed by four coiled-coil SNARE motifs (Jahn and Scheller, 2006). In view of its rapidity, enlargeosome exocytosis might be considered similar to the transmitter-release exocytoses of neurons and neurosecretory cells, which are triggered by two Q-SNAREs of the plasma membrane, syntaxin-1A (Stx1a) and SNAP25, together with the R-SNARE of the clear and dense-core vesicles, VAMP2. In the defective, enlargeosome-rich PC12-27 cells, however, these SNAREs, as well as the other proteins of the vesicles and of their exocytosis, are lacking (D’Alessandro et al., 2008; Malosio et al., 1999). Moreover, enlargeosome exocytosis is unaffected by tetanus toxin (Borgonovo et al., 2002), a result that excludes not only VAMP2, but also the other VAMP proteins that are sensitive to the toxin, i.e. VAMP1 and VAMP3, in this process. Therefore, enlargeosome exocytosis could be triggered only by other SNAREs, which, however, have remained unknown. The only protein that has been shown to be needed for the exocytosis of enlargeosomes is the actin-associated protein annexin-2, which is most probably active at a pre-fusion step (see Chasserot-Golaz et al., 2005; Knop et al., 2004), in resting PC12-27 cells, annexin-2 is bound to the cytosolic surface of these organelles (Lorusso et al., 2006).
In this study, using various experimental approaches, we monitored the possible involvement of several SNARE proteins in the exocytosis of enlargeosomes, investigated in the PC12-27-clone model. The advantages of using PC12-27 cells are multiple. Compared with the other cells mentioned above, they contain the highest concentration of this organelle. Moreover, in PC12-27 cells, other types of regulated exocytosis are not large enough to interfere with the revelation of enlargeosome exocytosis. However, in order to exclude that the results obtained with PC12-27 are specific only to that defective clone, and not to the enlargeosomes of other cells, we have extended some of the experiments to human SY5Y cells, which express enlargeosomes and, in addition, are also competent for neurosecretion. Our evidence demonstrates that enlargeosome exocytosis is mediated by a SNARE machinery that includes VAMP4, an R-SNARE that, so far, had never been shown to operate in a regulated exocytosis, but only in various membrane fusions taking place in the trans-Golgi network (TGN) area and in some forms of endocytosis (Ahras et al., 2006; Hinners et al., 2003; Moore et al., 2002; Peden et al., 2001; Steegmaier et al., 1999; Tooze et al., 2002; Tran et al., 2007; Wendler and Tooze, 2001; Zeng et al., 2003). Other SNAREs that might be involved are SNAP23, a ubiquitous Q-SNARE active in a large number of other exocytoses, regulated and constitutive (e.g. Bao et al., 2008; Castle et al., 2002; Feng et al., 2002; Predescu et al., 2005; Puri and Roche, 2006; Reales et al., 2005; Wang et al., 2007), and Stx6, another Q-SNARE, also known to operate in the TGN, in which it appears to interact with VAMP4 (Katsumata et al., 2007; Steegmaier et al., 1999; Wendler and Tooze, 2001). In the case of these two Q-SNAREs, however, the mechanism of involvement in enlargeosome exocytosis, whether direct or indirect, is still undefined.

Results
Expression and distribution of SNAREs in PC12-27 cells

Fig. 1 illustrates the expression of various SNARE proteins in PC12-27 cells, as revealed by western blotting. The results confirm that levels of the three neurosecretory SNAREs – VAMP2, Stx1a and SNAP25 – are hardly appreciable or inappreciable with the technique employed (Malosio et al., 1999). By contrast, other SNAREs are quite evident. In particular, the four additional VAMPs that were investigated, VAMP3, VAMP4, VAMP7 and VAMP8, exhibited prominent bands. Also prominent were the three syntaxins – Stx4, Stx6 and Stx13 – and SNAP23. In Fig. 1, the protein shown in the right-hand panel is annexin-2, which is abundant in PC12-27 cells compared with many other cells (Lorusso et al., 2006).

