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Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane

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The biogenesis of synaptic-like microvesicles (SLMVs) in neuroendocrine cells was investigated by studying the traffic of newly synthesized synaptophysin to SLMVs in PC12 cells. Synaptophysin was found to be sulfated, which facilitated the determination of its exit route from the trans-Golgi network (TGN). Virtually all [^{35}S]sulfate-labeled synaptophysin was found to leave the TGN in vesicles which were indistinguishable from constitutive secretory vesicles but distinct from immature secretory granules and SLMVs. [^{35}S]sulfate-labeled synaptophysin was rapidly transported from the TGN to the cell surface, with a $t_{1/2}$ of ~10 min in resting cells. After arrival at the cell surface, [^{35}S]sulfate-labeled synaptophysin cycled for at least 1 h between the plasma membrane and an intracellular compartment likely to be the early endosome. Up to ~40% of the [^{35}S]sulfate-labeled synaptophysin eventually (after 3 h and later) reached SLMVs, which could be distinguished from the other post-TGN compartments by their lower buoyant density in a sucrose gradient and their selective inclusion upon permeation chromatography using a controlled-pore glass column. Our results suggest that newly synthesized membrane proteins of SLMVs in neuroendocrine cells, and possibly of small synaptic vesicles in neurons, reach these organelles via the TGN → plasma membrane → early endosome.

Key words: neuroendocrine cells/sorting/synaptic vesicles/synaptophysin

Introduction

Small synaptic vesicles (SSVs) are 50 nm regulated secretory organelles of neurons which play a key role in the release of 'classical' neurotransmitters. Over the past few years, considerable progress has been made in the identification and characterization of proteins which are common to SSVs irrespective of their neurotransmitter content (Jahn and De Camilli, 1991; Südhof and Jahn, 1991; Trimble *et al.*, 1991). The observation that these membrane proteins are also present in endocrine cells led to the discovery that these cells contain a population of small vesicles, referred to as synaptic-like microvesicles (SLMVs) (Navone *et al.*, 1986; De Camilli and Jahn, 1990). SLMVs are thought to be the endocrine counterpart to the neuronal SSVs. SLMVs are distinct from endocrine secretory granules which are the counterpart to the neuronal large dense core vesicles (De Camilli and Jahn, 1990).

Despite the extensive molecular characterization of proteins characteristic of SSVs and SLMVs, surprisingly little is known about the subcellular site of biogenesis of these organelles and the sorting events involved in their biogenesis (Kelly, 1988). SSVs undergo multiple cycles of exocytosis–endocytosis at synaptic terminals. Thus, by definition, SSVs are regenerated at synaptic terminals. However, it is unclear whether this regeneration involves the *de novo* assembly of the characteristic components of the SSV membrane in the course of their endocytosis in the nerve terminal, or whether the SSV membrane is assembled elsewhere in the neuron and maintains its identity, e.g. as a membrane patch, throughout the exocytotic–endocytotic cycles. The colocalization of membrane proteins characteristic of SSVs and SLMVs with fluid phase markers of endocytosis (Johnston *et al.*, 1989; Clift-O'Grady *et al.*, 1990) and membrane proteins known to cycle between the plasma membrane and early endosomes (Cameron *et al.*, 1991) does not allow one to distinguish between these two possibilities, *de novo* assembly versus membrane patch maintenance. Thus, a key question concerning the biogenesis of SSVs/SLMVs is: from which compartment are newly synthesized SSV/SLMV membrane proteins first assembled into these organelles?

Concerning the origin of the SSV membrane, i.e. the site of its first assembly, several possibilities exist. First, the SSV membrane could be assembled in the trans-Golgi network (TGN), a major biosynthetic sorting compartment (Griffiths and Simons, 1986), reflecting the existence of a third exocytotic pathway from the TGN to the plasma membrane in addition to the constitutive secretory pathway and the secretory granule-mediated regulated secretory pathway. Such a biogenesis has been implicated from the subcellular localization of SSV membrane proteins in developing and adult neurons (Tixier-Vidal *et al.*, 1988; Janetzko *et al.*, 1989) and in transfected non-neuroendocrine cells (Leube *et al.*, 1989). However, a focal block of axonal transport does not lead to an accumulation of SSVs on the proximal side of the block (Tsukita and Ishikawa, 1980), which might be expected if SSVs were to originate from the TGN. Second, SSV membrane proteins could exit from the TGN into secretory granules and be assembled into an SSV membrane only after exocytosis of secretory granules, this assembly occurring either at the plasma membrane or in endosomes. Such a biogenesis has been considered in light of the observation that secretory granules contain low levels of membrane proteins which are characteristic of SSVs (Lowe *et al.*, 1988). However, in the neuroendocrine cell line PC12, a newly synthesized SLMV membrane protein was not found to pass through mature secretory granules on its way to SLMVs (Cutler and Cramer, 1990). These results exclude the possibility that SLMVs originate after exocytosis of mature secretory granules, but not that SLMV membrane proteins exit from the TGN with the regulated secretory pathway, since the formation of mature secretory granules

involves a short-lived intermediate, the immature secretory granule (S.A.Tooze, T.Flatmark, J.Tooze and W.B.Huttner, submitted; Tooze, 1991), from which the SLMV membrane could be assembled. In addition to this third possibility, a fourth possibility for the origin of SSVs and SLMVs exists, i.e. that SSV/SLMV membrane proteins exit from the TGN in constitutive secretory vesicles. This would imply that either the plasma membrane or an endosome is the site of first assembly of the SSV/SLMV membrane.

In the present study, we focused on the exit of newly synthesized SSV/SLMV membrane proteins from the TGN and their transport to SLMVs in PC12 cells. To investigate this issue, we used, as a model protein, synaptophysin (p38), a major component of SSVs and SLMVs (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Navone *et al.*, 1986). We exploited the observation, described below, that synaptophysin is sulfated. This has allowed us to make use of a recently established experimental system based on [^{35}S]sulfate pulse-chase labeling, which facilitates studying the exit of proteins from the TGN into various post-TGN vesicles (Tooze and Huttner, 1990).

Results

Synaptophysin is sulfated on carbohydrate residues

Sulfation of proteins on tyrosine and carbohydrate residues, both of which occur selectively in the TGN (Kimura *et al.*, 1984; Baeuerle and Huttner, 1987), has been found to be a powerful tool to study protein exit from the TGN (Tooze and Huttner, 1990; Miller and Moore, 1991). We therefore investigated whether synaptophysin was sulfated.

