

Retrieved constituents of large dense-cored vesicles and synaptic vesicles intermix in stimulation-induced early endosomes of noradrenergic neurons

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SUMMARY

Two storage compartments in cultured noradrenergic neurons derived from the superior cervical ganglion from fetal pig have been defined using sucrose density gradient centrifugation and electron microscopy: (1) large dense-cored vesicles (LDV) contain noradrenaline and dopamine- β -hydroxylase (D β H); (2) small electron-lucent vesicles contain acetylcholine and p38 and represent the noradrenergic small synaptic vesicles (SSV); no small dense-cored vesicles (SDV) could be detected. Our results demonstrate that internalized LDV membrane constituents are retrieved into early endosomes, as shown by the colocalization of retrieved D β H with the endosomal markers Rab5 and HRP in sucrose density gradients and on confocal microscopical images. Recycling of the SSV

membranes via an endosomal intermediate is also confirmed in noradrenergic neurons. Finally, colocalization of retrieved D β H and retrieved p38 in stimulated neurons indicates that the two sets of constituents intermix. These data provide the first experimental evidence for a common early endosome in which SSV and LDV membrane constituents are internalized after exocytosis and imply that endosomal sorting is an important process for the generation of different secretory vesicles in the noradrenergic nerve terminal.

Key words: Exocytosis, Endocytosis, Dopamine- β -hydroxylase, Synaptophysin, p38, Superior cervical ganglion

INTRODUCTION

It is now established that each neuron can secrete a variety of peptidergic and non-peptidergic/classical transmitters via at least two types of secretory organelles, i.e. the small synaptic vesicles (SSV) and the large dense-cored vesicles (LDV), also referred to as secretory granules. According to the current model, the classical neurotransmitters acetylcholine (ACh), noradrenaline (NA), glutamate and GABA are stored in, and released from, the SSV (for review see De Camilli and Jahn, 1990). These vesicles originate from the endosomal compartment (Régnier-Vigouroux and Huttner, 1993) and are continuously regenerated by local recycling. The neuropeptides, on the other hand, are stored in, and released from, the LDV (Winkler and Fischer-Colbrie, 1990), which are directly formed at the exit site of the trans-Golgi network and transported down the axon to their release sites. Exocytosis of SSV and LDV is considered to be differentially regulated and to take place at different release sites (Lundberg et al., 1994; Bruns and Jahn, 1995).

However, there are indications that the current model might be oversimplified and it is likely that different neuronal systems in the organism have developed variations along this common

theme of release. One such case is the noradrenergic neuron, which stores the classical transmitter NA not only in small dense-cored vesicles (SDV) but also in LDV. In addition, noradrenergic neurons contain another type of small, electron-lucent vesicle of unknown content, considered by some authors as the noradrenergic equivalent of SSV (Schwarzenbrunner et al., 1990; Annaert et al., 1995; Quatacker and De Potter, 1996). Moreover, our recent work on perfused organs demonstrated that NA in peripheral noradrenergic neurons is released from LDV. The SDV, despite their numerical majority in nerve terminals, are apparently not directly involved in the adrenergic neurotransmission (De Potter et al., 1995, 1997). This finding sharply contrasts with the general belief that classical transmitters are released from SSV and raises intriguing questions about the function of SDV and SSV and their relationship with LDV. In order to answer these questions, a better understanding of the biogenesis and the exo-endocytotic pathway of noradrenergic secretory vesicles is needed.

The recent work on SSV of the central nervous system unraveled the molecular mechanisms involved in vesicle recycling and provided a clear picture of the exo-endocytosis cycle (for review see Südhof, 1995). In particular, the characterization of membrane proteins on the SSV and the

plasmalemma has led to a general model of membrane fusion, i.e. the SNARE-hypothesis (Söllner et al., 1993; Rothman, 1994; Scheller, 1995). After exocytosis, SSV membranes are retrieved and recycled via clathrin-coated vesicles and endosomal intermediates (Heuser and Reese, 1973; Maycox et al., 1992; Takei et al., 1996).

In contrast, despite intensive studies on chromaffin cells (Patzak and Winkler, 1986), PC12 cells (Bauerfeind et al., 1993) and noradrenergic neurons (Thureson-Klein and Klein, 1990), the biogenesis of neuronal LDV remains poorly understood. It is generally assumed that LDV are formed at the exit site of the *trans*-Golgi network as immature vesicles and transported down the axon, during which maturation takes place. Alternatively, in view of the recent demonstration that the axonal reticulum in noradrenergic neurons represents a tubular neurosecretory system, extending from the Golgi apparatus located in the cell soma down to the nerve terminal, it has been suggested that the LDV can be formed locally in the nerve terminal from the axonal reticulum (Quatacker et al., 1995). After exocytosis, catalyzed by Rab3, LDV membrane constituents are retrieved in clathrin-coated vesicles (Annaert et al., 1997) and components of the LDV membranes can be transported back to the perikaryon (Annaert et al., 1994).

The biogenesis and fate of SDV and their relation with LDV remain highly controversial. It is speculated that the SDV membrane is formed, either directly (Winkler et al., 1987; Winkler and Fischer-Colbrrie, 1990) or indirectly (Bauerfeind et al., 1995), by retrieved constituents of LDV. However, if this is so, the different membrane composition between LDV and SDV (Willems and De Potter, 1983) indicates that the retrieved LDV membranes must undergo intensive sorting in endosomal structures. Clearly, more information about the exo-endocytotic cycles of the storage vesicles in noradrenergic neurons is required to clarify the functional connection between LDV and SDV.