The intracellular distribution of these SNARE proteins and of annexin-2 in relation to the distribution of desmoyokin (also known as Ahnak), a marker bound to the luminal face of the enlargeosome membrane (Borgonovo et al., 2002; Lorusso et al., 2006), was investigated by immunofluorescence of Triton-X-100-permeabilized cells. Fig. 2A-D shows typical deconvolved images acquired with a wide-field microscope from cells immunolabelled for three VAMPs and annexin-2, respectively. Double stainings of desmoyokin with the three syntaxins and SNAP23 are shown in Fig. 3. All the SNAREs that were expressed in the cells (left images) appeared widely distributed in the cytoplasm; out of these proteins...
and annexin-2, increased labelling of the surface area was evident for annexin-2 (Fig. 2), Stx13 and SNAP23 (Fig. 3). VAMP4, investigated using two polyclonal antibodies of commercial origin and two monoclonal antibodies produced in the laboratory, exhibited a strong labelling in the perinuclear area corresponding to the TGN, as previously reported by others (Hinners et al., 2003; Peden et al., 2001; Steegmaier et al., 1999; Zeng et al., 2003). This labelling was accompanied by a number of punctae scattered in the cytoplasm, including at the rim proximal to the plasma membrane. Interestingly, almost all punctae (>90%, counted in ten cells) positive for desmoyokin (Fig. 2, middle image) coincided with scattered puncta positive for VAMP4. Therefore, in the merged image to the right (Fig. 2), these punctae appeared not red but yellow. By contrast, many other cytoplasmic punctae, as well as the perinuclear structure heavily labelled for VAMP4, were negative for desmoyokin and therefore stained green in the merged image (Fig. 2A, right image). Also, Stx6 and, to a lesser extent, Stx13 exhibited strong labelling in the perinuclear area (Fig. 3B,C), in which desmoyokin was lacking (Fig. 2D). In the case of these two syntaxins, of Stx4 (Fig. 3A), VAMP7 and VAMP8 (Fig. 2B,C, respectively), but not of annexin-2 (Fig. 2D) (Lorusso et al., 2006), the apparent colocalization with desmoyokin in the punctae that were spread in the cytoplasm, counted in ten cells, remained below the level of significance (10%) (Cocucci et al., 2004), as shown by the segregation of green and red dots in the merged images (Figs 2 and 3). Near the surface, a moderate degree of apparent colocalization with desmoyokin was appreciable in the case of SNAP23 and also of Stx13 (Fig. 3C,D).

**Effects of antibody microinjection on enlargeosome exocytosis**

Evidence of enlargeosome exocytosis was first obtained by monitoring the rapid capacitance increases taking place in patch-clamped cells upon [Ca^{2+}]_{i} rise induced by photolysis of caged Ca^{2+} compounds (Cocucci et al., 2004; Kasai et al., 1999). In order to establish whether this approach was appropriate to reveal the role of single SNAREs, we carried out experiments in patch-clamped cells that had been microinjected, at least 2 hours earlier, with specific antibodies. The results obtained are illustrated in Fig. 4A-E. Photolysis of the caged Ca^{2+} compound NP-EGTA, which increased [Ca^{2+}]_{i} of the cells to 3-4 μM (Fig. 4A-D, lower traces), was found to increase the capacitance of non-microinjected control cells (Fig. 4A, upper trace) to reach average values corresponding to 10±2.7% increases of the cell surface (Fig. 4E). In cells microinjected with antibodies against proteins not expressed by the cells, i.e. a monoclonal antibody against the secretory protein chromogranin-B (ChgB) (Fig. 4B,E) and a polyclonal antibody against chromogranin-A (ChgA) (not shown), the results were almost identical to those in non-microinjected cells. By contrast, the capacitance increases induced by photolysis of NP-EGTA were markedly reduced in cells microinjected with the antibody against the Q-SNARE SNAP23 and also in those microinjected with the antibody against annexin-2 (~58±10.5% and ~32±21%, respectively, compared with non-microinjected cells, Fig. 4C-E). The result with annexin-2 confirms the participation of the protein in the regulation of enlargeosome exocytosis, already shown by different techniques (Lorusso et al., 2006).