After long-term (30 h) labeling of PC12 cells with [^{35}S]sulfate, prolonged exposure of the fluorogram (Figure 1, compare lanes 2 and 3) revealed the presence of a sulfated heterogeneous protein with the same electrophoretic mobility as authentic synaptophysin, immunoprecipitated with the monoclonal antibody SY38 (Wiedenmann and Franke, 1985) from PC12 cells after a 5 min pulse with methionine followed by a 2 h chase (Figure 1, lane 1). After pulse-labeling of PC12 cells with [^{35}S]sulfate for 5 min, the [^{35}S]sulfate incorporation into synaptophysin was found to be very low (Figure 1, lane 4); it was barely detectable after immunoprecipitation (Figure 1, lane 5) and was too low to allow transport studies involving pulse–chase labeling followed by subcellular fractionation.

[^{35}S]sulfate incorporation into synaptophysin as well as into other proteins including the hsPG and SgII was found to be greatly increased when PC12 cells were briefly (15 min) exposed to hypertonic medium containing 0.45 M sucrose prior to the 5 min [^{35}S]sulfate pulse (Figure 1, lanes 6 and 7). This hypertonic pretreatment was found to increase [^{35}S]sulfate uptake into the cells ~14-fold (data not shown). Alkaline hydrolysis of immunoprecipitated [^{35}S]sulfate-labeled synaptophysin revealed no detectable amount of tyrosine [^{35}S]sulfate, indicating that sulfation of synaptophysin occurred on carbohydrate residues (data not shown). Because of the more efficient [^{35}S]sulfate incorporation into synaptophysin after hypertonic pretreatment, we used this condition in all subsequent [^{35}S]sulfate-labeling experiments, unless indicated otherwise, to study the transport of synaptophysin from the TGN to its final destination.

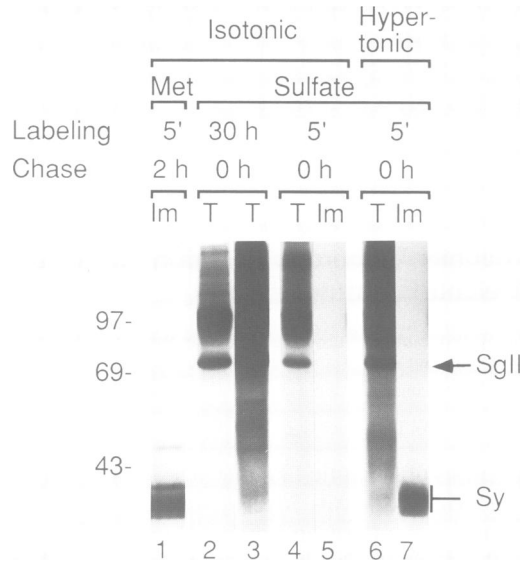

Fig. 1. [^{35}S]sulfate labeling of synaptophysin (Sy). PC12 cells were preincubated in isotonic or hypertonic medium, labeled with [^{35}S]methionine (Met, lane 1) or [^{35}S]sulfate (lanes 2–7), and aliquots of the total cleared lysates (T) or immunoprecipitates (Im), obtained from the cleared lysates using the monoclonal SY38 antibody, were analyzed by SDS–PAGE followed by fluorography, as specified in the figure. Lanes 2 and 3: fluorograms were exposed for 3 days and 22 days, respectively. Lanes 4–7 are from the same fluorogram (24 day exposure) showing 1/25 of the total cleared cell lysate (lanes 4 and 6) and all of the immunoprecipitated material (lanes 5 and 7).

Synaptophysin does not exit from the TGN in immature secretory granules

Cells producing synaptophysin, i.e. endocrine cells and neurons, are known to have two pathways of protein secretion, the constitutive and regulated pathway (Burgess and Kelly, 1987). It was previously shown (Tooze and Huttner, 1990) that in PC12 cells, constitutive secretory vesicles and immature secretory granules can be identified by the presence of a heparan sulfate proteoglycan (hsPG) and secretogranin II (SgII), respectively, using pulse–chase labeling with [^{35}S]sulfate. After a 5 min pulse with [^{35}S]sulfate, the hsPG and SgII are labeled selectively in the TGN; during a subsequent 15 min chase, the hsPG and SgII are packaged into constitutive secretory vesicles and immature secretory granules, respectively. These two vesicle populations can be separated from the TGN and from each other by sequential velocity–equilibrium sucrose gradient centrifugation (Tooze and Huttner, 1990).

When PC12 cells were pulse-labeled with [^{35}S]sulfate for 5 min, analysis by velocity and equilibrium sucrose gradient centrifugation showed that [^{35}S]sulfate-labeled synaptophysin was present in the TGN, being colocalized with the hsPG and SgII (Figure 2A,B,G; see also Tooze and Huttner, 1900). If the cells were chased for 7 or 15 min, the position of the [^{35}S]sulfate-labeled synaptophysin on both the velocity gradient and the equilibrium gradient coincided with that of the hsPG and was clearly distinct from that of SgII (Figure 2C–F, H). This indicated that synaptophysin did not exit from the TGN to any significant extent in immature secretory granules but, rather, left the TGN in vesicles that were very similar, if not identical, to constitutive secretory vesicles.