In this report, we first define the secretory compartments in cultured noradrenergic neurons derived from the superior cervical ganglion from the fetal pig (SCG neurons) and demonstrate that this cell culture is an appropriate model to address these questions. Dopamine- β -hydroxylase (DBH, EC 1.14.17.1), which catalyses the last step in the biosynthesis of NA (Kaufman and Friedman, 1965) is present in large amounts in the LDV of noradrenergic neurons (De Potter et al., 1970) and was used as a marker for LDV membranes. P38, also called synaptophysin, the most abundant integral membrane protein of SSV in almost all nerve terminals (Jahn et al., 1985; Wiedenmann and Franke, 1985), was used as a marker for SSV membranes. We next provide experimental evidence that LDV membrane constituents are retrieved via the same early endosomes that are involved in SSV membrane recycling. These data are finally discussed in relation to the biogenesis of SDV.

MATERIALS AND METHODS

Primary cultures of porcine superior cervical ganglia

Prenatal piglets (gestation stage between 6.5-10 weeks) were obtained from a local slaughterhouse. Superior cervical ganglia (SCG; usually 30 to 40) were dissected and transferred to sterile Ham's F12 medium (Life technologies, Merelbeke, Belgium) and finally cleaned of

remaining fat and small bloodvessels. All following steps were performed under sterile conditions. Ganglia were transferred to 0.2% collagenase (w/v) (Boehringer, Mannheim, Germany) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Locke's medium and incubated for 1 hour at 37°C with gentle stirring. Dissociated cells were collected by centrifugation at 700 *g* for 4 minutes and the collagenase supernatant was reused several times until ganglia were almost completely dissociated. Pooled cell pellets were resuspended in Ham's F12 medium by 2 passages through a 16 G/1.7 mm needle, sedimented again, resuspended in 40 to 50 ml F12 medium, plated in 2 tissue culture flasks and placed in the incubator for 2 hours (37°C, 5% CO_2).

After 2 hours, most non-neuronal cells became attached to the plastic surface while neuronal cells remained in suspension. These cells were gently removed, centrifuged at 170 *g* for 4 minutes, resuspended in a small volume of F12 medium supplemented with nerve growth factor (30 ng/ml final) and counted. They were then diluted in DMEM/F12 nutrient mix with Glutamax I (Life technologies) medium containing the following additions: 10% horse serum (Life technologies), 2 mg/ml insulin (Sigma Chemical Company, St Louis, MI, USA), 1 mg/ml transferrin (Sigma), 30 ng/ml nerve growth factor (Sigma), 2 mM PenStrep (Life technologies) and 2 mM gentamycin (Life technologies). For biochemical studies and electron microscopical experiments, cells were plated at a high density (0.5×10^6 cells/ml) in tissue culture flasks. For indirect immunofluorescence, cells were plated at a low density (10,000 cells/cover slip) in 24-well plates equipped with 10 mm coverslips. Medium was renewed on days 1, 2, and 4 after plating. Cells were processed no longer than 6 days after plating. To prevent proliferation of non-neuronal cells in the culture, antimetabolites (all from Sigma) were added to the medium: 10 mM fluorodeoxyuridine and 10 mM uridine throughout and 5 mM cytosine-araboside only at day 2.

Uptake of [^3H]NA and [^3H]choline

Cell monolayers were rinsed twice with HBS (135 mM NaCl, 5 mM KCl, 0.6 mM MgCl_2 , 2.5 mM CaCl_2 , 10 mM Hepes, 6 mM glucose, pH 7.4) and subsequently incubated with HBS containing 0.2 mM ascorbic acid and 40 nM [^3H]NA (Spec. act. 15 Ci/mmol, NEN-DuPont, Brussels, Belgium) or HBS containing 80 nM [^3H]choline (Spec. act. 60 Ci/mmol, NEN-DuPont). After incubation at 37°C for 1 hour, unaccumulated [^3H]NA or [^3H]choline was removed by washing the monolayer four times, each for 15 minutes, and cells were subjected to subcellular fractionation.

Subcellular fractionation

SCG cells were incubated in HBS (37°C) for 4 minutes (control condition) or stimulated for 4 minutes in modified HBS containing 120 mM KCl and 20 mM NaCl (37°C), chased for 5 minutes in HBS (37°C) and immediately placed on ice. All subsequent steps were performed at 4°C. The cell layer was rinsed once with ice-cold HBS and once with ice-cold homogenization buffer (250 mM sucrose, 5 mM Tris-HCl, 1 mM EGTA, pH 7.3). Cells (usually about 7×10^6 SCG cells per condition) were harvested using a rubber policeman and centrifuged at 170 *g* for 4 minutes. The cell pellet was resuspended in a small volume of homogenization buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin; Sigma) and homogenized using a ball bearing cell cracker (clearance 10 μm , EMBL, Germany). After one centrifugation (10,000 *g*_{max}, 15 minutes), the postmitochondrial supernatant was loaded on top of a continuous sucrose gradient (0.3 M-1.7 M sucrose in 5 mM Tris-HCl, 1 mM EGTA, pH 7.3) and centrifuged to equilibrium (300,000 *g*_{max}, 180 minutes, Kontron TST 41.14, Milan, Italy). Twelve fractions were collected from the top (Büchler Auto-densi Flow IIC, VEL, Belgium) and analyzed for the contents of the different markers.

In order to mark the position of early endosomes, cells were stimulated by 120 mM KCl at 37°C in the presence of horseradish peroxidase (HRP, 8 mg/ml, type VI, Sigma) for 4 minutes and chased for 5 minutes. After extensive washing according to the method of

Cupers et al. (1994; three times 30 seconds with ice-cold HBS, once for 5 minutes and once for 1 minute by HBS containing 10% horse serum, Life Technologies, and finally five times 30 seconds by HBS), cells were homogenized and fractionated as described. For each marker, the relative content (frequency) in all gradient fractions was calculated.

