Enlargeosome, an Exocytic Vesicle Resistant to Nonionic Detergents, Undergoes Endocytosis via a Nonacidic Route^D ^V

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Enlargeosomes, a new type of widely expressed cytoplasmic vesicles, undergo tetanus toxin-insensitive exocytosis in response to cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) rises. Cell biology of enlargeosomes is still largely unknown. By combining immunocytochemistry (marker desmoyokin-Ahnak, d/A) to capacitance electrophysiology in the enlargeosome-rich, neurosecretion-defective clone PC12-27, we show that 1) the two responses, cell surface enlargement and d/A surface appearance, occur with similar kinetics and in the same low micromolar $[\text{Ca}^{2+}]_i$ range, no matter whether induced by photolysis of the caged Ca^{2+} compound *o*-nitrophenyl EGTA or by the Ca^{2+} ionophore ionomycin. Thus, enlargeosomes seem to account, at least in large part, for the exocytic processes triggered by the two stimulations. 2. The enlargeosome membranes are resistant to nonionic detergents but distinct from other resistant membranes, rich in caveolin, Thy1, and/or flotillin1. 3. Cell cholesterol depletion, which affects many membrane fusions, neither disrupts enlargeosomes nor affects their regulated exocytosis. 4. The postexocytic cell surface decline is $[\text{Ca}^{2+}]_i$ dependent. 5. Exocytized d/A-rich membranes are endocytized and trafficked along an intracellular pathway by nonacidic organelles, distinct from classical endosomes and lysosomes. Our data define specific aspects of enlargeosomes and suggest their participation, in addition to cell differentiation and repair, for which evidence already exists, to other physiological and pathological processes.

INTRODUCTION

Regulated exocytosis is commonly envisaged as the final step of regulated secretion, i.e., the stimulation-induced fusion with the plasmalemma of the membrane of secretory granules and vesicles, followed by the release of their segregated content (for reviews, see Gerber and Sudhof, 2002; Burgoyne and Morgan, 2003). Regulated exocytoses, however, are not always secretory. The ultimate task of many of them is in fact the transfer of membrane patches, or of their components, from a cytoplasmic compartment to the cell surface. Processes of this type are widespread among cells.

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Abbreviations used: antibody, mouse monoclonal antibody raised against d/A; $[\text{Ca}^{2+}]_i$, cytosolic concentration of free calcium; cdx, methyl- β -cyclodextrin; d/A, desmoyokin/Ahnak; DG, dense granule; DM-nitrophen, dimethoxynitrophenamine tetrasodium salt; EEA1, early endosomal antigen 1; fF, femtoFarad, unit of capacitance; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; Lamp1, lysosomal membrane glycoprotein 1; NP-EGTA, *o*-nitrophenyl EGTA; PC12, rat pheochromocytoma cell line; SLMV, synaptic-like microvesicle; TeTx, tetanus toxin; TGN, trans-Golgi network; TX-100, Triton X-100; wt, wild-type.

Many exocytic, nonsecretory membrane transfers were first revealed by observations documenting the stimulation-induced appearance at the cell surface of specific integral membrane proteins (Sheng and Lee, 2001; Bryant *et al.*, 2002; Brown, 2003). In contrast, in two fibroblast lines (Chinese hamster ovary [CHO] and 3T3; Coorssen *et al.*, 1996; Nimomiya *et al.*, 1996) and in PC12-27 (Kasai *et al.*, 1999), a neurosecretory clone defective of regulated secretion (Malosio *et al.*, 1999; Grundschober *et al.*, 2002), the first observations, made by electrophysiological capacitance/patch-clamp assays, consisted in considerable (15–30%) expansions of the plasma membrane in response to large increases of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), induced by photolysis of caged Ca^{2+} compounds. In PC12-27, these responses start a few hundreds of milliseconds after stimulation and develop rapidly ($t_{1/2} < 1$ s), sustained by the tetanus toxin (TeTx)-insensitive exocytosis of small vesicles (diameter < 0.1 μm ; Kasai *et al.*, 1999). The latter results, which exclude the involvement of the TeTx target protein, the vSNARE vesicle-associated membrane protein 2 (Schiavo *et al.*, 1992), differentiate the process from the regulated exocytoses of neurosecretory cells, such as wild-type (wt) PC12 and chromaffin cells, which are TeTx sensitive (Xu *et al.*, 1998; Kasai *et al.*, 1999).

Knowledge about nonsecretory exocytosis is not limited to electrophysiology. Seminal cell biological studies have been carried out by the use of a monoclonal antibody (mAb) (indicated as antibody) specific for an exocytic vesicle marker (Borgonovo *et al.*, 2002). The latter, identified as a

form of the high-molecular-weight protein desmoyokin/Ahnak (d/A) (Shtivelman and Bishop, 1993; Hashimoto *et al.*, 1995) was shown 1) to be expressed by many, but not all types of cells, of both animal tissues and cultured lines, including those where nonsecretory capacitance responses had been reported: CHO, 3T3, and PC12-27; 2) to be localized at rest within vesicles distinct from other cytoplasmic organelles; and 3) to undergo rapid, TeTx-insensitive transfer to the surface of the cell upon stimulation with the Ca^{2+} ionophore ionomycin. Evidence suggested the d/A-positive vesicles to have a role in the surface enlargements occurring in important processes, such as cell differentiation and plasma membrane repair. Based on these considerations, the exocytic vesicles marked by d/A were given the name of enlargeosomes (Borgonovo *et al.*, 2002).

In our previous study (Borgonovo *et al.*, 2002), the electrophysiological and cell biological results were assumed to be both due to enlargeosome exocytosis. However, this identification remained open to question. In fact, the capacitance increases induced by caged Ca^{2+} photolysis seemed to require considerable $[\text{Ca}^{2+}]_i$ rises (Kasai *et al.*, 1999), much larger than those induced by the ionophore used in the cell biological studies. Moreover, no comparative results existed about many aspects of the exocytic responses induced in the same cells by the two types of stimulation. Therefore, the possibility that the responses were due to two parallel processes sharing some properties, such as TeTx insensitivity, could not be excluded, also because knowledge about the properties of enlargeosomes was still limited.

Here, we report about an integrated electrophysiological/cell biological investigation of enlargeosome exocytosis and the ensuing endocytic process, carried out in the defective PC12-27 clone. Our results, on the one hand, document that the Ca^{2+} dependence of the responses induced by photolysis and ionomycin is not as different as reported previously, but rather falls in the same range; on the other hand, they reveal new and unexpected properties of the enlargeosome and of its exocytic process and demonstrate for the first time the postexocytic endocytosis of d/A-positive membranes.