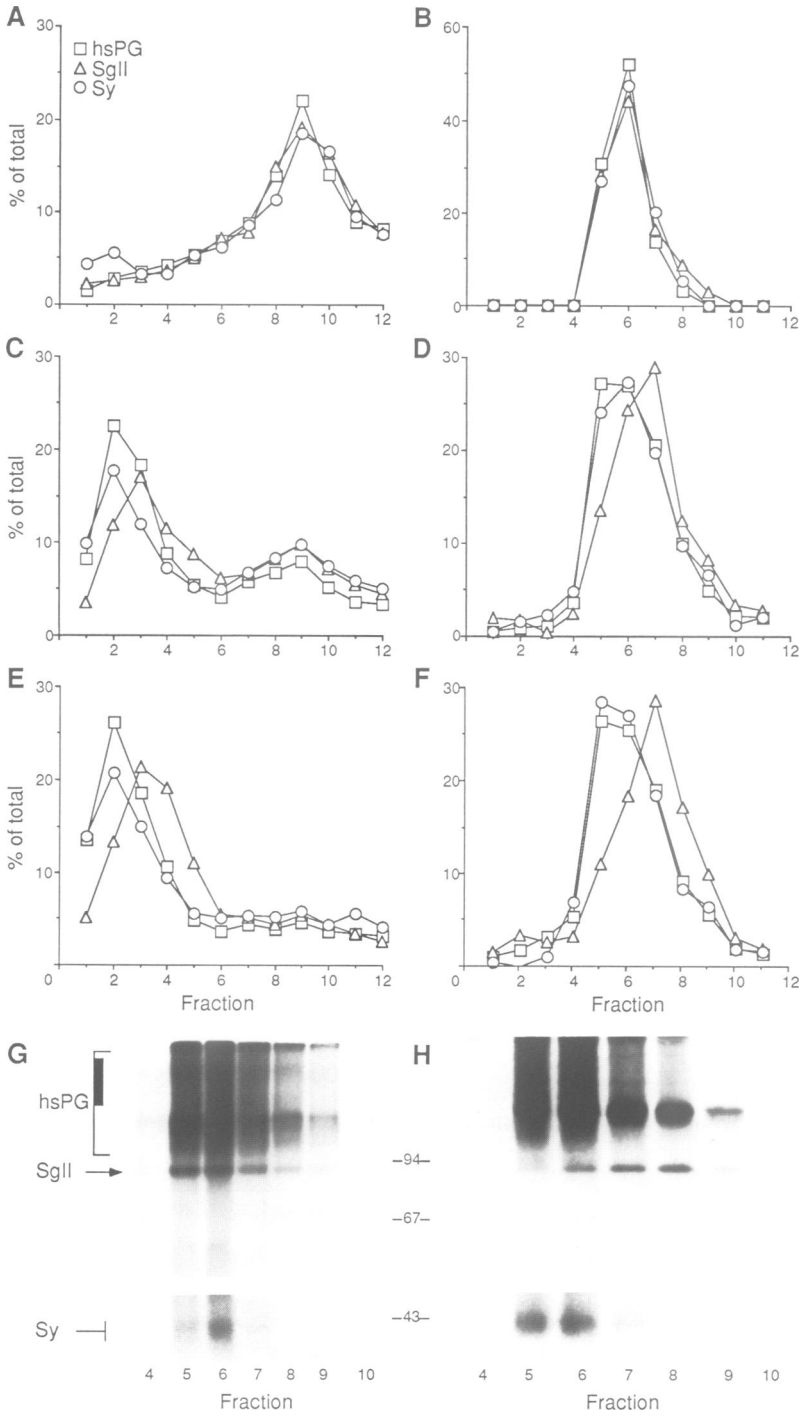

Fig. 2. Synaptophysin exits from the TGN into vesicles indistinguishable from constitutive secretory vesicles. PC12 cells were pulse-labeled for 5 min with [^{35}S]sulfate (panels A, B, G) or pulse-labeled and chased for 7 min (panels C,D) and 15 min (panels E,F,H). The post-nuclear supernatants were subjected to velocity sucrose gradient centrifugation (panels A,C,E). The pooled fractions 8−10 (panel A) and fractions 2−4 (panels C,E) were centrifuged to equilibrium on a second sucrose gradient (panel B and panels D,F, respectively). Fractions from each gradient (fraction 1 = top) were analyzed by SDS−PAGE followed by fluorography and quantification of the radioactivity in the hsPG (squares; thick bar in panel G indicates the area used for quantification), SgII (triangles) and synaptophysin (Sy, circles). Data are expressed as percent of the total (sum of fractions 1−12) [^{35}S]hsPG, [^{35}S]SgII and [^{35}S]synaptophysin. Panels G and H show fluorograms corresponding to panels B and F, respectively (panel H is from a different experiment than panel F). To allow the visual comparison of all three markers, a shorter exposure for the hsPG and SgII (upper part, 10 day exposure) than for synaptophysin (Sy; lower part, 21 day exposure) is shown.

Immunoisolation of constitutive secretory vesicles with anti-synaptophysin antibody

The above results did not allow us to distinguish between two possibilities concerning the exit of synaptophysin from the TGN: (i) that synaptophysin leaves this compartment in constitutive secretory vesicles; and (ii) that synaptophysin leaves the TGN in SLMVs if these vesicles were formed from the TGN and were indistinguishable from constitutive secretory vesicles under the centrifugation conditions used. If the first is the case, one would expect that constitutive

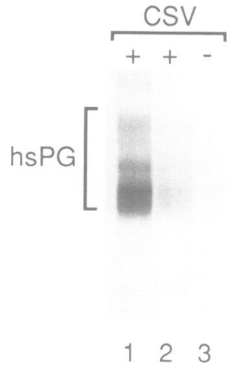

Fig. 3. Immunoisolation of PC12 cell constitutive secretory vesicles (CSV) with the monoclonal SY38 antibody against synaptophysin. PC12 cells were labeled with [^{35}S]sulfate for 5 min followed by a 15 min chase. The post-nuclear supernatant was subjected to velocity sucrose gradient centrifugation, and fractions 1–4 were subjected to equilibrium sucrose gradient centrifugation. Fractions from the equilibrium gradients in constitutive secretory vesicles (fractions 5–6) were subjected to two cycles of immunoisolation in the presence (+; lanes 1 and 2) and absence (−; lane 3) of the anti-synaptophysin antibody. The immunoisolated material was subjected to SDS–PAGE followed by fluorography.

secretory vesicles can be immunoisolated using an anti-synaptophysin antibody.

PC12 cells were pulse-labeled with [^{35}S]sulfate for 5 min under isotonic conditions and chased for 15 min. Fractions containing constitutive secretory vesicles, as identified by the presence of the [^{35}S]sulfate-labeled hsPG, were obtained by sequential velocity–equilibrium gradient centrifugation and subjected to immunoisolation using the monoclonal anti-synaptophysin antibody SY38 (Figure 3, lanes 1 and 2). Analysis of the immunoisolated material indicated that constitutive secretory vesicles could be immunoisolated with the anti-synaptophysin antibody whereas this was not the case when the immunobeads were coated only with the secondary antibody, but not with the primary anti-synaptophysin antibody (Figure 3, lane 3). Comparison of the starting amount of [^{35}S]sulfate-labeled hsPG with that recovered after immunoisolation and that remaining in the unbound material revealed that most (>80%) of [^{35}S]sulfate-labeled hsPG was recovered with the immunoisolated material. Nearly quantitative immunoisolation of vesicles containing [^{35}S]sulfate-labeled hsPG was also observed if the chase was carried out for 7 rather than 15 min (data not shown). When fractions from the equilibrium gradient containing immature secretory granules, as identified by the presence of [^{35}S]sulfate-labeled SgII after a 15 min chase, were subjected to immunoisolation using the anti-synaptophysin antibody, we found that immature secretory granules could also be immunoisolated. However, the efficiency of immunoisolation of immature secretory granules was much less than that of the constitutive secretory vesicles, being 20% or less (data not shown). These results show that synaptophysin is present in most, if not all, of the constitutive secretory vesicles but only in the minority of immature secretory granules.