Measurement of [³H]NA and [³H]ACh

The 12 gradient fractions were diluted in 20 ml of homogenization buffer and sedimented by centrifugation (280,000 *g*_{max}, 45 minutes) using a Beckman 60 Ti fixed angle rotor (Beckman Instruments, Palo Alto, CA, USA) to remove soluble material. For [³H]NA measurement, pellets were resuspended in water and counted by liquid scintillation. For [³H]ACh measurement, pellets were subjected to acid-acetone extraction to separate the [³H]ACh from the [³H]choline incorporated in the phospholipids (Bauerfeind et al., 1993). Briefly, pellets were quickly resuspended in 300 µl of ice-cold 1 N formic acid-acetone (15:85), mixed with 200 µl H₂O and transferred into conical glass tubes on ice. Subsequently, diethylether (2 ml) was added and the samples were mixed vigorously, kept on ice for 60 minutes and centrifuged for 10 minutes at 100 rpm in a Heraeus centrifuge (Heraeus, Osterode am Harz, Germany). The organic phase was discarded and the radioactivity in the aqueous phase was counted.

Indirect immunofluorescence

SCG cells were cultured for 6 days at low density in 24-well plates containing 10 mm round glass coverslips that had been coated with 1 mg/ml poly-DL-ornithine (Sigma) for 6 hours at room temperature, followed by 1 µg/ml laminin (Sigma) treatment for 2 hours at room temperature. Cells were first rinsed 2 times 15 minutes with HBS at 37°C, then stimulated at 37°C for 4 minutes in modified HBS containing 120 mM KCl and one of the following primary antibodies: affinity-purified rabbit anti-DβH IgGs (20 µg/ml; De Potter et al., 1988); biotinylated Fab-fragments thereof (20 µg/ml) or rabbit anti-p38 IgGs (10 µg/ml; Annaert et al., 1993). Fab-fragments were generated using the Pierce Immunopure Fab preparation kit (Pierce, Rockford, IL, USA) and were biotinylated according to the method of Bayer and Wilchek (1990) using biotinyl-N-hydroxysuccinimide ester as reagents. Immediately (exocytotic spot labelling) or after a 5 minute chase period in HBS at 37°C, cells were transferred to 4°C, washed 3× 5 minutes with ice-cold HBS and fixed in HBS containing 4% formaldehyde for 10 minutes at 4°C, followed by 20 minutes at room temperature. Subsequently, cells were rinsed with 100 mM glycine in HBS, pH 8.5, for 2× 5 minutes to quench the residual formaldehyde cross-linking activity. Cells were finally permeabilized with 0.25% Triton X-100 in HBS. Subsequently, cells were blocked with HBS containing 10% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA). Where indicated, cells were incubated in a humid chamber with monoclonal anti-Rab5 (5 µg/ml) (clone 621.2; Fischer von Mollard et al., 1994), which was kindly provided by R. Jahn (Yale, New Haven, USA). After overnight incubation with these antibodies at 4°C, cells were washed 3× 5 minutes and incubated with secondary antibodies for 1 hour at room temperature. In control staining experiments, cells were washed twice with HBS prior to fixation and permeabilized as described above. Subsequently, cells were incubated in a humid chamber with monoclonal antibodies to p38 (4 µg/ml; clone SY38; Boehringer). After overnight incubation with these antibodies at 4°C, cells were washed and incubated with secondary antibodies.

Polyclonal antibodies were visualized with FITC-conjugated donkey anti-rabbit antibodies, TRITC-conjugated goat anti-rabbit antibodies, Fc fragment-specific or FITC-conjugated streptavidin (Jackson ImmunoResearch). Monoclonal antibodies were visualized using biotin-conjugated donkey anti-mouse antibodies followed by TRITC-conjugated streptavidin (Jackson ImmunoResearch). Finally the coverslips were washed 3× with PBS, 3× with H₂O and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

The samples were first observed at a 63× magnification (NA 1.25) in a conventional fluorescence microscope (Axioskop, Zeiss, Germany), followed by analysis with a confocal laser scanning microscope (MRC-600, Bio-Rad Laboratories, Richmond, CA, USA). The cells were illuminated with 488 nm light from an argon ion laser, and fluorescence was split into a red and green signal by a beam splitter of 565 nm combined with a long band pass filter of 600 nm and a barrier filter of 540 nm. Confocal images were printed out with a Sony UP-3000p color Videoprint.

Electron microscopy

SCG cells were plated at high density (7×10⁷ cells/flask) in poly-DL-ornithine/laminin coated flasks and cultured for 6 days. The neuron cultures were fixed by 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate, pH 7.3, for 1 hour at 4°C, washed once in 0.1 M cacodylate, pH 7.3, harvested using a rubber policeman and spun down at 170 *g* for 4 minutes. In order to evaluate the ultrastructure of the neurons, the cell pellet was washed in 0.1 M cacodylate, pH 7.3, for 30 minutes, followed by a 1 hour incubation in 1% OsO₄ in 0.1 M cacodylate, pH 7.3. Next, cell pellets were extensively washed in 0.1 M cacodylate, pH 7.3, dehydrated (in 75%, 90%, 95%, 99% and twice 100% ethanol for 15 minutes each) and processed for Epon embedding. Ultrathin sections were examined in a JEOL 1200EX electron microscope at 80 keV.

Miscellaneous

Trypsin digestion of the SSV enriched fractions and of Triton X-100 treated SSV enriched fractions was carried out as described by Jahn et al. (1985). SDS-PAGE and western blotting were performed according to established procedures followed by enhanced chemiluminescence (Renaissance; NEN-DuPont). Immunoreactive bands were quantified by laser densitometric scanning with an Ultrascan XL (Pharmacia-LKB, Uppsala, Sweden). HRP enzymatic activity was quantified in a 96-well ELISA plate using o-dianisidine as a substrate (Cupers et al., 1994). Absorptions were measured with a Titertrek Multiscan MCC/340 spectrophotometer (Labsystems, Helsinki, Finland).