MATERIALS AND METHODS

Cloned PC12-27, wild-type (wt) PC12, HeLa cells, and monoclonal antibodies, IgG_{2a} anti-d/A antibody and IgG_{2a} anti-chromograninB ClRO, were as in Borgonovo *et al.* (2002). TeTx and the IgG1 anti-lysosomal membrane glycoprotein 1 (Lamp1) monoclonal were gifts from C. Montecucco (University of Padua, Italy) and I. Mellman, (Yale University, New Haven, CT) respectively. The IgG1 anti-TGN 38 monoclonal and the anti-EEA1 polyclonal were from Affinity Bioreagents (Golden, CO); the IgG1 anti-transferrin receptor monoclonal was from Zymed Laboratories (San Francisco CA); the IgG1 anti-G58K monoclonal was from Abcam (Cambridge, United Kingdom); the anti-caveolin1 polyclonal and the IgG1 anti-flotillin1 monoclonal were from BD Biosciences Pharmingen (Heidelberg, Germany); the IgG1 anti-Thy1 monoclonal was from Serotec (Oxford, United Kingdom); cholera toxin-Alexa fluor488, *o*-nitrophenyl EGTA (NP-EGTA), and Fura-6F were from Molecular Probes (Eugene, OR); fluorescein isothiocyanate (FITC)-conjugated and tetraethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse, goat anti-rabbit, and goat anti-mouse IgG subclasses were from Southern Biotechnology Associates (Birmingham, AL); the E2-Link activated peroxidase kit and the Immunopure Fab kit were from Pierce Chemical (Rockford, IL); CypHer5 was from Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom; ionomycin was from Calbiochem (Schwalbach, Germany); and other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell Cultures

Media were supplemented with 2 mM L-glutamine, 100 U ml⁻¹ penicillin, and streptomycin (Cambrex Bio Science Verviers S.p.A., Verviers, Belgium). PC12 wt and PC12-27 were grown in DMEM with 10% horse serum (Euroclone, Wetherby, United Kingdom) and 5% fetal clone III serum (Hyclone Laboratories, Logan, UT); HeLa cells in the same medium without horse serum and with 10% fetal clone III serum; hybridoma cells in Iscove's mod-

ified Dulbecco medium medium with 10% fetal clone I serum, 5% macrophage conditioned medium, and 50 μM β -mercaptoethanol.

Patch-Clamp Experiments

Patch pipettes were pulled from borosilicate glass capillaries (GC150F-15; WPI, Sarasota, FL) with a horizontal puller (P-97; Sutter Instrument, Novato, CA) to a resistance of 1–4 M Ω in a solution containing 100 mM KCl, 10 mM tetraethylammonium-Cl, 40 mM KOH/HEPES, 2 mM Na₂ATP, 2 mM MgCl₂, 3.6 mM CaCl₂, 4 mM NP-EGTA, and 0.5 Fura-6F, pH 7.3. In ionomycin experiments, CaCl₂ and NP-EGTA were omitted. The bath contained 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 30 mM NaOH/HEPES, 4 mM CaCl₂, and 6 mM glucose, pH 7.3. All recordings were made at room temperature.

Membrane capacitance (C_m) and access conductance (G_a), compensated by C_m and G_a control, were measured by using a SWAM II C (Celica, Ljubljana, Slovenia), operating at 800-Hz lock-in frequency and by applying a sine voltage of 11-mV rms. The phase angle setting was determined by applying 1-pF pulses and monitoring their projection from the C (signal proportional to C_m) to G outputs of the lock-in amplifier. C_m , G_a , membrane current, and membrane potential were recorded unfiltered into a PC via an A/D converter (PCI-6035E; National Instruments, Austin, TX). WinWCP software (John Dempster, Strathclyde University, Glasgow, United Kingdom) was used to acquire and analyze data. Graphs were drawn using the Sigma Plot (SPSS, Chicago, IL).

$[\text{Ca}^{2+}]_i$ Measurements in Flash Photolyzed and Ionomycin-stimulated Cells

A UV flash from a Xe arc flash lamp (JML-C2; Rapp Opto-Electronic, Hamburg, Germany) was delivered, through an optical fiber to a 40 $\times$ fluor oil immersion objective of a Zeiss Axiovert 200 microscope, to whole-cell patch-clamped PC12 or PC12-27 loaded with both NP-EGTA and Fura-6F. The optical pathway included a combination of two mirrors, the first to merge 85% of flash light and 15% of fluorescence excitation light (M70-85/15 Rapp; Opto-Electronic, Hamburg, Germany); the second, a dichroic mirror (395 nm), to reflect both lights through the objective to the cell, with the emitted Fura-6F fluorescence passing back to the photomultiplier through a 420-nm filter. The Ca^{2+} sensor was excited by a monochromator at 380 nm, and the emitted light was acquired by a Hamamatsu photomultiplier (Till Photometry System, Gräfelfing, Germany); the signal was recorded after filtering (300 Hz, 4-pole Bessel). Ionomycin was applied directly to the extracellular solution. This implied a delay of the effects of ~ 30 s that was corrected in the traces. In each cell, $[\text{Ca}^{2+}]_i$ was calibrated by measuring autofluorescence (in the cell-attached configuration) and fluorescence (in a resting whole-cell recording) (Kreft *et al.*, 1999).

Differential Centrifugation

Suspensions of PC12-27 in 0.32 M sucrose, 5 mM HEPES, pH 7.4, and protease inhibitors were gently homogenized in a cell cracker (Borgonovo *et al.*, 2002), and the homogenates were centrifuged at 500 rpm for 5 min. The postnuclear pellet and the final supernatant were obtained by centrifuging the first supernatant at 40,000 rpm for 60 min.

Resistance to Nonionic Detergents

The postnuclear pellet, carefully resuspended in 0.32 M sucrose, 5 mM HEPES, pH 7.4, and protease inhibitors, was supplemented with Triton X-100 (TX-100), 0.5% final concentration. After 30 min in ice, the preparation was applied to either floatation (Schuck *et al.*, 2003) or top-down (Gagnoux-Palacios *et al.*, 2003) gradients. In the first, loading was in a 1.23 M sucrose cushion (2 ml) at the bottom of a SW 50.1 tube, covered with 2 ml of 1.10 M and then with 0.15 M sucrose to volume. In the second, the preparation in 0.32 M sucrose was applied in the same type of tube over two cushions of 1 and 1.3 M sucrose. After 18 h at 46,000 rpm, five fractions and the pellet were collected from either gradient and processed for Western blotting.

Monolayers of PC12-27 cells in phosphate-buffered saline (PBS), exposed in the cold to 1% TX-100 as described above, were fixed and processed for immunolabeling.

Cholesterol Depletion

Monolayers in the serum-containing medium were treated with 3.8 mM methyl- β -cyclodextrin (cdx) for 10–50 min, and then 0.5 $\mu\text{g}/\text{ml}$ cholera toxin-Alexa fluor488 was added (to monitor its endocytosis; Wolf *et al.*, 2002) and the incubation was pursued for 10 min. After 5-min treatment with 3 μM ionomycin or its solvent, cells were fixed and immunolabeled. For SDS-PAGE, cell processing was similar, but cholera toxin and ionomycin were not added.

SDS-PAGE and Western Blotting

Resuspended monolayers were solubilized in ice-cold medium containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 15 mM MgCl₂, 1 mM EGTA, 10% mM glycerol, and 1% TX-100, and then quickly centrifuged at 20,000 $\times g$ for 5 min to eliminate nuclei. Protein was assayed with bicinchoninic acid. For detergent resistance experiments, fixed volumes of the gradient fractions were

loaded for SDS-PAGE; otherwise, fixed amounts of protein were loaded. For Western blotting, gels transferred to nitrocellulose filters were first blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline (TBS), and then incubated for 3 h with the primary antibody diluted in PBS with 3% bovine serum albumin (BSA), washed in TBS (5-fold for 10 min), incubated for 1 h with the peroxidase-conjugated secondary antibody ($1 \mu\text{g ml}^{-1}$), washed again in TBS as described above and once in PBS, and developed by chemiluminescence (ECL Western blotting detection; Amersham Biosciences UK). Signals were acquired by Personal Densitometer SI and Image Quant (Amersham Biosciences).