A second approach to investigate the exocytosis of enlargeosomes in microinjected cells was based on the surface appearance of the organelle marker desmoyokin, revealed by immunofluorescence of non-permeabilized cells fixed upon treatment with the Ca^{2+} ionophore ionomycin (3 μM, 5 minutes). Also, in this case, the responses in groups of 20-25 cells (Fig. 4N) were the same in the two types of control cells, non-microinjected and microinjected with anti-ChgB (Fig. 4F) and anti-ChgA (not shown). Likewise, no change was observed in the cells microinjected with antibodies against VAMP7, VAMP8 or Stx4 (Fig. 4J,K,L,N). By contrast, and in agreement with the results obtained by patch-clamping (compare Fig. 4N to 4E), the responses in the cells microinjected with the anti-SNAP23 and anti-annexin-2 antibodies (Fig. 4G,H) were smaller (~64±10% and ~52±13%, respectively) compared with those in non-microinjected cells. Similar decreases were observed in cells.
microinjected with antibodies against VAMP4 (one polyclonal and one monoclonal) and against Stx6 (Fig. 4I,M; on average, –74±23% and –63±9%, see Fig. 4N). Interestingly, an analogous decrease (–59±8.7%) was observed in cells incubated for 12 hours with botulinum toxin E (Fig. 4O), which, in addition to its main target, SNAP25, is known to cleave SNAP23 of several animal species, including rat (Banerjee et al., 2001; Rao et al., 2004; Vaidyanathan et al., 1999).

Downregulation of SNAREs with siRNAs – effects on enlargeosome exocytosis

The first series of these experiments was carried out on PC12-27 cells transfected with siRNAs specifically targeting VAMP4 and Stx6, two SNAREs that the previous results had suggested have a role in enlargeosome exocytosis. siRNA targeting VAMP8 was used as a negative control. A scrambled siRNA failed to induce any effect on the levels of the three SNAREs (see supplementary material Fig. S1), whereas each of the three specific siRNAs induced a consistent downregulation of their respective target (on average, –50/60%; Fig. 5A). Enlargeosome exocytoses induced by ionomycin, revealed by the surface appearance of desmoyokin, was investigated in single transfected cells by a two-step protocol; i.e. after fixation, the non-permeabilized cells were first immunodecorated with anti-desmoyokin, then permeabilized and immunodecorated for each of the three SNAREs. The variability among the cells not exposed to the siRNAs was moderate (Fig. 5B,D,F, blue circles, triangles and squares for VAMP4, Stx6 and VAMP8, respectively). This situation changed dramatically in the downregulated cells. In terms of expression of their target SNARE, the cells of the populations exposed to each of the three siRNAs...
appeared distributed in two groups of similar size, the first with cells (most likely transfected) showing levels lower or much lower than the minimum observed in the untreated cells, the other with cells (most likely non-transfected) exhibiting levels in the range of untreated cells (Fig. 5B,D,F, red circles, triangles and squares for VAMP4, Stx6 and VAMP8, respectively). Most cells exhibiting normal SNARE levels also exhibited strong ionomycin-induced (3 μM, 1 minute) surface responses of desmoyokin (examples shown in Fig. 5C,E,G); those exhibiting low levels of either VAMP4 (Fig. 5C') or Stx6 (Fig. 5E') exhibited low or undetectable responses, i.e. they had little or no enlargeosome exocytosis. By contrast, those downregulated for VAMP8 exhibited similar enlargeosome-exocytosis responses in the whole population (Fig. 5F), independently of the levels of their SNARE expression (Fig. 5G,G', compare the surface red labelling). Overall, the correlation between SNARE expression and enlargeosome exocytosis was high for VAMP4 and Stx6 (>0.95 and >0.83), and below significance for VAMP8.

The PC12-27 cells employed in the present studies lack the classical SNAREs of neurosecretory cells, i.e. VAMP1, VAMP2, SNAP25 and Stx1a (see Fig. 1) (Borgonovo et al., 2002). Therefore, we cannot exclude that the involvement of unusual SNAREs, in particular VAMP4, in enlargeosome exocytosis is not in relation to a property of the organelle but results from the replacement of the classical SNAREs lacking in the defective cells. To investigate this possibility, we repeated the VAMP4 downregulation experiment in the neuroblastoma SH-SY5Y, a human cell line competent for neurosecretion and thus expressing VAMP2 and the other classical SNAREs, which in addition exhibits a good number of enlargeosomes (E.C. and J.M., unpublished). Panels A (right blots), H, I and I' of Fig. 5 illustrate the results obtained with SH-SY5Y cells transfected with siRNA against human VAMP4 siRNA, notice the decrease of VAMP4 (by 40%), with no change in VAMP2 levels. The top band (calnexin) is shown to certify that equal protein loads were seeded in the slots.