RESULTS

Biochemical evidence for the presence of LDV and SSV

Post-mitochondrial supernatants of SCG neurons loaded with [³H]NA were layered on continuous sucrose density gradients and centrifuged to equilibrium. As shown in Fig. 1A, a broad NA-peak was detected towards the bottom of the gradient, at densities centered on 1.15 g/ml, as expected for LDV (Annaert et al., 1995). DβH-immunoreactivity displayed a sharper peak at similar densities (Fig. 1B).

In contrast, when SCG neurons were loaded with [³H]choline, an ACh-peak was detected in the top of the gradient (Fig. 1C), at densities from 1.02 to 1.05 g/ml, as expected for SSV (Fried et al., 1981). Distribution of the immunoreactivity of the typical SSV marker p38 was comparable (Fig. 1D). Since each gradient fraction was pelleted after dilution in homogenization buffer and only sedimentable material was counted, the low-density ACh peak was not due to free ACh derived from broken vesicles or from leakage out of vesicles that would contaminate the loading zone.

Electron microscopical evidence for the presence of LDV and SSV

The SCG cell bodies show long extensions running parallel in

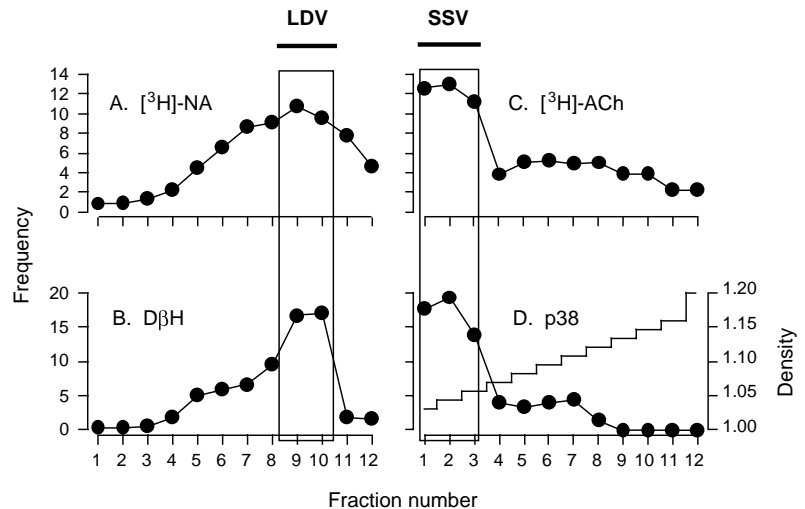


Fig. 1. Resolution of LDV and SSV by subcellular fractionation of control SCG neurons. SCG neurons were loaded with [^3H]NA (A,B) or [^3H]ACh (C,D) as described in Materials and Methods and postmitochondrial supernatants were equilibrated in sucrose gradients. Distributions of the neurotransmitters in pelleted fractions (A,C) are compared to those of the endogenous protein markers D β H (B) and p38 (D). Density of the fractions is given in D. Recoveries varied between 78% and 133%. Results are the means of 3 independent experiments.

bundles, along which varicosities of different sizes can be seen. At the ultrastructural level, these varicosities contain numerous LDV (organelles with an obvious dense core; Fig. 2A). In addition, clusters of small electron-lucent vesicles can be detected (Fig. 2B).

Membrane constituents of both LDV and SSV are retrieved into early endosomes

Post-mitochondrial supernatants obtained from control neurons or from neurons that had been stimulated with 120 mM KCl and chased for 5 minutes were equilibrated in sucrose density gradients. Stimulation resulted in a decrease of 35% of D β H-activity in the total post-mitochondrial supernatant, indicating exocytosis of LDV upon stimulation. The residual membrane-bound fraction of the enzyme was primarily recovered at an intermediate density from 1.07 g/ml to 1.12 g/ml (Fig. 3A). Upon stimulation, the membrane-bound SSV marker p38 almost disappeared from the low-density peak, indicating SSV exocytosis, and was also recovered primarily in the intermediate density region from 1.07 g/ml to 1.12 g/ml (Fig. 3C), largely overlapping with the D β H distribution.

The subcellular distributions of D β H and p38 in stimulated SCG neurons was next compared with distributions of an endogenous marker for early endosomes, Rab5 (Chavrier et al., 1990; Fig. 3B) and the exogenous tracer HRP (Courtoy, 1991;

Fig. 3D). These endosomal markers were primarily recovered at the same intermediate density region as for the D β H and the p38 peak, indicating that after stimulation, LDV constituents are retrieved into early endosomes, as is known for those of SSV membranes (Fischer von Mollard et al., 1994).

In order to follow the retrieved LDV and SSV membranes after exocytosis by confocal laser scanning microscopy, antibodies to the luminal epitopes of D β H and p38 can be used. When the cells are stimulated, the luminal domains of D β H and p38 become exposed at the cell surface where they can be reached by the corresponding antibodies. Whereas D β H is a partially soluble and membrane-bound enzyme in LDV, p38 has four transmembrane domains and a carboxy-terminal cytoplasmic tail. Well-characterized monoclonal antibodies, like SY38, bind to the cytoplasmic domain of p38 (Knaus and Betz, 1990). However, polyclonal antibodies to p38 might recognize additional epitopes in the luminal domains. Therefore, the epitope topology of the polyclonal antibody to p38 used in this study was first checked.