Immunofluorescence and Immunoelectron Microscopy

For cell surface immunofluorescence, monolayers plated on poly-(L-lysine)-coated coverslips, at rest or after ionomycin treatment ($0.1\text{--}5 \mu\text{M}$; $0.5\text{--}5 \text{ min}$), treated with or without drugs and/or cholera toxin as indicated in figure legends, were fixed on ice for 10 min with 4% paraformaldehyde in PBS, pH 7.4, quenched with 0.1 M glycine, and then washed in PBS containing 0.3% BSA and 20% goat serum. The latter solution also was used for further washes and to dissolve antibodies. Exposure to the primary antibodies was for 2 h at 22°C , and then monolayers were washed extensively, exposed for 2 h to FITC- or TRITC-conjugated secondary antibodies, washed, mounted and analyzed. For quantifications, monolayers were processed as described above except that nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI). For establishing the cell percentage that had undergone exocytosis in 5-min application of various concentrations of ionomycin, d/A-positive and -negative cells were counted under blind conditions; for measuring quantitatively the d/A surface labeling at rest and after stimulation, the intensity of the signal in individual surface-labeled cells was established making reference to an arbitrary scale of six values, and the average score $\pm$ SE of 30–40 cell groups was calculated and expressed as percentage of the top fluorescence value. For the time course of the ionomycin-induced exocytic responses, parallel monolayers were fixed by rapid addition of paraformaldehyde (4% final concentration) at different times after application of $3 \mu\text{M}$ ionomycin and processed as described above. The labeling intensity of cell groups was established as described above.

For whole-cell immunofluorescence, the monolayers, fixed and quenched as described above, were washed with the PBS-BSA-goat serum solution with 0.3% TX-100. Treatment with antibodies was as for surface immunolabeling, except that the solution and washing PBS always contained 0.3% TX-100. In some experiments, the cells were processed by a combination of surface and whole-cell immunolabeling, i.e., they were first surface immunolabeled with antibody, and then permeabilized and whole-cell immunolabeled with other primary antibodies.

Immunofluorescence experiments of endocytosis were carried out as for surface immunolabeling, except that $1\text{--}5 \mu\text{M}$ ionomycin was administered at 37°C in the presence of antibody for 5–30 min. Monolayers were then washed, fixed, quenched, permeabilized, and labeled by the secondary antibody. In some surface and endocytosis experiments, the cells, after d/A immunolabeling and fixation, were washed with the PBS-BSA-goat serum supplemented with 0.3% TX-100 and then dually labeled for another marker following the whole-cell immunofluorescence protocol. Dual immunolabeled samples were analyzed quantitatively for colocalization of d/A with specific markers by using the ImageJ software to establish the fraction of pixels dually labeled above a threshold, according to the formula (dually labeled)/(d/A-labeled) $^{-1} \times 100$.

Immunofluorescent cells were studied using Bio-Rad MRC 1024 and Leica SP2 AOBs confocal microscopes. For image deconvolution aimed at blur removal and tridimensional cell reconstruction, optical sections, taken every 150 nm with a wide field microscope on the Delta Vision system, were analyzed with the soft WoRx Deconvolve software (Applied Precision, Washington, DC).

Electron microscopy of immunoperoxidase endocytosis was carried out as for immunofluorescence, except that antibody was conjugated to horseradish peroxidase whereas fixation was with 2% glutaraldehyde–4% paraformaldehyde. The diaminobenzidine reaction was carried out according to Ochs and Press (1992), except that H_2O_2 (0.03%) was included in the mixture, and the reaction was arrested after 20 min by washing with Tris buffer. Reacted monolayers were washed with cacodylated buffer, postfixed in 1% OsO_4 for 10 min, dehydrated, embedded in Epon, and examined in a Hitachi H7000 electron microscope.

RESULTS

The clone of the rat pheochromocytoma PC12 cell line used here, PC12-27, is advantageous for the present studies: it is rich with enlargeosomes (Borgonovo *et al.*, 2002); it maintains most of the molecular and structural properties typical of wt PC12, a neurosecretory line devoid of enlargeosomes, which can therefore be used for reference; it is specifically defective of the neurosecretion program, in particular it fails to express both types of neurosecretory organelles, synaptic-

like microvesicles (SLMVs) and dense granules (DGs), as well as the plasmalemma and soluble proteins instrumental to their exocytic discharge (Malosio *et al.*, 1999; Grundschober *et al.*, 2002); and it is very poor of lysosome exocytosis (Borgonovo *et al.*, 2002). Therefore, the participation of lysosomes to the cell surface enlargement induced by stimulation is negligible.

Ca^{2+} Dependence and Kinetics of the Nonsecretory Exocytosis

Our first aim was to establish whether in PC12-27 the rapid surface increase induced by photolysis of a caged Ca^{2+} compound, revealed by patch-clamp capacitance assay, occurs only in response to the high $[\text{Ca}^{2+}]_i$ rises ($50 \mu\text{M}$ or above) previously used by Kasai *et al.* (1999). These rises are much higher than those (a few micromolar only) induced by the Ca^{2+} ionophore ionomycin, which nevertheless are sufficient to trigger enlargeosome exocytosis, as revealed by the surface appearance of d/A, the enlargeosome marker (Borgonovo *et al.*, 2002).

To answer this question we replaced the previously used caged Ca^{2+} compound dimethoxynitrophenamine tetrasodium salt (DM-nitrophen) with another compound of lower Ca^{2+} affinity, NP-EGTA, whose induced $[\text{Ca}^{2+}]_i$ changes can be kept low, similar to those attained with ionomycin (Yang *et al.*, 2002). The capacitance responses induced by photolysis of NP-EGTA (maximal increases $\sim 8\%$ above the resting surface area; Figure 1c) were investigated in both the neurosecretory wt PC12 and in the defective PC12-27. The responses of the first were biphasic, composed by an initial small peak, possibly due to the exocytosis of SLMV, followed by the slower, higher, and more persistent increase due to DGs (Figure 1a). Both these increases were almost completely blocked by the intracellular perfusion of TeTx (Figure 1c). In contrast, in the defective PC12-27 the increases of capacitance were rapid, monophasic (Figure 1b), and unaffected by TeTx (our unpublished data). PC12-27 cells stimulated by photolysis of NP-EGTA also were processed for surface immunofluorescence to reveal enlargeosome exocytosis. As can be seen in Figure 1d, a cell fixed with paraformaldehyde a few seconds after photolysis seemed already surface positive for the specific marker d/A, documenting that enlargeosome exocytosis had quickly taken place.

Figure 2 shows results obtained in PC12-27 cells treated with $3 \mu\text{M}$ ionomycin. With this drug, the $[\text{Ca}^{2+}]_i$ rises were slow and were followed by delayed increases of capacitance, irregular in their outline (possibly due to the coexistence of some endocytosis), reaching plateaus within 3–4 min (Figure 2a; see also Huang and Neher, 1996). These traces were compared with the quantized surface immunolabeling responses induced by the ionophore, administered for different times (30 s to 5 min), followed immediately by quick fixation and immunodecoration of the cells. As can be seen in Figure 2b, the increase of the surface d/A signal induced by ionomycin followed a kinetics similar to that of the capacitance responses. As far as the concentration dependence, d/A became appreciable at the surface of a few PC12-27 cells already at $0.1 \mu\text{M}$ ionomycin (average $[\text{Ca}^{2+}]_i \sim 1.0 \mu\text{M}$). At $3 \mu\text{M}$ and above, the majority of the cells seemed positive (Figure 2c), most often showing strong immunolabeling signals (Figure 2d; Borgonovo *et al.*, 2002).