(B,D,F,H) The correlation between surface desmoyokin (d/A), documenting enlargeosome exocytosis, and the total level of individual SNAREs in single PC12-27 (B,D,F) and SH-SY5Y (H) cells non-transfected (blue signs) or transfected (red signs) with the siRNAs specific for the indicated SNAREs and then stimulated with ionomycin (3 μM, 1 minute) is shown. After fixation, the cells were first immunolabelled for surface desmoyokin, then permeabilized and immunolabelled for VAMP4 (circles, B and H), Stx6 (triangles, D) and VAMP8 (squares, F). (B) Notice that, in the population of PC12-27 cells exposed to VAMP4 siRNA (red circles), cells with levels of the SNARE that were distinctly lower than those of the untreated cells (blue circles) (by 90±1.7% on average in a group of 20 cells) exhibited a parallel decrease of enlargeosome exocytoses, revealed by the low level of surface appearance of desmoyokin (decreased by 89.9±1.8% compared with non-transfected cells). (H) A similar, although less intense, result was obtained in the SH-SY5Y cells transfected with the human siRNA (~94.7±1.9%) was dissociated from the surface appearance of desmoyokin, which was almost unchanged (~7.8±7.6%). (C,E,G,I) Examples of PC12-27 (C,E,G) and SH-SY5Y (I) cells in the populations of B, D, F and H, i.e. exposed to the siRNAs for VAMP4, Stx6 and VAMP8 and then stimulated with ionomycin, chosen because of their high (C,E,G) and low (C',E',G',I') labelling for the three SNAREs. Notice the clear desmoyokin surface labelling of C, E, G and I (yellowish in C and I because of the co-discharge of VAMP4) and its lack in C', E' but not in G'. Scale bar: 5 μm.
antigens from the surface. After stimulation with ionomycin, clear
very low, documenting the almost complete exclusion of the two
surface. The cells in B and C, also non-permeabilized, were fixed after stimulation with ionomycin (3 μM, 5 minutes) and then decorated with anti-EGFP and anti-desmoyokin antibodies, respectively. The yellowish labelling at the surface documents the incorporation of VAMP4 in the plasma membrane (B’), and its colocalization with desmoyokin (C’). (D,D’) PC12-27 cells were stimulated, permeabilized and immunolabelled for desmoyokin. Notice that the juxtanuclear EGFP labelling (the TGN) is green, whereas many punctae scattered in the cytoplasm in the proximity of the cell surface are yellowish, documenting the colocalization of VAMP4 and desmoyokin. Only a few green and red punctae are visible in the cytoplasm and also at the surface.

Discussion
Enlargeosomes are cytoplasmic organelles that are competent for a rapid regulated exocytosis, and have been recognized in a variety of cells and characterized in many respects, including the regulation and kinetics of the discharge and the recycling (Borgonovo et al., 2002; Cocucci et al., 2004; Cocucci et al., 2007; Kasai et al., 1999). So far, however, the information about the machinery of their exocytosis, in particular about the possible involvement of SNAREs, was only negative: no participation of the neurosecretory SNAREs VAMP2, Stx1a and SNAP25, and insensitivity of the process to tetanus toxin (Borgonovo et al., 2002; Malosio et al., 1999). By providing the first positive demonstration of the participation of SNAREs, the present results clarify a few mechanistic aspects of enlargeosome exocytosis. Although mostly carried out in the cell model known as the richest in enlargeosomes, the 27 clone of the rat PC12 pheochromocytoma cell line, our studies failed to reveal an exclusive or prevalent localization of any SNAREs in the enlargeosome membrane. The SNAREs necessary for the exocytosis to take place, in particular VAMP4 and Stx6, are in fact concentrated and operative also in other structures, in particular in the TGN.
Because of this wide distribution, classical experiments in the field, such as the co-immunoprecipitation and the co-recovery of the complexed SNAREs from single subcellular fractions, yielded inconclusive results (not shown). We therefore used an alternative approach, based on the inhibition (obtained by microinjection with specific antibodies and incubation with botulinum E toxin) and downregulation of several SNAREs expressed in PC12-27 cells and in another, neurosecretion-competent cell line, SH-SY5Y. Using this approach, we could exclude the involvement of a few SNAREs, i.e. VAMP7, VAMP8 and Stx4, in enlargeosome exocytosis and concentrate our attention on the others.