Trypsin digestion of the SSV enriched gradient-fractions (fractions 1 to 3) generates a p38-derived fragment with an apparent molecular mass of 25 kDa, as described by Jahn et al. (1985). Whereas this fragment was no longer recognized by the monoclonal antibody (SY38), indicating the loss of the cytoplasmic epitope, it still strongly reacted with the

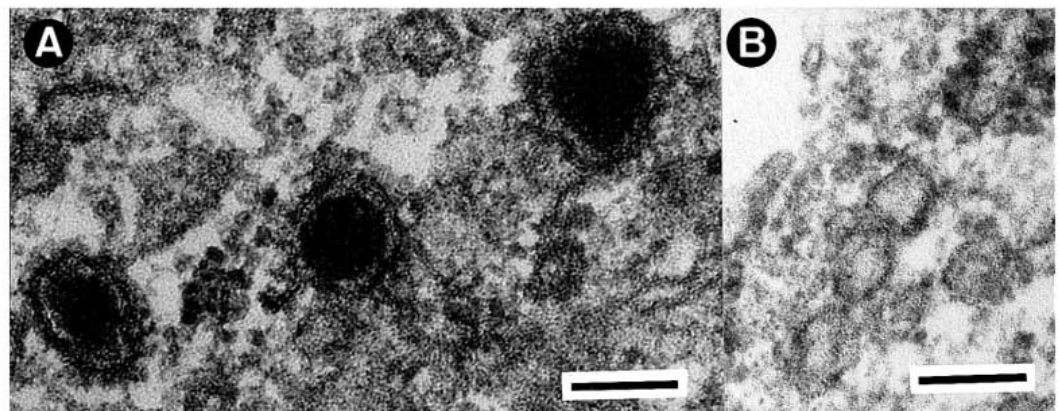
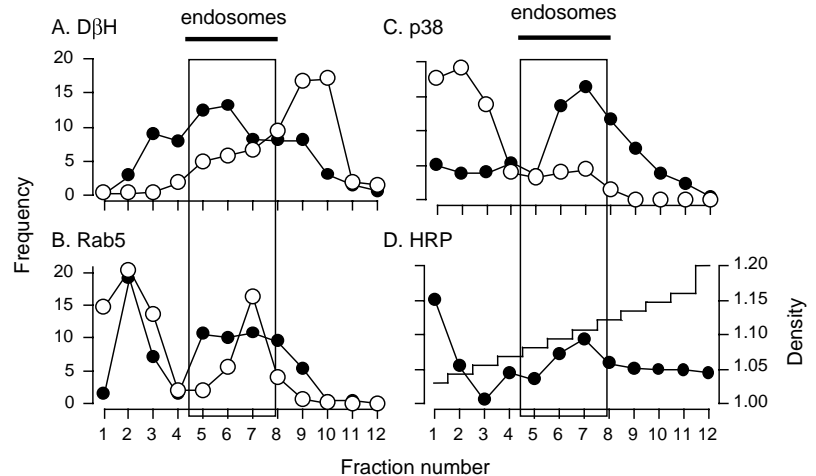


Fig. 2. Electron microscopical evidence for the presence of LDV and SSV in control SCG neurons. At the ultrastructural level, varicosities can be detected containing numerous LDV (organelles with an obvious dense core; A). In addition clusters of small electron-lucent vesicles can be detected (B). Bars, 100 nm.

Fig. 3. Merging of D β H and p38 into endosomes upon stimulation of SCG neurons. Postmitochondrial supernatants of control (open circles) and stimulated (filled circles) SCG neurons were equilibrated in sucrose density gradients. The distribution (by semiquantitative western blotting) is shown for D β H (A), Rab5 (B) and p38 (C). The distribution of the fluid phase marker HRP is shown together with the density in each fraction (D). The distributions for D β H and p38 in control conditions are the same as in Fig. 1B,D and are given here for comparison purposes. Notice that stimulation displaces the bulk of D β H and p38 from opposite regions of the gradient to the same intermediate region, where endosomes equilibrate (boxed; compare with the distributions of Rab5 and the peak of HRP). Results are the means of 2 to 3 independent experiments.



polyclonal antibody, suggesting that this antibody recognizes one or more luminal epitopes of p38. As a control, TX-100 lysis of the vesicles prior to addition of trypsin led to the total degradation of the fragment (data not shown). In support of these data, a SSV exocytotic spot labelling was performed with either the polyclonal antibody to p38, or with the monoclonal antibody to the cytoplasmic domain of p38 (SY38). Whereas a clear SSV exocytotic spot labelling was demonstrated with the polyclonal antibody to p38, it was not possible to demonstrate any exocytotic spot labelling with the monoclonal antibody, although this monoclonal antibody recognizes the antigen in permeabilized material (data not shown). Accessibility on intact cells thus complements trypsin sensitivity with or without detergent lysis on isolated vesicles and demonstrates that the polyclonal antibody recognizes one or more luminal epitopes of p38.

The biochemical evidence that membrane constituents of both LDV and SSV are retrieved into early endosomes was confirmed by confocal laser scanning microscopy. We took advantage of the recognition by antibodies to luminal epitopes of D β H and p38 to follow the retrieved LDV and SSV membranes after exocytosis. Neurons were stimulated in the presence of antibodies to D β H or to p38, chased for 5 minutes, fixed and immunolabeled for Rab5. The immunolabelling pattern for D β H in the varicosity shown in Fig. 4A,C is similar to that of Rab5 (Fig. 4B,C). In addition, immunolabelling for p38 (Fig. 4D,F) also matches the Rab5 immunolabelling (Fig. 4E,F). Combined with the quantitative subcellular fractionation data, this provides direct morphological evidence that LDV membrane constituents, as for those of SSV membranes, are retrieved into early endosomes.

Intermixing of retrieved LDV and SSV membranes in stimulation-induced early endosomes

Since Rab5 catalyzes the homotypic fusion between early endosomes, its presence on the retrieved LDV membranes indicates their fusion capacity with other endosomal organelles. Therefore, it was interesting to determine whether intermixing occurred between the early endosomes involved in LDV retrieval and early endosomes involved in SSV membrane recycling.