The Enlargeosome Membrane Is Resistant to Nonionic Detergents

So far, little was known about the enlargeosome membranes. In particular, their resistance to nonionic detergents, a prop-

Figure 1. NP-EGTA photolysis-induced exocytosis in wt PC12 and PC12-27 cells. (a and b) $[Ca^{2+}]_i$ increases and the ensuing plasma membrane capacitance responses induced by photolysis of the caged Ca^{2+} compound in representative wt PC12 and PC12-27 cells, respectively. The biphasic capacitance trace of the wt PC12 (a) is probably accounted for by the rapid exocytosis of SLMVs followed by the slow exocytosis of DGs; the rapid response of the PC12-27 cell (b) may be sustained, entirely or in part, by the exocytosis of enlargeosomes. In agreement with this possibility, PC12-27 cells fixed a few seconds after photolysis and then immunolabeled, show an intense surface positivity for d/A (d). (c) On average, the maximal capacitance increase (expressed as percentage of the cell surface area at rest) was approximately the same in wt and defective PC12 cells and in wt PC12, this response was blocked by tetanus toxin ($4 \mu M$ inside the pipette; Xu *et al.*, 1998). The bars in d and in all immunofluorescence panels of the subsequent Figures correspond to $10 \mu m$.

erty dependent on the cholesterol and phospholipid composition, had not been investigated. A postnuclear particulate fraction was therefore resuspended in 0.5% TX-100 at $4^\circ C$ and analyzed 30 min later by both floatation (Figure 3) and top-down (our unpublished data) sucrose gradient centrifugation, with consistent results. As can be seen in Figure 3,

c and d, the markers of organelles known to be mostly solubilized by TX-100, i.e., transferrin receptor (recycling endosomes; Figure 3d) and G58K (Golgi cisternae; Figure 3c), were recovered almost completely in the dissolved, nonfloated fractions 4 and 5, whereas the endoplasmic reticulum (ER) chaperone proteins calreticulin and calnexin

Figure 2. Ionomycin-induced exocytosis in PC12-27 cells. (a) Increase of capacitance that follows the $[Ca^{2+}]_i$ rise in a representative cell exposed to ionomycin ($3 \mu M$, arrow). Notice the slow rate of both responses and the irregular outline of the capacitance trace, which could be due to concomitant membrane endocytosis. The capacitance bar of 500 fF corresponds to $\sim 2.5\%$ of the initial cell surface area. (b) Time course of the d/A surface appearance, given for each time point as the average fluorescence intensity in a population of 30–40 cells exposed to ionomycin for the indicated times, and then rapidly fixed and surface immunolabeled without permeabilization. Notice that the curve of this panel, expressed in percentage of the top value of the scale, resembles roughly the capacitance trace of a. The last point of the trace corresponds to 300 s of stimulation. (c) Concentration dependence of the ionomycin-induced d/A surface appearance, expressed as the percentage of positive cells in a population of scanned PC12-27. In the positive cells, the intensity of the surface fluorescence signal did not remain the same but increased considerably as a function of the ionomycin concentration, in parallel to the percentage of cell positivity, reaching high values such as those of d, which shows cells exposed to $3 \mu M$ ionomycin for 5 min.

Figure 3. Effects of TX-100 on the distribution of d/A in a floatation gradient, compared with other membrane markers, and on the d/A immunolabeling of PC12-27 cells. The fractions of the floatation gradient (a–d) were analyzed by Western blotting. Of the fractions, P is the pellet, 5 and 4 include the components solubilized by TX-100, 3 and 2 (shadowed) are the detergent-resistant fractions, and 1 is the top, 0.15 M sucrose layer. The SDS-PAGE slots of all lanes except 1 were loaded with 100- μ l samples of the fractions recovered from the gradient. Values are given as percentage of recovery of the markers in the various fractions. The traces in a refer to d/A and total protein (tp); those in b to caveolin (Cav), Thy1 and flotillin1 (Flot); those in c to the Golgi (G58K) and TGN (TGN38) markers; those in d to the transferrin receptor (TFR), and the endoplasmic reticulum proteins calnexin (Cnx) and calreticulin (CR). (e and f) d/A immunolabeling of PC12-27 cells fixed before (e) and after (f) treatment with TX-100 (1%, 30 min at 4°C) and then processed for whole-cell immunofluorescence.

(Figure 3d), and especially the *trans*-Golgi network (TGN) marker TGN38 (Figure 3c), exhibited a dual distribution, with similar recovery in the dissolved and in the floated 2 and 3 fractions (shadowed areas in Figure 3). In contrast, well known markers of resistant membranes, caveolin1 (van Deurs *et al.*, 2003) and Thy1 (also known as CD90) (Simons and Toomre, 2000), were recovered mostly (>65%) in the floated fractions (Figure 3b) together with only 20% of total protein (Figure 3a). In the case of flotillin1, another recognized resistance marker (Bickel *et al.*, 1997), the recovery in the dissolved fractions 4 and 5 (~30%) was higher and that in the resistant fractions 2 and 3 (<50%) lower than those of caveolin1 and Thy1 (Figure 3b). The recovery of d/A, on the other hand, was similar to that of the latter two resistance markers (Figure 3a). Consistently, the d/A immunofluorescence pattern was apparently unchanged by the TX-100 treatment applied to PC12-27 cells before fixation (compare in Figure 3, f and e), whereas in the same cells the transferrin receptor immunofluorescence was completely removed by the treatment (our unpublished data).

The detergent resistance of d/A opened the possibility of enlargeosomes to be one of the known organelles specific for the resistance markers. Previous studies with anti-TGN38 and anti-ER markers had already excluded the colocalization of antibody with the corresponding antigens (Borgonovo *et al.*, 2002). As far as the other markers (Figure 4, a–c; quantitative data in Figure 4d), an apparent minor d/A colabeling was observed in PC12-27 with both Thy1 (Figure 4b) and flotillin1 (Figure 4c), whereas with caveolin1 (investigated in HeLa cells, which are also rich of enlargeosomes; Borgonovo *et al.*, 2002), because the available antibody recognized poorly the fixed rat protein of PC12-27, the immunolabeling pattern seemed almost completely distinct from that of the enlargeosome marker, which was similar to that of PC12-27 (Figure 4a).

Immunolabeling of membrane-resistant markers was carried out also in ionomycin-stimulated cells. To focus on the

surface-exposed d/A and compare its distribution with that of the other markers, which are not exposed to the extracellular space, we used the combined protocol of surface and whole-cell immunolabeling, i.e., fixed cells were first exposed to antibody, then permeabilized and finally exposed to the primary antibody against one of the three investigated markers. As can be seen in the images of Figure 4, e–g, and in the corresponding quantitative data of Figure 4h, no coincidence was observed between exocytized d/A and caveolin1 (Figure 4, e and h). In contrast, some of the stimulation-induced d/A surface labeling seemed to coincide with Thy1 (Figure 4, f and h) and especially with flotillin1 (Figure 4, g and h). Part of this coincidence remained evident after deconvolution of the images (see Supplemental Material Video I for a tridimensional rendering of the surface interaction between Thy1 and d/A).