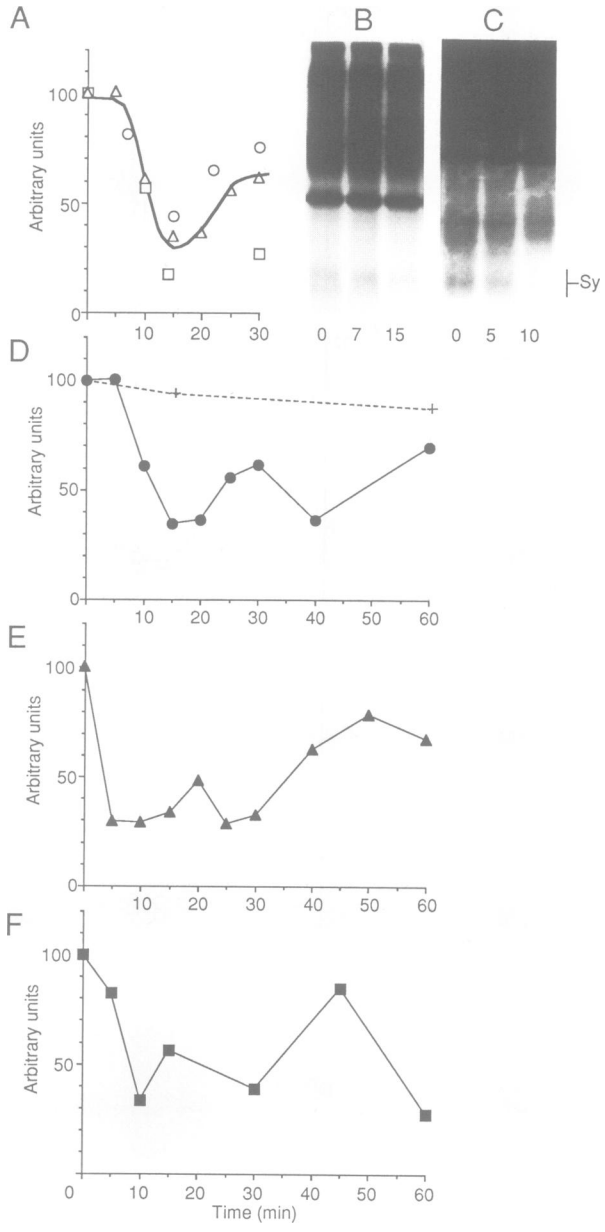

Fig. 4. After exit from the TGN, synaptophysin (Sy) rapidly appears at the cell surface and is subsequently internalized. PC12 cells were [^{35}S]sulfate-labeled for 5 min and chased for various times, followed by either cell surface biotinylation or cell surface endoglycosidase F treatment. The cleared lysates were either subjected to streptavidin–agarose precipitation to remove the biotinylated proteins followed by analysis of the unbound material by SDS–PAGE and fluorography (**panel B**), or in the case of endoglycosidase F treatment were directly analyzed by SDS–PAGE and fluorography (**panel C**). [^{35}S]Synaptophysin was quantified, and data are expressed as arbitrary units, with the t = 0 value being set to 100. The data represent the amount of [^{35}S]synaptophysin resistant to either cell surface biotinylation (**panels A,D,E**) or endoglycosidase F treatment (**panel F**). Panel A shows the results of three independent experiments up to 30 min of chase. Panels D and E show the results of two independent experiments with chase times up to 60 min. The dashed line in panel D shows the amount of [^{35}S]synaptophysin present in the post-nuclear supernatant.

Constitutive exocytosis of TGN-derived synaptophysin

If newly synthesized synaptophysin leaves the TGN in constitutive secretory vesicles, one would expect that the [^{35}S]sulfate-labeled synaptophysin should rapidly appear at the cell surface, without stimulation of regulated secretion. To investigate this possibility, PC12 cells pulse-labeled with [^{35}S]sulfate for 5 min and chased for up to 60 min were subjected either to cell surface biotinylation followed by

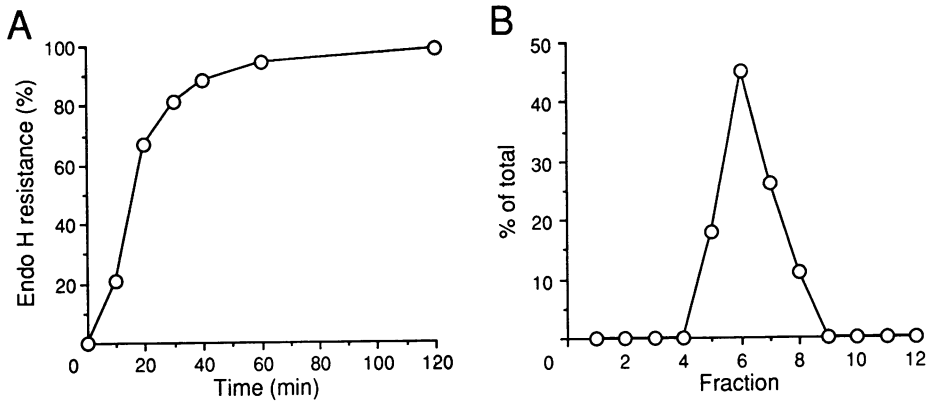

Fig. 5. Synaptophysin exit from the TGN into constitutive secretory vesicles does not depend on hypertonic sucrose pretreatment. **Panel A:** PC12 cells were [^{35}S]methionine-labeled for 5 min and chased for various times. Synaptophysin was immunoprecipitated, incubated in the presence of endoglycosidase H (Endo H), and analyzed by SDS−PAGE followed by fluorography. The endoglycosidase H-sensitive and -resistant forms were quantified, and the endoglycosidase H-resistant form is expressed as percent of the sum of endoglycosidase H-sensitive plus -resistant forms. **Panel B**: PC12 cells were [^{35}S]methionine-labeled for 5 min and chased for 35 min. The post-nuclear supernatant was subjected to velocity sucrose gradient centrifugation. The pooled fractions 2−4 were centrifuged to equilibrium on a second sucrose gradient. Fractions of the second gradient (fraction 1 = top) were analyzed by SDS−PAGE followed by fluorography. [^{35}S]Synaptophysin was quantified and is expressed as a percentage of the total (sum of fractions 1−12) [^{35}S]synaptophysin. The same distribution across the equilibrium gradient was observed when [^{35}S]synaptophysin was immunoprecipitated from the fractions and quantified (data not illustrated).

binding of biotinylated proteins to streptavidin−agarose, or to cell surface endoglycosidase F treatment to remove carbohydrate-linked [^{35}S]sulfate. The total [^{35}S]sulfate-labeled synaptophysin remained largely constant over the 60 min chase period (Figure 4D, dashed line), indicating the lack of any significant desulfation and/or proteolysis during this time. Within 10−15 min of chase, the majority of the [^{35}S]sulfate-labeled synaptophysin became biotinylated and could be removed from the lysate by streptavidin−agarose (Figure 4A,B), indicating that synaptophysin was rapidly transported from the TGN to the cell surface. The $t_{1/2}$ of cell surface appearance of [^{35}S]sulfate-labeled synaptophysin was very similar to that of the hsPG (not apparent in Figure 4B because of over-exposure) and was found to be ~10 min, a value identical to that determined previously for the cell surface appearance of a constitutively secreted [^{35}S]sulfate-labeled protein in fibroblasts (Friederich *et al.*, 1988). Cell surface treatment with endoglycosidase F after various times of chase also revealed the rapid appearance of the majority of the [^{35}S]sulfate-labeled synaptophysin at the plasma membrane (Figure 4F). Following its initial appearance at the cell surface, the amount of [^{35}S]sulfate-labeled synaptophysin sensitive to cell surface biotinylation and endoglycosidase F digestion did not remain constant, but varied with time in a manner consistent with synaptophysin undergoing multiple cycles of endocytosis−exocytosis (Figure 4D−F).