When neurons were stimulated in the presence of both

biotinylated anti-D β H Fab-fragments and anti-p38 IgGs, chased for 5 minutes and processed for double immunofluorescence using streptavidin and Fc fragment-specific anti-rabbit secondary antibodies, respectively, a clear colocalization between the retrieved D β H (Fig. 5A,C) and p38 (Fig. 5B,C) was observed, indicating that LDV membrane constituents are retrieved via the same early endosomes that are involved in SSV membrane recycling. No cross-labelling of biotinylated anti-D β H Fab-fragments with Fc fragment-specific anti-rabbit secondary antibodies was detected (Fig. 6A,B).

DISCUSSION

Two storage vesicle populations coexist in cultured SCG neurons

The combined subcellular fractionation and electron microscopical analyses reveal two distinct vesicle populations in resting SCG neurons: one population, containing both D β H and NA and equilibrating at a density around 1.16 g/ml, represents LDV; the second population, made of electron-lucent vesicles, containing p38 and equilibrating below a density of 1.05 g/ml, represents SSV. The subcellular distribution of NA does not fully coincide with that of D β H, as it is rather broad, extending down to 1.12 g/ml. Since immature LDV in the splenic nerve axon equilibrate at a significantly lower density than the mature LDV in the spleen itself (De Potter et al., 1970; Chubb et al., 1970), the NA content of the lighter fractions may represent immature LDV. Only a minor amount of the NA is recovered at the SDV equilibrium density (density 1.07 g/ml; Fillenz, 1990), without a well-defined peak, indicating that, if SDV are present in SCG cells, their number should be rather low. Morphometric analysis of the size distribution of the vesicles in SCG neurons shows that the large vesicles display a diameter range from 80 nm to 100 nm, whereas the small vesicles are distributed in a very narrow range around 45 nm (Annaert et al., 1997). In the literature, there is a general agreement concerning the size of LDV in noradrenergic neurons being ~85 nm (Fillenz, 1990), and SSV are known to be highly homogenous in size, being ~50 nm (De Camilli and Jahn, 1990).

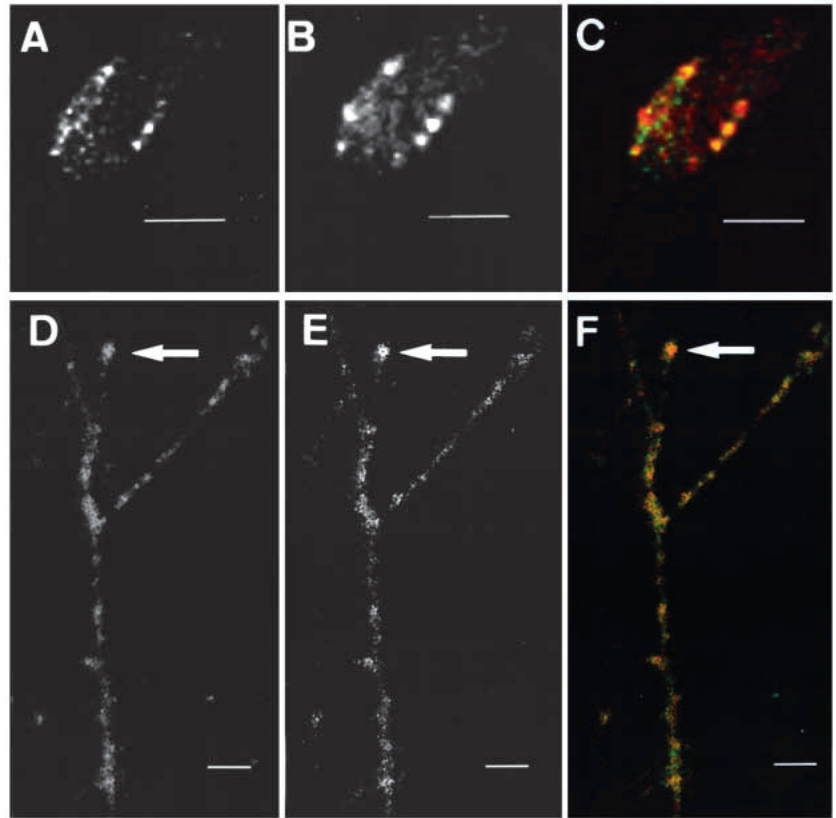


Fig. 4. Colocalization of retrieved D β H (A,C) or p38 (D,F) with Rab5 (B,C,E,F) in stimulation-induced early endosomes of SCG neurons. SCG cells were stimulated with 120 mM KCl in the presence of polyclonal antibodies to D β H (A,C_{green}) or p38 (D,F_{green}) and chased for 5 minutes. After washing, fixation and permeabilization, the cells were incubated with monoclonal antibodies to Rab5 (B,C_{red}, E,F_{red}). D β H and p38 antibodies were then visualized with FITC-conjugated donkey anti-rabbit antibodies and Rab5 antibodies were visualized with biotinylated anti-mouse antibodies followed by TRITC-conjugated streptavidin. (A,B,C) A varicosity in which a striking colocalization (C_{yellow}) between the retrieved D β H (A,C_{green}) and Rab5 (B,C_{red}) is shown. (D,E,F) A neurite with several varicosities. Arrows point to colocalization (F_{yellow}) between retrieved p38 (D,F_{green}) and Rab5 (E,F_{red}). Bars, 5 μ m.