Cholesterol Depletion Does Not Inhibit Enlargeosome Exocytosis

In view of their detergent resistance, enlargeosome membranes seem to be rich in cholesterol. We therefore investigated whether, and to what extent, the structure and function of the vesicle are affected by incubation of PC12-27 cells with the cholesterol-extracting agent cdx (3.8 mM) for up to 60 min. During the last 10 min of the cdx treatment, cholera toxin-Alexa fluor488 was added to monitor an independent process, toxin endocytosis (Wolf *et al.*, 2002). In PC12-27 cells, the latter process is inhibited almost completely by 1-h incubation with cdx (compare Figure 5, a and c; see also Supplemental Material Figure 1), in parallel with the drop of cholesterol revealed by cell labeling with filipin (our unpublished data). In contrast, endocytosis of the transferrin receptor is unaffected by cdx (our unpublished data).

Cholesterol-depleted PC12-27 cells exhibited some structural alterations, such as flattening and/or shrinkage of the cell body with redistribution of the enlargeosomes from the

ionomycin -

ionomycin +

Figure 4. Dual immunolabeling of HeLa and PC12-27 cells for d/A (red) and raft markers (green). (a–c) HeLa (a) and PC12-27 (b and c), investigated by whole-cell immunolabeling while at rest. d/A puncta, localized primarily in the superficial layers of the cytoplasm, show very little apparent colocalization with caveolin1 (a), localized primarily at the cell surface. With Thy1 (b), and, especially, with flotillin1 (c), which are distributed to the whole cytoplasm, the apparent colocalization is still low but more pronounced than with caveolin1. The flotillin1 immunolabeling of the nucleus is an artifact. In d, the apparent colocalization of the three raft markers with d/A in the cytoplasm of groups of 10 resting cells is given in quantitative terms. The HeLa cells (e) and the PC12-27 cells (f and g) were stimulated with ionomycin (3 μ M, 5 min). After fixation they were first surface immunolabeled for d/A, and then permeabilized and whole-cell labeled for the other markers. Under these conditions, therefore, apparent colocalization can be observed only at the cell surface. Notice that such a colocalization of d/A with caveolin1 (e) is very low, with Thy1 (f) is larger but variable, whereas with flotillin1 (g) it is considerable, as confirmed by the quantitative analyses of h, which refers only to the cell surface. For the Thy1-d/A surface colocalization, also see Supplemental Material Video I, showing a tridimensional rendering.

subplasma membrane area to the whole cytoplasm, accompanied by their partial aggregation in one or a few large clumps (Supplemental Material Figure 1, compare e and f to d; and Video II for a deconvolution analysis). Within living cells, however, most enlargeosomes were not disrupted, as documented by the good preservation of the d/A band in Western blots prepared from total cell preparations (Supplemental Material Figure 1g; Borgonovo *et al.*, 2002). In contrast, in Western blots prepared from gently homogenized, cdx-pretreated cells (but not from control cells), the d/A band seemed converted into a ladder (Supplemental Material Figure 1h). This suggests that the cholesterol-depleted organelles had not resisted the homogenization insult, releasing the marker that was then cleaved by cytosolic proteases.

Exocytosis of enlargeosomes was not blocked by cholesterol depletion. Compared with control cells, where cholera toxin endocytosis was considerable (Figure 5, a and b) and only little surface d/A labeling was visible before stimulation (Figure 5, a and e), the cdx-treated cells showed a progressive decrease of cholera toxin endocytosis (Figure 5, c and d; Supplemental Material Figure 1, a–c) accompanied

by an increase of the resting d/A surface signal (Figure 5, c and e). The latter, however, was still reinforced by ionomycin (3–5 μ M), applied for 5 min after 60 min of cdx treatment, to an extent similar to that of cdx-untreated cells (compare Figure 5, b and d; Figure 5e). We conclude that cholesterol depletion affects the traffic of enlargeosome membranes at the surface of resting cells without inhibiting significantly the responses induced by the Ca^{2+} ionophore.

Postexocytic Endocytosis

In the previous patch-clamp studies, the capacitance increases triggered in PC12-27 cells by photolysis of DM-nitrophen persisted in all cases until the end of the recordings (Kasai *et al.*, 1999). Whether this was due to a lack of endocytosis or whether it was an artifactual consequence of the high $[\text{Ca}^{2+}]_i$ rises induced in the cells to stimulate exocytosis remained unclear. To investigate this point, a systematic analysis was carried out in PC12-27 cells stimulated at lower $[\text{Ca}^{2+}]_i$ by photolysis of NP-EGTA. Figure 6a illustrates a photolyzed cell ($[\text{Ca}^{2+}]_i \sim 4 \mu\text{M}$), representative of an $\sim 60\%$ subpopulation of analyzed cells, in which the increased capacitance remained almost stable during the first 30 s after

Figure 5. Effects of cholesterol depletion on enlargeosome exocytosis. PC12-27 cells, pretreated (c and d) or not (a and b) with cdx for 60 min, were all exposed to cholera toxin-Alexa488 (green) during the last 10 min of the pretreatment, followed (b and d) or not (a and c) by ionomycin stimulation ($3 \mu\text{M}$, 5 min). After fixation the cells were surface immunolabeled for d/A (red). Notice that in the cells not treated with cdx (a and b), the endocytosis of cholera toxin (green) was intense. The surface d/A labeling was inappreciable in the cells in a, as in all resting PC12-27 cells (Borgonovo *et al.*, 2002), and became clearly evident after ionomycin (red and yellow labeling of b). After cholesterol depletion (c and d), the general PC12-27 phenotype was altered, with flattening and or/shrinkage of the cell body. Endocytosis of cholera toxin was blocked (lack of green labeling). A clear surface d/A signal (red) was visible already in the resting cells (c) and was increased by ionomycin to levels similar to those of stimulated controls (compare d with b). (e) Quantitative summary (as in Figure 2b) of the surface d/A-labeling results obtained in groups of 10 cells from the populations illustrated in a–d.

photolysis and was largely unaffected by the application of further photolysis flashes inducing additional $[\text{Ca}^{2+}]_i$ increases. On the contrary, the cells in Figure 6, b and c, representative of the remaining subpopulation, showed appreciable declines of the NP-EGTA photolysis-induced capacitance increases, which were accelerated by further photolysis flashes (Figure 6c). These observations suggested that, at least in the second subpopulation, postexocytic endocytosis is regulated by $[\text{Ca}^{2+}]_i$ in the $3\text{--}5 \mu\text{M}$ range. Indeed, in the cells where photolysis had induced relatively low $[\text{Ca}^{2+}]_i$ rises (average $3.76 \pm 0.25 \mu\text{M}$; $n = 5$) the subsequent capacitance decreases were slow ($\sim 30 \text{ fF/s}$) and only partial during the first minute (Figure 6, d and e), whereas in those with higher $[\text{Ca}^{2+}]_i$ rises (average of $4.82 \pm 0.24 \mu\text{M}$; $n = 5$), the rate of decline was almost fourfold faster, and the values reached at 0.5–1 min after stimulation were close to resting (Figure 6, b–e).