The SNARE shown to participate in enlargeosome exocytosis by both short-term and long-term experiments is VAMP4. This R-SNARE is expressed by a variety of cells. Its preferential localization in the TGN (Steegmaier et al., 1999) is due to specific motifs in its N-terminal cytoplasmic domain (Hinners et al., 2003; Peden et al., 2001; Zeng et al., 2003). In the TGN, VAMP4 together with Stx6 and synaptotagmin-4 participates in the maturation of secretion vesicles (Ahras et al., 2006; Moore et al., 2002; Tooze et al., 2002; Wendler and Tooze, 2001; Williams and Pessin, 2008). In addition, VAMP4 has been shown to cycle to the plasma membrane via constitutive exocytic vesicles, and back to the TGN via early and recycling endosomes (Tran et al., 2007). So far, however, VAMP4 had never been shown to participate in a regulated exocytosis.

Our results confirmed the main localization of VAMP4 in the TGN and, in addition, revealed the existence, in PC12-27 cells, of a population of punctae, which were identified as enlargeosomes by their co-labelling for desmoyokin. The harvesting of VAMP4 in the enlargeosome membrane was further documented by the almost complete absence, revealed by immunofluorescence, of its EGFP-tagged construct from the surface of resting cells and by its co-appearance with desmoyokin upon stimulation, with generation of dually labelled patches. The partial segregation of the two proteins that was observed after a few minutes of ionomycin stimulation, and the appearance of desmoyokin-positive and VAMP4-EGFP-negative punctae observed in the cytosol of the cells, could be due to events that follow the exocytic fusion, such as the separate sorting of the two proteins in the plasma membrane and their recycling by two different endocytic systems (Tran et al., 2007; Cocucci et al., 2004; Cocucci et al., 2007).

The presence of VAMP4 in the enlargeosome membrane correlated well with the results of functional experiments, in particular with extensive inhibition of the regulated exocytic process observed in two conditions: after microinjection of either a polyclonal or a monoclonal anti-VAMP4 antibody and after downregulation of the SNARE. The latter result, obtained first in PC12-27 cells, was duplicated in the human neurosecretion-competent cell line SH-SY5Y, known to express the enlargeosomes together with the classical neuroexocytic SNAREs (VAMP2, Stx1a, SNAP25) missing in the defective PC12-27 clone. Taken together, these results strongly suggest that the targeting motifs that keep VAMP4 away from the mature dense-core vesicles of neurosecretory cells (Hinners et al., 2003) do not preclude the transport of the protein to the enlargeosomes, making possible the direct participation of the SNARE in their regulated exocytosis, not only in the PC12-27 clone, but in another, and possibly in many, cell types in which the organelle is expressed. Enlargeosomes are known to differ from neurosecretory vesicles in many respects, including the luminal pH and the detergent resistance of their membranes (Cocucci et al., 2004). Whether these or other features are responsible for the different targeting of VAMP4 remains to be investigated.

Evidence suggesting participation to enlargeosome exocytosis was also obtained with two other SNAREs. In the case of SNAP23, the exocytic process, revealed by the capacitance increase and by the surface appearance of the marker, desmoyokin, was strongly inhibited in the cells that were microinjected with the specific antibody or pre-incubated with botulinum toxin E, which, in addition to SNAP25, is known to also cleave the murine SNAP23 (Banerjee et al., 2001; Rao et al., 2004; Vaidyanathan et al., 1999). SNAP23 is a ubiquitous Q-SNARE that is moderately overexpressed in PC12-27 cells (Grundschober et al., 2002). Previous studies had already shown SNAP23 to participate in a variety of exocytoses, both constitutive and regulated (e.g. Bao et al., 2008; Castle et al., 2002; Feng et al., 2002; Predescu et al., 2005; Puri and Roche, 2006; Reales et al., 2005; Wang et al., 2007), and to be able to replace, at least in part, its homologue SNAP25 in neurotransmitter release from neurons and chromaffin cells (Delgado-Martinez et al., 2007; Sorensen et al., 2003). Inhibition of enlargeosome exocytosis after antibody microinjection and downregulation was observed also with Stx6, a Q-SNARE known to participate in multiple membrane-fusion processes, in the TGN (maturity of secretory organelles) and in the endocytic pathway (Kuliawat et al., 2004; Watson and Pessin, 2000; Wendler et al., 2001; Wendler and Tooze, 2001). A role of Stx6 in the regulated exocytosis of human neutrophil granules had also been reported (Martin-Martín et al., 2000).