The presence of p38-positive small electron-lucent vesicles in the bovine splenic nerve and vas deferens has been reported by several investigators (Schwarzenbrunner et al., 1990; Annaert et al., 1995; Quatacker and De Potter, 1996), who classified them as noradrenergic equivalents of SSV. This study demonstrates, using subcellular fractionation and electron microscopy, the presence of equivalents of SSV in cultured noradrenergic neurons. However, so far the corresponding neurotransmitter remains unknown. A good candidate is acetylcholine, since this neurotransmitter was localized to small synaptic like microvesicles in PC12 cells (Bauerfeind et al., 1993). Moreover, it is well known that sympathetic ganglia exhibit significant phenotypic lability when their presynaptic input is severed *in vivo* (Jonakait, 1993). Cultured fetal pig SCG neurons change from an adrenergic to a cholinergic phenotype with time in culture (Wang et al., 1995). Data of the present report using sucrose density gradient centrifugation techniques allow us to conclude that noradrenergic equivalents of SSV in these cultured cells contain ACh. The minor part of ACh equilibrating at the density of endosomes is in agreement with an active ACh uptake system in the early endosomes, as shown for PC12 cells (Bauerfeind et al., 1993).

The role of SSV in noradrenergic neurons *in vivo* and *in vitro*

If noradrenergic equivalents of SSV in cultured SCG neurons do not contain detectable amounts of NA, the question about their role in noradrenergic neurons immediately arises. A possibility might be that in noradrenergic neurons SSV have lost their function as major neurotransmitter organelles upon neuronal differentiation to a fully noradrenergic phenotype,

resulting in a residual population of vesicles. It is known that neuronal differentiation is controlled by transsynaptic regulation (Comb et al., 1987). It can, therefore, be expected that upon arrival at the target cell, the neuronal growth cone receives information to differentiate to the desired phenotype. For instance, the SSV pathway would be up-regulated when the target cell is a skeletal muscle, while noradrenergic differentiation would favour LDV as the most important storage organelle. This interpretation would also account for the phenotypic lability of the non-differentiated fetal pig SCG neurons. In this study, all experiments were performed on fetal pig SCG neurons cultured for 6 days, when cells remain predominantly adrenergic but cholinergic characteristics become detectable (e.g. expression of choline-acetyltransferase (ChAT) and substance P; Wang et al., 1995).

It may seem contradictory that although very low ChAT values are reported on day 6 in SCG neurons, significant amounts of ACh can be measured. This apparent contradiction can be explained by the different manner data are expressed in the two studies. Wang et al. (1995) measured the enzyme activity by incubating an homogenate of 10^6 cells mixed with substrate solution containing [1- 14 C]acetyl-coenzyme A at 36°C for 1 hour and ChAT activity was expressed as pmol [14 C]acetylcholine formed. Since [14 C]acetyl-choline counts were low but significant and reproducible, the corresponding enzyme activity was accordingly also low (ranging from 2 to 5 pmol ACh/hour per million cells). In the present study, formed [3 H]acetyl-choline was counted and directly expressed as the relative content (frequency) in each fraction and not as enzyme activities. The corresponding enzyme activity, based on the [3 H]acetyl-

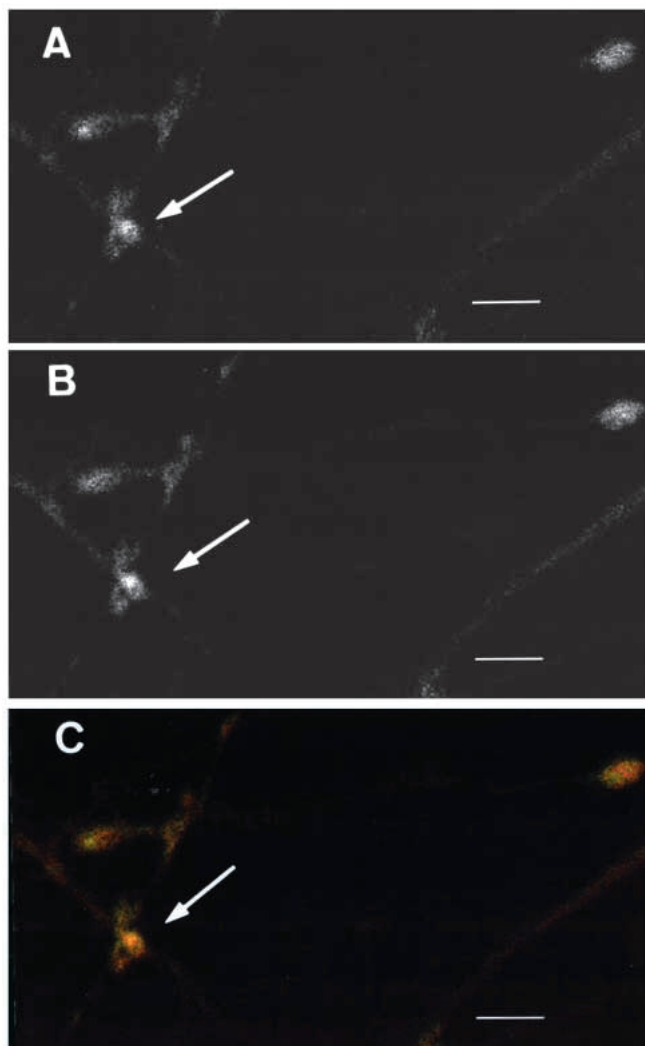


Fig. 5. Colocalization of retrieved D β H (A,C) with retrieved p38 (B,C) in stimulation induced early endosomes of SCG neurons. SCG cells were stimulated with 120 mM KCl in the presence of biotinylated Fab-fragments of polyclonal antibodies to D β H (A,C_{green}) together with polyclonal antibodies to p38 (B,C_{red}) and chased for 5 minutes. After washing, fixation and permeabilization, the biotinylated Fab-fragments were visualized with FITC-conjugated streptavidin and anti-p38 was visualized with TRITC-conjugated goat anti-rabbit antibodies, specific to Fc fragments. A network of neurites is shown with a varicosity in which retrieved D β H (A,C_{green}) and retrieved p38 (B,C_{red}) colocalize (C_{yellow}; arrows). Bars, 5 μ m.

choline counts found in the present study is 2 pmol ACh/hour per million cells, which is perfectly comparable with the previous reported values.