In the cells stimulated with ionomycin, on the other hand, a large poststimulatory capacitance decline was never observed as long as the recording could be pursued (several minutes). Rather, the rate was slow and the decline was only partial. Irregularities of the traces, already mentioned during the initial capacitance rise, persisted and increased in the cells during decline (Figure 6f). Together with the data of Figure 2a, these results may suggest that, in the course of the ionomycin treatment, exo- and endocytosis take place concomitantly, with predominance of exocytosis during the first few minutes and of endocytosis thereafter.

d/A Endocytosis by Nonacidic Endosomes

The capacitance results of Figure 6 document that, in a significant fraction of stimulated PC12-27 cells, poststimulatory endocytosis takes place within the first minute. However, they provide no information about the nature and properties of the endocytic vesicles. To investigate whether the membrane exhibiting the enlargeosome marker d/A participates in the endocytosis, living cell monolayers were first exposed to antibody, its monovalent fragment (Fab-antibody), or an unspecific antibody applied with or without ionomycin ($3 \mu\text{M}$) for 0.5, 5, and 30 min at 37°C , and then cooled at 4°C , washed extensively, and finally fixed. The intracellular distribution of the endocitized antibodies was revealed by staining with the secondary antibody applied after membrane permeabilization with TX-100 and staining of nuclei with DAPI. No intracellular immunolabeling was appreciable in nonstimulated cells incubated for up to 30 min with either the antibody or the control, anti-chromograninB antibody (our unpublished data). Likewise, no labeling was observed when the latter antibody was applied as described above, however together with ionomycin (Figure 7d). In contrast, with antibody and Fab-antibody, similar patterns were observed, composed of puncta, initially small and distributed close to the surface of a few cells, later more and more numerous, enlarged and much more frequent in the cell population (Figures 7, b and c, and Figure 8). We conclude that, in PC12-27 cells, the enlargeosome marker d/A is involved in the poststimulatory endocytosis.

Differential properties of the d/A-positive endosomes were investigated by dual immunofluorescence with respect to specific markers: the early endosome antigen 1 (EEA1; Figure 8b) and the transferrin receptor (Figure 8c), known to reside in the two classes of the coated vesicle-derived endosomes, the sorting and the recycling endosomes, respectively (Maxfield and McGraw, 2004); TGN38 (Figure 8a) and Lamp1 (Figure 8d), the markers of TGN and lysosomes, where endosomes and endosomal proteins are often addressed. In all analyzed cells the d/A-positive puncta (red) were preferentially distributed to the cytoplasmic layers in the proximity of the cell surface, with no obvious overlapping with the other markers (green in Figure 8, a–d). The latter, on the other hand, exhibited their well known distribution: clustering in juxtanuclear areas for TGN38 (Figure 8a) and the transferrin receptor (Figure 8c); the superficial cytoplasmic layers for EEA1 (Figure 8b); a wide scattering throughout the cytoplasm for Lamp1 (Figure 8d). The lack of colocalization of these markers with d/A puncta was confirmed by a quantitative analysis of groups of 10 cells selected at random. In these groups, in fact, apparent coincidence did not exceed average values ($<10\%$) attributable to the low resolution power of confocal microscopy.

Subsequent experiments were carried out to establish whether the lumen of the d/A-positive endosomes is acidic, as it is the case of classical endosomes and lysosomes, or whether, in contrast, it is neutral. To investigate the prob-

Figure 6. Time course of capacitance after the stimulation-induced rise in PC12-27 cells: Ca²⁺ dependence of endocytosis. The three representative cells in a–c show jumps of [Ca²⁺]_i to ~4 μM induced by NP-EGTA photolysis causing parallel capacitance rises of ~600–2000 fF. In the cell of a, the increased capacitance remains largely stable, insensitive to further photolysis-induced [Ca²⁺]_i spikes. This cell belongs to the subpopulation in which endocytosis is delayed. In contrast, b and c show cells of the subpopulation in which endocytosis begins during the first minute. Notice that the rate of the process is faster in c, where subsequent photolysis spikes prevent the decrease of the [Ca²⁺]_i. The data in d and e illustrate the Ca²⁺ dependence of the endocytic process. Compared with a group of five cells reaching maximal [Ca²⁺]_i levels <4 μM (average 3.76 ± 0.25 μM), five cells reaching levels >4.5 μM (average 4.82 ± 0.24 μM) exhibit endocytic rates more than fourfold faster (d). Therefore, 30 s after stimulation, the average capacitance values of these cells correspond to an 82% decrease from the exocytic peak (e), i.e., they have returned near the resting values, whereas the average values of the lower [Ca²⁺]_i group have decreased of only 38%. (f) Time course of the capacitance in a representative cell stimulated with ionomycin (3 μM) administered at the arrow. Notice the irregularities of the trace which declines slowly and remains away from the resting values for the whole recording time (several minutes).

lem, antibody was coupled to a pH-sensitive dye, CypHer5, which is unstained in the neutral and turns red in the acidic environment (Adie *et al.*, 2003). Parallel experiments were carried out for reference with the dye coupled to the antibody against the lysosomal marker Lamp1. Figure 9a shows PC12-27 cells stimulated for 30 min with ionomycin in the presence of antibody-CypHer5, subsequently fixed, permeabilized, and stained with the FITC-conjugated secondary antibody. As can be seen, these cells exhibited numerous puncta that were all green. The results indicate that, in the endocytic organelles reached by d/A during the 30-min incubation, CypHer5 remains unstained, i.e., that the or-

ganelle lumen is neutral. In contrast, when the 30-min ionomycin stimulation was carried out in the presence, not of antibody but of Lamp1-CypHer5, the color of puncta was yellow (Figure 9b), as expected by the combination of the green of the FITC-secondary antibody with the red acquired by CypHer5 in the acidic environment of lysosomes. Similar results—green puncta with antibody-CypHer5 and yellow puncta with anti-Lamp-CypHer5—were obtained in further, longer incubation experiments carried out with and without stimulation by ionomycin. The results obtained in unstimulated PC12-27 cells incubated for 24 h with either antibody-CypHer5 or anti-Lamp1-CypHer5 are shown in Figure 9, c

Figure 7. d/A endocytosis in ionomycin-stimulated PC12-27 cells. The panels show the cellular distribution of antibody applied to living cells together with ionomycin for 0.5 (a), 5 (b), and 30 (c) min. Notice that at the early time point, only a small fraction of cells shows intracellular accumulation of d/A-positive puncta. Later, the number of positive cells increases and the number and intensity of their puncta also increase, reaching considerable apparent size at 30 min. With a control mAb, against a protein not expressed by PC12-27 cells (chromogranin-B), incubated with ionomycin (d) or without (our unpublished data), no intracellular labeling was observed.