The above results indicated that most, if not all, of the [^{35}S]sulfate-labeled synaptophysin left the TGN in constitutive secretory vesicles. The hypertonic pretreatment used to increase the efficiency of [^{35}S]sulfate labeling affected neither the kinetics of exit of the [^{35}S]sulfate-labeled hsPG and SgII nor the sorting of these molecules from each other (Figure 2; cf. Tooze and Huttner, 1990). It was therefore unlikely that this pretreatment interfered with the sorting of proteins in the TGN and caused the packaging of synaptophysin into constitutive secretory vesicles. Nevertheless, we investigated whether under isotonic conditions, the bulk of newly synthesized synaptophysin was present in constitutive secretory vesicles after leaving the TGN. When PC12 cells were pulse-labeled for 5 min with [^{35}S]methionine and chased for various times, the labeled synaptophysin became resistant to endoglycosidase H, indicating its arrival in the medial Golgi, with a $t_{1/2}$ of ~20 min (Figure 5A), consistent with a previous report (Cutler and Cramer, 1990). To identify [^{35}S]methionine-labeled synaptophysin in early post-TGN vesicles, PC12 cells pulse-labeled for 5 min with [^{35}S]methionine were chased for 35 min, the post-nuclear supernatant was subjected to velocity sucrose gradient centrifugation, and fractions 2−4, which are known to contain post-TGN vesicles but not TGN membranes (Tooze and Huttner, 1990), were collected. Analysis of these vesicles by equilibrium sucrose gradient centrifugation revealed a distribution of this [^{35}S]methionine-labeled synaptophysin (Figure 5B) which was very similar to that of the constitutive secretory hsPG and clearly distinct from immature secretory granules identified by SgII (see Figure 2D and F). Thus, the exit of synaptophysin from the TGN into constitutive secretory vesicles was not caused by the hypertonic pretreatment.

Fate of synaptophysin after arrival at the plasma membrane

We investigated the fate of [^{35}S]sulfate-labeled synaptophysin following its exit from the TGN in constitutive secretory vesicles and its arrival at the plasma membrane. The observation that the amount of [^{35}S]sulfate-labeled synaptophysin sensitive to cell surface biotinylation and endoglycosidase F digestion varied during the first 60 min of chase (Figure 4D−F) suggested a cycling of [^{35}S]sulfate-labeled synaptophysin, after its initial arrival at the cell surface, between the plasma membrane and an intracellular compartment. We therefore investigated the fate of [^{35}S]sulfate-labeled synaptophysin in relation to early and late endosomes. The bulk of [^{35}S]sulfate-labeled synaptophysin appears in fractions 1−4 of the velocity gradient within 15 min of chase (see Figure 2E), concomitantly with its arrival at the cell surface (see Figure 4A). When PC12 cells were pulse-labeled with [^{35}S]sulfate for 5 min and chased for up to 60 min, the bulk of

[^{35}S]sulfate-labeled synaptophysin was found in fractions 1–4 at 30 min of chase (Figure 6A), a time at which a portion of the [^{35}S]sulfate-labeled synaptophysin molecules had apparently been endocytosed (see Figure 4A). In fact, at any of the chase times investigated, the vast majority of [^{35}S]sulfate-labeled synaptophysin was found in fractions 1–4 of the velocity gradient (Figure 6D). This indicated that these fractions contained the bulk of both the plasma membrane and the intracellular compartment(s) involved in the cycling of [^{35}S]sulfate-labeled synaptophysin.

PC12 cells were allowed to take up horseradish peroxidase, a fluid phase marker of endocytosis, for 7 min. Fractionation of the post-nuclear supernatant by velocity sucrose gradient centrifugation revealed a peak of peroxidase activity in the top fractions of the gradient (Figure 6B). (Further centrifugation of fractions 2–4 revealed that virtually all of the peroxidase present in these fractions was particulate; however, we assume that at least some of the peroxidase in fraction 1 was soluble, probably as a result of cell homogenization.) In contrast, when PC12 cells were chased for 53 min following a 7 min horseradish peroxidase uptake, the peak of peroxidase in the top fractions decreased and a second peak of peroxidase was found in the bottom half of the velocity gradient (Figure 6C). These results suggested that early endosomes and the vesicles mediating transport between early endosomes and the plasma membrane, identified by the 7 min uptake of horseradish peroxidase, were largely present in the top fractions of the gradient, whereas late endosomes, identified by the 53 min chase of internalized horseradish peroxidase, were largely present in the bottom half of the gradient. From the comparison of the distribution across the velocity gradient of early endosomes (Figure 6B), late endosomes (Figure 6C) and the TGN (cf. Figure 2A) with that of [^{35}S]sulfate-labeled synaptophysin chased for up to 60 min, it can be concluded that during this time newly synthesized endocytosed synaptophysin was not transported to any major extent to late endosomes and the TGN, but may have passed through early endosomes. Consistent with this conclusion, preliminary experiments with PC12 cells exposed to horseradish peroxidase for 7 min show that peroxidase-containing vesicles can be immunoisolated by an anti-synaptophysin antibody (data not shown).

Transport of synaptophysin to a population of light vesicles

We investigated the transport of newly synthesized synaptophysin to its final destination. In the brain, the final destination of synaptophysin are the SSVs, which can be highly enriched by differential centrifugation followed by equilibrium sucrose gradient centrifugation (Huttner *et al.*, 1983). We used a similar centrifugation protocol to isolate SLMVs, the presumed final destination of synaptophysin in PC12 cells. The post-nuclear supernatant from PC12 cells was centrifuged for 20 min at 25 000 *g* to remove large membraneous structures (e.g. plasma membrane and endosome fragments), and the vesicles remaining in the supernatant were pelleted. This pellet, referred to as 'P3 fraction', was subjected to equilibrium sucrose gradient centrifugation followed by immunoblotting for synaptophysin. The distribution of synaptophysin immunoreactivity (peak in fraction 5, density of 1.099 g/cm^3; Figure 7A) revealed that PC12 cell SLMVs had a lower buoyant density than, and therefore could be separated from,