Our previous studies demonstrated that NA, neuropeptide Y and ACh are released from these neurons in culture in a Ca²⁺-dependent manner (Annaert et al., 1997; unpublished results). Moreover, release from LDV and SSV can be visualized by confocal laser scanning microscopy upon exposing stimulated neurons to antibodies to D β H and p38 prior to fixation. It can thus be concluded that these neurons in culture are functionally active and that both LDV and SSV undergo regulated exocytosis. Taken together, these data indicate that this

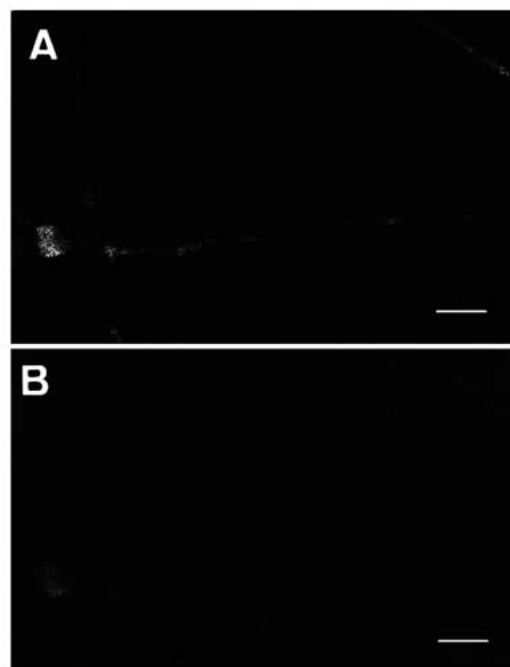


Fig. 6. Specificity of immunolabelling. SCG cells were fixed, permeabilized and incubated with biotinylated Fab-fragments of polyclonal antibodies to D β H alone. The biotinylated Fab-fragments were visualized with FITC-conjugated streptavidin (A) or with TRITC-conjugated goat anti-rabbit antibodies, Fc fragment-specific (B). No cross-labelling of biotinylated anti-D β H Fab-fragments with Fc fragment-specific anti-rabbit antibodies was detected. Bars, 5 μ m.

experimental system is an appropriate model to study the relation between exo-endocytotic pathways of LDV and SSV, so as to clarify the biogenesis of SDV.

LDV membranes are retrieved into early endosomes

While retrieval of SSV constituents is well documented (for a review see Südhof, 1995), data on the internalization of noradrenergic LDV remain fragmentary. Most of our knowledge concerning recycling of LDV comes from studies on chromaffin cells, where retrieved secretory granule constituents have been shown to recycle via the *trans*-Golgi network (Patzak and Winkler, 1986; Philips, 1987; Hurtley, 1993). After stimulation of SCG neurons in primary culture, LDV constituents enter the endocytotic pathway via clathrin-coated pits (Annaert et al., 1997) and a large part of these retrieved membranes is supposed to be retrogradely transported to the cell body (for a review, see Thureson-Klein and Klein, 1990). In splenic nerve, this retrograde transport of LDV membrane constituents occurs via multivesicular bodies (Annaert et al., 1994). However, no information is available on intermediates between the coated pit and the multivesicular bodies. Therefore, it is important to clarify whether internalized LDV membrane constituents directly evolve into multivesicular bodies or are first delivered to an endosomal compartment where sorting could occur between the retrograde transport pathway and a recycling pathway whereby some membrane constituents are re-utilized for the biogenesis of other storage organelles i.e. the SDV.

This study demonstrates that LDV membrane constituents

can be retrieved into early endosomes after exocytosis, as can be concluded from their colocalization with the endosomal markers Rab5 and HRP in subcellular fractionation analysis and their colocalization with Rab5 in confocal microscopy. Rab5 is a specific marker for early endosomes (Chavrier et al., 1990) and its occurrence on synaptic vesicles provided evidence that SSV membrane constituents recycle via an endosomal intermediate (Fischer von Mollard et al., 1994; de Hoop et al., 1994). In full agreement with these studies, we here demonstrate that noradrenergic SSV membrane constituents also recycle via an endosomal intermediate.

Retrieved constituents of LDV and SSV intermix in stimulation-induced early endosomes of SCG neurons

Rab5 regulates the early steps of the endocytotic pathway, i.e. internalization at the plasma membrane and homotypic fusion of early endosomes with exchange of contents (Gorvel et al., 1991; Bucci et al., 1992). Rab5 overexpression increases the endocytotic activity and the size of the early endosomes, while overexpression of a mutant defective in GTP binding decreases endocytotic uptake and results in the accumulation of endocytotic vesicles (Bucci et al., 1992). Therefore, the presence of Rab5 on both retrieved LDV and SSV membranes in the stimulated SCG neurons indicates their possible intermixing. It has been recently speculated that the SSV marker p38 and the LDV marker D β H meet in early endosomes after endocytotic membrane retrieval (Bauerfeind et al., 1995). The present study, in which the colocalization of retrieved D β H and retrieved p38 in stimulated neurons is demonstrated by fractionation data and on confocal microscopical images, offers direct evidence supporting this hypothesis.

Endosomal sorting of LDV and SSV membrane constituents

LDV- and SDV-membranes differ substantially in composition (Willems and De Potter, 1983). This implies that if SDV are derived from retrieved LDV membranes upon stimulation, the retrieved LDV membranes must pass through a common sorting endosomal compartment (Courtoy, 1991; Gruenberg et al., 1989), in which part of the LDV membranes are sorted into multivesicular bodies and transported back to the cell body, while the transmitter-uptake machinery and possibly other constituents are sorted into SDV. While the present study provides evidence that LDV membranes are indeed retrieved into early endosomes, the sorting process and the stimulation-induced SDV formation await further investigation.

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