Figure 8. Endocytic organelles positive for d/A are distinct from the TGN, classical endosomes, and lysosomes. The panels show groups of PC12-27 cells exposed to ionomycin (3 μ M) together with antibody (red) for 30 min, and then fixed and whole-cell labeled (green) for the TGN marker TGN38 (a), the sorting endosome marker EEA1 (b), the recycling endosome marker transferrin receptor (c), and the lysosome marker Lamp1 (d). Merging of the images of the dually labeled cells reveals only very low levels of apparent coincidence, documenting a lack of colocalization of d/A with the investigated markers. Notice also that the overall distribution of the d/A-positive endosomes in the cytoplasm is mostly peripheral not only with respect to the TGN and recycling endosomes but also to sorting endosomes and lysosomes. The green staining of nuclei in b is an artifact of the polyclonal anti-EEA1 antibody used.

and d, respectively. The neutral lumen, therefore, is not only a property of the d/A-positive, postexocytic endosomes but also of the organelles where the enlargeosome marker is recovered long time after endocytosis. Together with the dual labeling data of Figure 8, these results confirm that the d/A-rich endocytic/recycling organelles are distinct from classical endosomes/lysosomes.

The investigation of d/A endocytosis was pursued at the ultrastructural level. Previous attempts of enlargeosome immunoelectron microscopy had been largely unsuccessful because, after fixation with even low concentrations of glutaraldehyde, the antigen is poorly recognized by antibody (Borgonovo *et al.*, 2002). In the present study, we focused on the distribution and morphology of the organelles labeled by peroxidase-coupled antibody applied for 5–30 min to living PC12-27 cells during ionomycin stimulation. With the control mAb (against chromograninB, which is not expressed by PC12-27), administered at the same concentration and for the same times of antibody, no detectable intracellular labeling was observed (our unpublished data). In contrast, with antibody cytoplasmic organelles were labeled, although to variable extent. In the intensely positive cells, weakly labeled vesicles were accompanied by more numerous, distinctly larger (diameters between 80 and 150 nm), and strongly labeled profiles, often of irregular shape (Figure 10, a and c), that could be clustered in groups in the proximity to plasma membrane (Figure 10b). Deeper in the

Figure 9. Distribution of either CypHer-antibody or CypHer-anti-Lamp1 applied to PC12-27 cells during stimulation with ionomycin for 30 min (a and b) or while resting for 24 h (c and d). Notice that the organelles where antibody and anti-Lamp1 antibody accumulate exhibit different color labeling because of their different luminal pH. The d/A-positive puncta (a and c) are all green, due to the antibody immunolabeling only. In these puncta, therefore, CypHer is unstained, i.e., the lumen of the organelle is neutral. In contrast, the Lamp1-positive organelles, the lysosomes, are yellow (b and d), resulting from the merge of the green of the Lamp1 immunolabeling with the red that CypHer acquires in the acidic environment of the lumen.

cytoplasm, the organelle labeling was intense, concentrated primarily into vacuoles (300–600 nm in diameter), some of which containing discrete vesicles (Figure 10, c–f). These vacuoles often showed small, coated bulges, looking as budding vesicles (Figure 10, d and e). In the Golgi/TGN area, d/A-positive organelles were absent.

In the labeling conditions used, the diamino benzidine precipitate was always distributed in direct contact with the inner face of the organelle limiting membrane. Interestingly, the decoration seemed not random but distributed according to a pattern composed by rows of puncta aligned at ~ 40 nm, center-to-center distance from each other (Figure 10, a–e). This decoration pattern might be related to the structure of the d/A protein, composed by two terminal domains connected by >30 internal repeats (Shtivelman and Bishop, 1992), each including a site for antibody binding (Borgonovo *et al.*, 2002).

DISCUSSION

The occurrence in various cell types of regulated, nonsecretory exocytoses, taking place in response to large $[Ca^{2+}]_i$ increases and inducing considerable surface enlargements, had been shown several years ago by patch-clamp capacitance studies (Coorssen *et al.*, 1996; Ninomiya *et al.*, 1996; Kasai *et al.*, 1999). More recently, the search for the corresponding vesicles lead to the discovery of enlargeosomes, identified by their luminal marker, the high-molecular

Figure 10. Ultrastructure of d/A-positive endocytic organelles decorated by antibody-peroxidase. PC12-27 cells were incubated with antibody-peroxidase together with ionomycin as in Figure 7. Various forms of the d/A-positive organelles are shown in the panels. In a, three positive organelles, 100–300 nm in diameter, are distributed individually in the subplasmalemma area, at 1–1.5 μm distance from each other; in b, small (80–100 nm), lightly labeled profiles are clustered in the proximity of the plasma membrane together with larger, more intensely labeled vesicles; c shows at the top a small positive vesicle, with an organelle of 150 nm diameter and a larger irregular vacuole containing a few segregated vesicles located in the proximity, but not inside the Golgi/TGN area. Even larger, irregular vacuoles containing vesicles and with coated bulges, likely corresponding to budding vesicles (asterisks), are shown in d and e. The latter panel includes also another positive organelle and a vesicle. Finally, f shows an enlargement of the vacuole of e, illustrating the distribution of the DAB precipitate apposed to the luminal face of the limiting membrane. Examples of the periodic pattern of peroxidase labeling of the vesicle and vacuole luminal membrane surface are visible in all panels. In contrast to the d/A-positive organelles, the coated pits and vesicles (arrows and arrowheads in c and e) are all d/A negative. Bars, 0.5 μm , except in d and f, where bars are 0.2 μm . GC, Golgi complex.

weight, peripheral membrane protein d/A (Shtivelman *et al.*, 1992; Shtivelman and Bishop, 1993; Hashimoto *et al.*, 1995), which upon exocytosis remains bound to the cell surface (Borgonovo *et al.*, 2002). So far, however, the exocytoses revealed by capacitance and those revealed by immunocytochemistry were investigated separately. On the one

hand, some of their properties seemed common: both are due to small vesicles and both are Ca^{2+} dependent and insensitive to TeTx; on the other hand, one had been triggered rapidly ($t_{1/2} < 1$ s) by high $[\text{Ca}^{2+}]_i$ jumps (50 μM and above; Kasai *et al.*, 1999), the other was much slower and occurred already at low micromolar $[\text{Ca}^{2+}]_i$ (Borgonovo *et al.*, 2002).

Although very suggestive, therefore, the possibility that they are due to the same process was still open to question.

The results we have obtained by a combined, capacitance/immunocytochemical investigation strengthen the single process hypothesis, solving in particular the problem of the different Ca^{2+} dependence. By replacing the high-affinity caged Ca^{2+} compound DM-nitrophen with the lower affinity NP-EGTA, we showed that $[\text{Ca}^{2+}]_i$ rises much lower than those reported previously are sufficient to trigger the capacitance responses, i.e., they fall in the same range of those induced by ionomycin, the Ca^{2+} ionophore mostly used in the immunocytochemical studies. Moreover, the d/A exocytic responses induced by the ionophore were shown to develop concomitantly to slow, but considerable capacitance rises, following the $[\text{Ca}^{2+}]_i$ responses. It should be mentioned, however, that because the increase of the cell surface area induced by exocytosis of the d/A-positive vesicles could not be evaluated quantitatively, we cannot exclude that only part of the capacitance responses are accounted for by enlargeosomes, the rest being due to other, so far unknown exocytic organelles, exocytized in parallel and with similar properties.