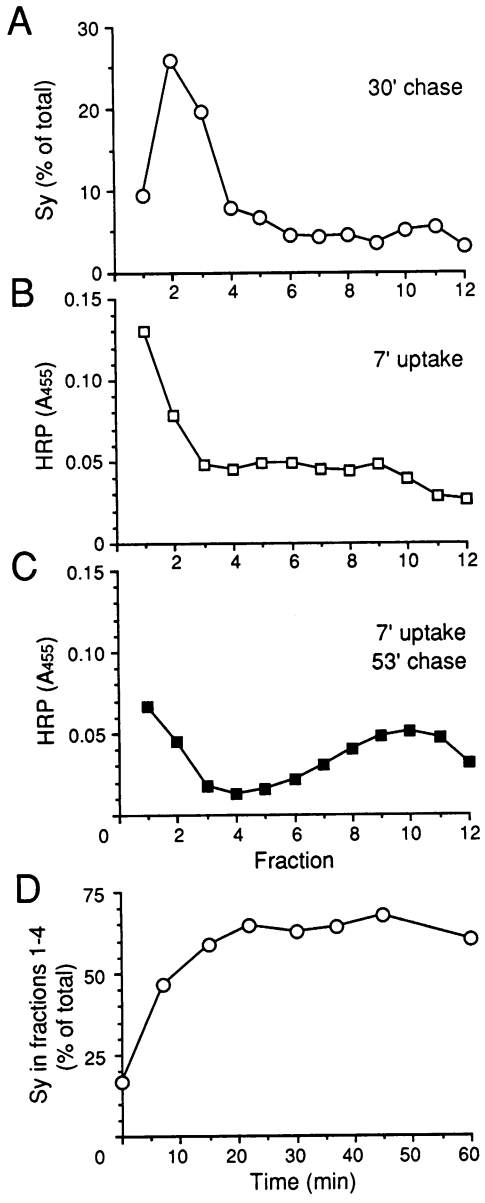

Fig. 6. Distribution of post-TGN synaptophysin in the velocity gradient in comparison to the distribution of early and late endosomes. PC12 cells were [^{35}S]sulfate-labeled for 5 min and chased for various times, with horseradish peroxidase being present in the medium during the first 7 min of chase. The post-nuclear supernatants were subjected to velocity sucrose gradient centrifugation, and gradient fractions (fraction 1 = top) were either analyzed by SDS–PAGE followed by fluorography and quantification of the radioactivity in synaptophysin, or assayed for horseradish peroxidase activity. **Panel A**: distribution of [^{35}S]synaptophysin (Sy) across the gradient after 30 min of chase. Data are expressed as a percentage of the total (sum of fractions 1–12) [^{35}S]synaptophysin. **Panels B and C**: distribution of horseradish peroxidase (HRP) activity across the gradient after 7 min of uptake (**panel B**) and after 7 min of uptake followed by 53 min of chase (**panel C**). Note that the scale of the ordinates is the same. **Panel D**: time course of [^{35}S]synaptophysin (Sy) distribution across the velocity gradient. The sum of [^{35}S]synaptophysin present in fractions 1–14 is expressed as percent of the total (sum of fractions 1–12) [^{35}S]synaptophysin.

constitutive secretory vesicles, identified by the presence of the [^{35}S]sulfate-labeled hsPG chased for 7 min (peak in fraction 6, density of 1.116 g/cm^3; Figure 7A). When the post-nuclear supernatant from PC12 cells [^{35}S]sulfate-labeled for 5 min and chased for various time periods was subjected to the same differential and equilibrium gradient

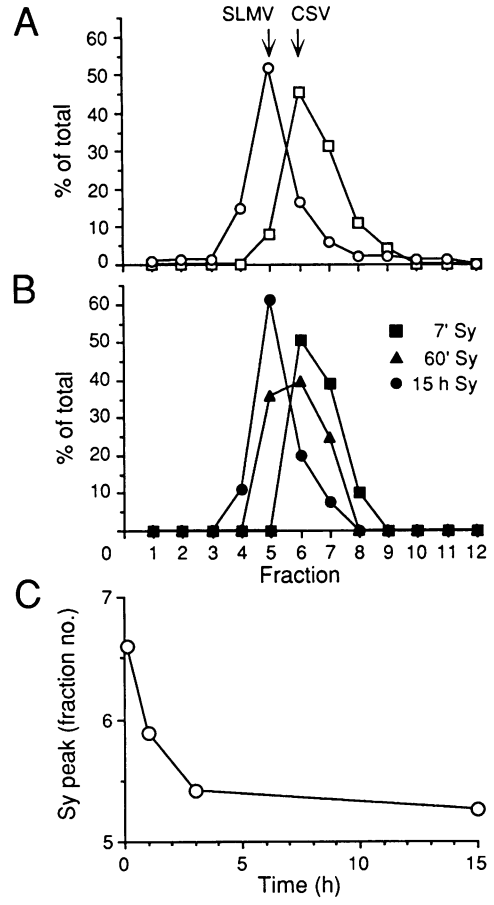

Fig. 7. Transport of synaptophysin to its final destination—analysis by equilibrium sucrose gradient centrifugation. PC12 cells were [^{35}S]sulfate-labeled for 5 min and chased for 7 min, 60 min, 3 h and 15 h. The post-nuclear supernatants were subjected to differential centrifugation followed by equilibrium sucrose gradient centrifugation. The fractions from the sucrose gradients (fraction 1 = top) were analyzed by SDS–PAGE followed by either fluorography or immunoblotting for synaptophysin using [^{125}I]protein A. The [^{35}S]hsPG, [^{35}S]synaptophysin and synaptophysin immunoreactivity were quantified and are expressed as a percentage of the total (sum of fractions 1–12) [^{35}S]hsPG, [^{35}S]synaptophysin and [^{125}I]synaptophysin, respectively. **Panel A**: distribution across the gradient of the [^{35}S]hsPG after a 7 min chase (constitutive secretory vesicles (CSV), open squares) and of synaptophysin immunoreactivity (SLMV, open circles) **Panel B**: distribution across the gradient of [^{35}S]synaptophysin (Sy) after a 7 min chase (closed squares), 60 min chase (closed triangles) and 15 h chase (closed circles). **Panel C**: Time course of arrival of [^{35}S]sulfate-labeled synaptophysin in SLMVs. The position of the peak of [^{35}S]synaptophysin (Sy) in the equilibrium gradient was calculated for the 7 min, 60 min, 3 h and 15 h chase, and is expressed in relation to fraction number.

centrifugation, the position of [^{35}S]sulfate-labeled synaptophysin was found to shift with time from that of constitutive secretory vesicles (7 min chase) via an intermediate position (60 min chase) to that of the SLMVs (15 h chase; Figure 7B). The time course of this shift revealed that most of the [^{35}S]sulfate-labeled synaptophysin recovered in the P3 fraction was present in SLMVs after 3 h of chase (Figure 7C).