Our evidence regarding SNAP23 and Stx6, however, does not include any short-term results, such as those documenting the stimulation-induced surface appearance of patches co-labelled by VAMP4-EGFP and desmoyokin. Therefore, the possibility cannot be excluded that the role of these SNAREs in enlargeosome exocytosis is indirect, mediated by other, so far unidentified, processes that take place during the time necessary for the action of the antibody, botulinum E toxin or siRNA to occur. This possibility is strengthened by at least two considerations. First, a few hours inhibition of a SNARE can indirectly affect various membrane-trafficking processes. For example, caveolar endocytosis was dramatically reduced following inhibition of Stx6; however, this was not due to the direct participation of the SNARE to the process but upon the block of membrane microdomain delivery to the plasmalemma (Choudhury et al., 2006). Moreover, the SNAP motif of Stx6 appears structurally and functionally more homologous to the Qc motif of the SNAPs than to the Qa motif of other syntaxins (Bock et al., 2001; Misura et al., 2002). Therefore, a SNAP complex including both Stx6 and SNAP23 might be unlikely because it would be composed, together with the Qb motif of SNAP23 and the R motif of VAMP4, by two Qc and no Qa motifs.

In conclusion, our results strongly suggest that the regulated exocytosis of enlargeosomes involves a SNARE machinery that includes VAMP4, an R-SNARE believed so far to be involved only in constitutive membrane fusions, primarily in the TGN. Two other SNAREs, SNAP23 and Stx6, are also needed for enlargeosome exocytosis; however, their mechanisms of action, whether direct or indirect, are still undefined. Other SNAREs expressed in PC12-27 cells and known to work in both intracellular and surface-membrane fusions (Ishiki and Klip, 2005; Lippert et al., 2007; Proux-Gillardieux et al., 2005; Wang et al., 2007), i.e. VAMP3, VAMP7, VAMP8 and Stx4, appear not to be involved. Our data add a new chapter not only in the field of regulated exocytosis but also in that of VAMP4, which seems to be promiscuous in the choice of its partners, triggering several membrane fusions different not only in terms of location (at the surface and within the cell) but also of...
control (regulated and constitutive) and speed (fast and slow). These results appear consistent with the general conclusion by Jahn and Scheller (Jahn and Scheller, 2006) that the properties of the various membrane fusions are determined not only by the pairing of their SNAREs, but especially by additional layers of control that, in the case of enlargosomes, remain to be identified.

**Materials and Methods**

Cloned PC12-27 cells, the IgG2a-purified anti-desmoyokin and anti-ChgB antibodies, and two anti-VAMP monoclonal antibodies were produced and characterized in our laboratory as in Borgonovo et al. (Borgonovo et al., 2002). The monoclonal anti-Stx1a antibody was purchased from Sigma-Aldrich (Milan, Italy), the monoclonal IgG1, anti-annexin-2 from BD Biosciences Pharmingen (Heidelberg, Germany). The rabbit anti-ChgA and anti-VAMP7 polyclonal antibodies and botulinum toxin E were the kind gifts of Andrea Laslop (Dept. Pharmacology, University of Innsbruck, Austria), Troy Galli (Jacques Monod Institute, Paris, France), and Cesare Montecucco (Dept. Biomedical Science, University of Padua, Italy), respectively. Rabbit anti-VAMP2, anti-VAMP4, anti-Stx3, anti-Stx4, anti-Stx6, anti-Stx13, anti-SNAP23 and anti-SNAP25 polyclonal antibodies were purchased from Synaptic Systems (Goettingen, Germany); rabbit anti-VAMP3, anti-VAMP8 and a second anti-VAMP4 polyclonal antibody from AbCam (Cambridge, UK); rabbit polyclonal anti-calsequestrin from Streissgen Biotech. (Vicenza, Italy). Fluorescein isothiocyanate-conjugated and rhodamine-conjugated goat anti-mouse antibodies, goat anti-rabbit antibodies and goat anti-mouse IgG subclasses were purchased from Southern Biotechnology Ass. (Birmingham, AL), ionomicron was from Calbiochem (Schwalbach, Germany), the bicinchoninic acid (BCA) protein assay kit from Pierce Biotechnology (Rockford, IL). The cDNA of VAMP4-EGFP was the kind gift of Wanjin Hong (Institute of Molecular and Cellular Biology, Singapore). The siRNAs for VAMP4 (murine and human), VAMP8 and Stx6, and the scrambled siRNA were purchased from Ambion Europe (Cambridge, UK). All other chemicals were from Sigma-Aldrich (Milan, Italy).