Our results extend significantly our knowledge about enlargeosomes and their cellular role. The low micromolar $[\text{Ca}^{2+}]_i$ dependence of their exocytosis expands the range of physiological and pathological processes in which these vesicles could be involved. Some of these processes, cell differentiation and membrane repair, have already been identified (Borgonovo *et al.*, 2002; Cerny *et al.*, 2004). Moreover, the enlargeosome membranes were found to be resistant to nonionic detergents, a property attributed to the existence, in the plane of their membrane, of small microdomains (<100 nm in diameter), the rafts, rich in cholesterol and sphingolipids (Pralle *et al.*, 2000; Simons and Toomre, 2000; Anderson and Jacobson, 2002; Parton and Hancock, 2004), where specific proteins accumulate in a dynamic equilibrium with the other membrane domains (Kenworthy *et al.*, 2004). Extensive evidence in various organelles and processes (especially the TGN; endocytosis; constitutive exocytosis) has demonstrated the key role of rafts in membrane dynamics (fusions, fissions, trafficking, and interactions: for reviews, see Nichols, 2003; Helms and Zurzolo, 2004; Mayor and Rao, 2004). The abundance of rafts, as observed in enlargeosomes, is not common among the organelles competent for regulated exocytosis and could therefore play specific roles at various steps of the new vesicle life, including (in addition to exocytosis, which is discussed below): generation, presumably at specific domain(s) of the TGN (Gkantiragas *et al.*, 2001; Maxfield, 2002; Gleeson *et al.*, 2004); traffic and possible interactions, homologous and possibly also heterologous with other membrane systems. The recognition of the extensive detergent resistance, the first general property of the enlargeosome membrane so far identified, could serve in the study of these processes, for example, by providing criteria for the isolation and characterization of the vesicles together with important cues and tools for the interpretation of future results.

As a direct fall-out of membrane resistance results, we exposed the cells to cholesterol depletion, believed to disassemble the rafts and to reveal therefore their role in specific processes. Interestingly, this treatment had been reported to inhibit all types of regulated exocytosis investigated so far (Lang *et al.*, 2001; Ohara-Imaizumi *et al.*, 2004; Salaun *et al.*, 2004). In PC12-27 cells, depletion was amply effective, as revealed by the blockade of the cholera toxin endocytosis. Also, the enlargeosomes were affected, showing increased fragility, redistribution from the peripheral areas to the

whole cytoplasm, formation of clumps and vacuoles, accumulation of d/A at the surface of resting cells, possibly due to disturbance of the trafficking to and/or from the plasma membrane. However, the enlargeosome exocytic responses induced by ionomycin were unchanged, suggesting their possible independence from the existence of rafts. After the insensitivity to tetanus toxin, this seems to be another property that distinguishes the regulated exocytosis of enlargeosomes from those of other exocytic organelles.

The proposal of the enlargeosome as a new type of organelle was initially based on its distinction from the classical cytoplasmic structures, the ER, Golgi complex, TGN, sorting and recycling endosomes, lysosomes, glut4-rich vesicles, and constitutive secretory vesicles. The detergent resistance of the enlargeosome membrane opened now the possibility of a link to the other detergent-resistant organelles, in particular to caveolin-rich vesicles, caveosomes, and other known raft-rich vesicles trafficking between the TGN and the plasma membrane. Of the three raft markers investigated, however, caveolin1 was found to lack any colocalization with d/A, whereas Thy1 and also flotillin1, which is localized not in a single type of membrane but is distributed to various types (Morrow *et al.*, 2002; Kokubo *et al.*, 2003), exhibited only a low degree of colocalization. These results exclude the identification of enlargeosomes with organelles specific for the three markers. A considerable colocalization of d/A with flotillin1 became apparent at the cell surface after stimulation of exocytosis. Because, however, the resolution of confocal images is low compared with the size of the raft microdomains, the significance of the observation, in particular whether it was due to real intermixing of the exocytized d/A-rich membrane domains with those rich in flotillin1, remains to be established.

A property of the enlargeosome system that so far was completely unknown is endocytosis. In the previous patch-clamp and immunocytochemical studies (Ninomiya *et al.*, 1996; Kasai *et al.*, 1999; Borgonovo *et al.*, 2002; Cerny *et al.*, 2004), the process had not been specifically investigated. Now, we have found that a low-rate endocytosis of d/A-rich patches occurs even in resting cells, presumably as a consequence of spontaneous enlargeosome exocytic events, and that intense endocytosis follows the stimulation-induced exocytic responses. Also in the stimulated cells, however, endocytosis is markedly asynchronous: it is appreciable within one or a few minutes from the stimulation only in a fraction of cells, whereas in others it requires longer times to become well appreciable. For obvious technical reasons, only the cells of the first group could be studied by both patch clamping and immunocytochemistry, whereas with the others only the second experimental approach could be used.

Several aspects of the enlargeosome endocytosis are of interest. Among the PC12-27 cells investigated by patch clamping, those that responded to stimulation with $[\text{Ca}^{2+}]_i$ rises $>4.5 \mu\text{M}$ exhibited capacitance decrease rates several-fold faster than those that had reached values $<4 \mu\text{M}$. The enlargeosome endocytosis seems therefore to be regulated, a property that, in other cells and secretory systems, has already attracted great interest (Sankaranarayanan and Ryan, 2001; Sorkin and Von Zastrow, 2002; Maxfield and McGraw, 2004). Moreover, the intracellular vesicles positive for d/A maintained a neutral pH in their lumen. This result excludes the involvement of the endosomes generated from coated pits and vesicles, characterized by their classical acidic lumen (Maxfield and McGraw, 2004); however, it seems insufficient for the identification of the organelles involved. Various types of nonacidic endosomes have in fact been

described, trafficking independently or coordinately with each other and with acidic endosomes along multiple intracellular pathways (Nichols and Lippincott-Schwartz, 2001; Nabi and Le, 2003; Nichols, 2003; Parton and Richards, 2003; Massol *et al.*, 2004). Finally, a sequence of the events occurring within 30 min after the generation of the d/A-positive endocytic vesicles could be deduced from the ultrastructural study of cells that had internalized antibody in the course of ionomycin stimulation. Early vesicles, relatively small and moderately immunolabeled, developed into more heavily labeled organelles, i.e., larger vesicles and then vacuoles with a few segregated vesicles in their lumen and some vesicles budding from their cytosolic surface. Interestingly, the TGN seemed completely excluded from the d/A-positive endocytic pathway. Further work is needed to investigate the numerous issues still open in the field, in particular to identify the nature of the d/A-positive endosomes, to map in more detail their intracellular pathway, and to clarify the possible role of this recycling system in the regeneration of enlargeosomes competent for regulated exocytosis.

In conclusion, the enlargeosomes that emerge from the present study are significantly more detailed than those known until now (Borgonovo *et al.*, 2002). Working on the PC12-27 cell model, we have shown that this new type of vesicle does indeed participate in the regulated exocytic responses and does account, at least in large part, for the surface enlargements revealed by patch-clamp capacitance, induced by both photolysis of caged Ca^{2+} compounds and the Ca^{2+} ionophore ionomycin; that its membrane, resistant to nonionic detergents, is different from other membranes sharing this property; that cholesterol extraction does not block its exocytosis; and that after exocytosis its membrane is recycled by a nonacidic form of endocytosis. Enlargeosomes seem therefore to have a specific profile, compatible with a wide role in physiology and possibly also in pathology. In addition to their intrinsic interest, these results promise to be instrumental for future studies on multiple aspects of the enlargeosome cell biology that are still unknown, including the biogenesis and intracellular transport of the organelles, the molecular mechanisms of their exocytic membrane fusion, and the nature and pathways of their endocytic system.

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