Separation of SLMVs from other post-TGN organelles by permeation chromatography using controlled-pore glass

A characteristic feature of brain SSVs is their uniform small size, which allows these vesicles to be recovered as an 'included' peak upon permeation chromatography using controlled-pore glass (CPG) beads, in contrast to other membrane vesicles which are found in the void volume (Nagy *et al.*, 1976; Huttner *et al.*, 1983). The typical optical density profile and distribution of synaptophysin immunoreactivity upon CPG chromatography of partially purified rat brain SSVs is shown, as reference, in Figure 8 (panels A and E, and panel B, respectively). Confirming a previous study (Jahn *et al.*, 1985), synaptophysin immunoreactivity coincided with the included peak of optical density (fractions 8–13). When SLMVs isolated from PC12 cells by differential and equilibrium gradient centrifugation were subjected to CPG chromatography, most of the synaptophysin immunoreactivity was included in the column (Figure 8C). The synaptophysin found in the position of the void volume probably represented membranes larger than SLMVs which had not been removed by the differential and equilibrium gradient centrifugation. The included peak of synaptophysin (fractions 6–11, Figure 8C) did not coincide with that of the rat brain SSV synaptophysin (Figure 8B) but was slightly shifted towards the void volume. Taken together, these results indicate that PC12 cell SLMVs, like rat brain SSVs, are included in the column upon CPG chromatography but are slightly larger than the SSVs, which is consistent with previous morphological observations (Navone *et al.*, 1986).

We investigated the behaviour of constitutive secretory vesicles and plasma membrane fragments upon CPG chromatography, using as a marker the [^{35}S]sulfate-labeled hsPG chased for 7 min (Figure 8F) and 22 min (Figure 8G), respectively. For both chase times, the [^{35}S]sulfate-labeled hsPG peaked in the void volume with an overall distribution typical of a particle excluded from the column. Exclusion from the CPG column was also observed for immature secretory granules identified by the presence of [^{35}S]sulfate-labeled SgII chased for 7 min (Figure 8H). (The exclusion of constitutive secretory vesicles and immature secretory granules was observed not only under these conditions (Figure 8F and H) but also when the top fractions of the velocity gradient were subjected directly to CPG chromatography (data not shown)). These data show that of the post-TGN membranes of PC12 cells analyzed, only SLMVs are included in the CPG column.

Transport of synaptophysin to its final destination, the SLMV

Because of the selective inclusion of SLMVs in the CPG column, it was possible to analyze the appearance of [^{35}S]sulfate-labeled synaptophysin in SLMVs during the chase by CPG chromatography. When the peak of [^{35}S]sulfate-labeled synaptophysin chased for 60 min, obtained after differential and equilibrium sucrose gradient centrifugation (fractions 5 and 6; see Figure 7B), was subjected to CPG chromatography, [^{35}S]sulfate-labeled synaptophysin was found in both the void volume and the fractions following immediately thereafter (Figure 8D), in a profile quite different from that of the SLMV synaptophysin immunoreactivity (Figure 8C). In contrast, when the peak of [^{35}S]sulfate-labeled synaptophysin chased for 15 h was subjected to CPG chromatography, [^{35}S]sulfate-labeled synaptophysin was included in the column (Figure 8D), its distribution coinciding with that of the SLMV synaptophysin immunoreactivity (Figure 8C). Thus, consistent with the results of the analysis by equilibrium sucrose gradient centrifugation (see Figure 7B),