**Cell culture, antibody microinjection, loading with botulinum toxin E** and transfection of VAMP4-EGFP and of siRNAs

PC12-27 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum (Euroclone, Wetherby, UK), 5% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (BioWhittaker, Verviers, Belgium) in a humidified 5% CO2 atmosphere. SH-SY5Y cells were cultured in a 1:1 mixture of DMEM and Ham’s F12 media supplemented with 10% fetal bovine serum. IgG immunoglobulins, purified by incubation with Sepharose–protein-G beads and washed extensively and then eluted with 0.1 M glycine buffer, pH 2.5, were buffered at pH 7.3 with Tris, diluted to 2-20 mg/ml in the microinjection mixture (150 mM K-gluconate, 2 mM MgCl2, 10 mM HEPES containing 6 mg/ml Na-fluorescein) and delivered to at least 30 cells per coverslip by the Femtojet microinjection system (Eppendorf), by applying 20-50 pA pulses for 0.5-1 seconds, with a compensation pressure of 1 pA. Microinjection, the cells were cultured for at least 2 hours to recover. Control cells received dilution buffer only. Botulinum toxin E (30 nM, dissolved in PBS) or PBS alone were added to the culture medium for 12 hours at 37°C.

Cells were transfected with the VAMP4-EGFP plasmid using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. Forty-eight hours after transfection, the cells were fixed by FACs cells. Transfection of siRNAs of rat and human VAMP4, of rat VAMP8 and syntaxin, and of scrambled siRNA was performed as in Lorusso et al. (Lorusso et al., 2006). In brief, the cells were transfected twice, 24 hours apart, with 100 nM siRNA and used 72 hours after the first transfection. The cells processed by these three treatments were stimulated with ionomicrin (3 μM, 1 minute) and then processed for desmoyokin surface immunofluorescence.

**SDS-PAGE, western blotting and biochemical assays**

Protein concentration of the samples was determined by the BCA method. Arginine transport was assayed using a gel stained with Coomassie Blue. Other gels were transferred to nitrocellulose filters that were processed at 22°C, first by blockade for 1 hour with 5% non-fat dry milk in TBS, then by incubation for 3 hours with the primary antibody diluted in PBS plus 3% BSA, followed by five 10 minute washes in TBS and incubation for 1 hour with the appropriate HRP-conjugated secondary antibody (1 μg/ml). After further washes in TBS and PBS, the filters were developed photographically by chemiluminescence, using the ECL western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK). Signals were acquired by the Personal Densitometer SI and Image Quant (Amersham Biosciences, Little Chalfont, UK). Quantitation of signals was made using the ImageJ program. 

**Patch clamping and [Ca2+]i measurements**

PC12-27 cells, incubated and patched as in Cocucci et al. (Cocucci et al., 2004), were recorded at 37°C in continuous perfusion. Compensated membrane capacitance (Cm), access conductance (Gm), membrane current and membrane potential were recorded and then analyzed. Whole patch-clamped PC12-27 cells, loaded with both NP-EGTA and Fura-6F, were illuminated with a monochromated light at 380 nm (Polychrome IV, Till Photonmetry System, Gräfelfing, Germany), short-pass filtered at 410 nm and reflected in the perfusion chamber by a dichroic mirror centred at 400 nm. A continuous illumination was sufficient to both uncage the NP-EGTA and motivate the [Ca2+]i ([Cocucci et al., 2004]. The [Ca2+]i sensitivitity of enlargosome exocytosis was assessed by ramp [Ca2+], increases generated by 30-second continuous illumination at 380 nm (Yang et al., 2005). The emitted fluorescence, transmitted through the dichroic mirror and filtered through a 470 nm barrier filter, was acquired by a Hamamatsu photomultiplier (Till Photonmetry System). The signal was recorded after filtering (300Hz, 4-pole Bessel). [Cav]i was calibrated as in Rupnik et al. (Rupnik et al., 2000).

We thank Ilaria Prada for her contribution; Tom Kirchhausen (Harvard Medical School) for hospitality and suggestions; Wanjin Hong (Institute of Molecular and Cell Biology, Singapore) for the gift of the VAMP4-EGFP construct; Andrea Laslop and Thierry Galli for antibodies; and Cesare Montecucco for the botulinum toxin E. During a short-term stay in Maribor, E.C. was the recipient of fellowships from FEBS, and the Italian Ministry of Research, MIUR.

**References**