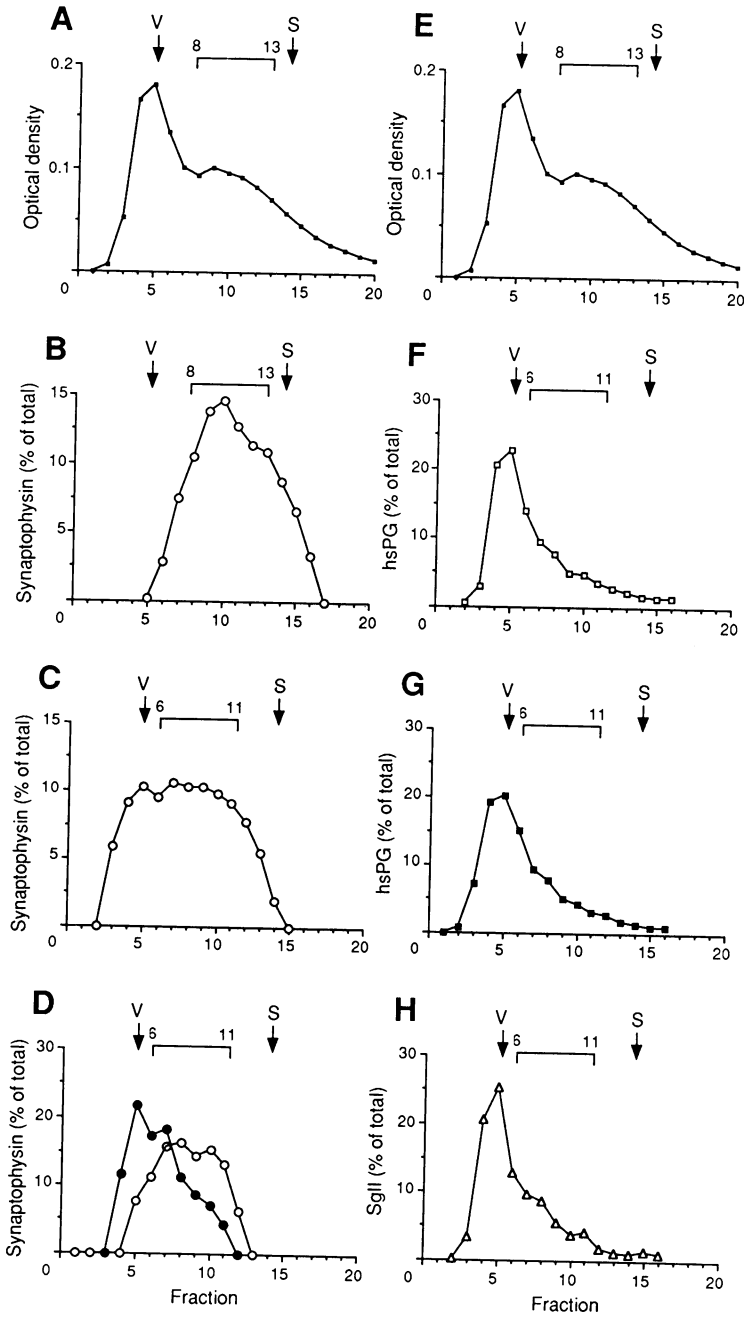

Fig. 8. Transport of synaptophysin to its final destination—analysis by CPG permeation chromatography. Panels A, B, E: a rat brain SSV preparation (SG-V, see Huttner *et al.*, 1983) was subjected to CPG chromatography. **Panels A and E**: the same optical density profile (280 nm), shown as a reference. **Panel B**: SSVs; distribution of synaptophysin immunoreactivity determined by immunoblotting using peroxidase. Panels C, D, F, H: post-nuclear supernatants from PC12 cells [^{35}S]sulfate-labeled for 5 min and chased for various times were subjected to differential centrifugation followed by equilibrium sucrose gradient centrifugation, and fractions 5 and 6 of the equilibrium gradient were subjected to CPG chromatography. **Panel C**: SLMVs; distribution of synaptophysin immunoreactivity determined by immunoblotting using peroxidase. **Panel D**: transport of synaptophysin to SLMVs; distribution of [^{35}S]synaptophysin after a 60 min chase (closed circles) and 15 h chase (open circles). **Panel F**: constitutive secretory vesicles; distribution of [^{35}S]hsPG after a 7 min chase. **Panel H**: immature secretory granules; distribution of [^{35}S]SgII after a 7 min chase. **Panel G**: plasma membrane-derived vesicles; the post-nuclear supernatant from PC12 cells [^{35}S]sulfate-labeled for 5 min and chased for 22 min was subjected to velocity sucrose gradient centrifugation, fractions 2–4 were subjected to CPG chromatography, and the distribution of the [^{35}S]hsPG was determined. For each marker quantified, the results are expressed as percent of the total (sum of all fractions analyzed) hsPG, SgII and synaptophysin. V: peak position of the void volume (fraction 5) as revealed by optical density; S: beginning of the peak of the soluble marker dye phenol red (fraction 14). The positions of the included peaks of SSVs (fractions 8–13) and SLMVs (fractions 6–11) are indicated.

most of the [^{35}S]sulfate-labeled synaptophysin recovered in the P3 fraction was present in SLMVs after 15 h of chase.

The CPG chromatography of PC12 cell SLMVs shown in Figure 8 involved enriching the SLMVs by differential centrifugation and equilibrium sucrose gradient centrifugation prior to the permeation chromatography (see Materials and methods). When a post-nuclear supernatant from PC12 cells pulse-labeled with [^{35}S]sulfate and chased for 15 h was subjected to velocity gradient centrifugation, and fractions 2–4, which contained the bulk of the [^{35}S]sulfate-labeled synaptophysin, were directly subjected to CPG chromatography, the majority of the [^{35}S]sulfate-labeled

synaptophysin was found in the void volume (data not shown). This suggested the presence of relatively large synaptophysin-containing membranes in the post-nuclear supernatant, which obscured the presence of SLMVs upon CPG chromatography. Most of these larger membranes were presumably pelleted with the P2 fraction upon differential centrifugation and therefore absent from the P3 fraction. Consistent with this interpretation, we found that only ~40% of the [^{35}S]sulfate-labeled synaptophysin present in the post-nuclear supernatant was recovered in the P3 fraction.

The observations that the bulk of [^{35}S]sulfate-labeled synaptophysin was constitutively transported from the TGN to the plasma membrane (see Figures 2, 3 and 4), and that the kinetics of appearance of [^{35}S]sulfate-labeled synaptophysin at the cell surface were considerably faster than the kinetics of appearance in SLMVs (cf. Figures 4 and 7), suggested that [^{35}S]sulfate-labeled synaptophysin moved from the plasma membrane to SLMVs. To address this point directly, PC12 cells were pulse-labeled with [^{35}S]sulfate for 5 min, chased for 15 min to allow for the delivery of [^{35}S]sulfate-labeled synaptophysin to the cell surface (cf. Figure 4), subjected to cell surface biotinylation, and chased for 3 h to allow for the transport of biotinylated [^{35}S]sulfate-labeled synaptophysin to SLMVs (cf. Figure 7C). SLMVs were then isolated by differential centrifugation followed by equilibrium sucrose gradient centrifugation, and [^{35}S]sulfate-labeled synaptophysin was analyzed for its ability to bind to streptavidin–agarose. We found that ~90% of the [^{35}S]sulfate-labeled synaptophysin chased into SLMVs had become biotinylated at the cell surface. This showed that virtually all of the newly synthesized synaptophysin delivered to SLMVs had taken the TGN → plasma membrane → SLMV route.

Discussion

In the present study, pulse–chase labeling with [^{35}S]sulfate was used to investigate the exit of newly synthesized synaptophysin from the TGN and its transport to its final destination, the SLMVs. It is unlikely that sulfation occurred on a (hypothetical) form of synaptophysin that had recycled to the TGN, for the following reason. The half-life of [^{35}S]sulfate-labeled synaptophysin was in the range of 1–2 days (data not shown), a value similar to that reported for total cellular synaptophysin (Johnston *et al.*, 1989; Wiedenmann and Huttner, 1989). Thus, turnover of the [^{35}S]sulfate label occurred concomitantly with protein turnover, implying that newly synthesized synaptophysin was the substrate for sulfation. In addition, since [^{35}S]sulfate-labeled synaptophysin was eventually found in SLMVs, it can be concluded that the intracellular traffic of [^{35}S]sulfate-labeled synaptophysin was representative of that of newly synthesized synaptophysin in general.

Synaptophysin traffic—from TGN to plasma membrane

We conclude from the results that most, if not all, of the newly synthesized synaptophysin is transported from the TGN to the plasma membrane in constitutive secretory vesicles (Figure 9, step 1). This conclusion is based on three independent lines of evidence. First, the bulk of [^{35}S]sulfate-labeled synaptophysin left the TGN in vesicles which were indistinguishable by velocity and equilibrium sucrose gradient centrifugation from constitutive secretory vesicles, but distinct from immature secretory granules and SLMVs. Second, constitutive secretory vesicles could be efficiently immunoisolated using an anti-synaptophysin antibody. Third, the bulk of [^{35}S]sulfate-labeled synaptophysin reached the cell surface of resting cells with a $t_{1/2}$ characteristic of delivery by the constitutive secretory pathway. Our finding that newly synthesized synaptophysin leaves the TGN in constitutive secretory vesicles does not support the idea that SLMVs arise from the TGN, as has been discussed e.g. by Leube *et al.* (1989), nor does it support the possibility that the regulated pathway of protein secretion plays a major role in the delivery of newly synthesized synaptophysin to its final destination (Lowe *et al.*, 1988).

The [^{35}S]sulfate labeling used in the present study involved a hypertonic pretreatment of cells with 0.45 M sucrose. This treatment was used since it greatly increased [^{35}S]sulfate uptake and thereby the efficiency of [^{35}S]sulfate labeling. On the other hand, this treatment is known to inhibit receptor-mediated, but not fluid-phase, endocytosis by blocking clathrin-coated pit formation (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). Thus, if one would hypothesize that SLMVs form from the TGN in a clathrin-dependent process, it could be argued that this process would be inhibited by the hypertonic treatment, resulting in the exit of newly synthesized synaptophysin from the TGN, by default, to the constitutive secretory pathway. Several lines of evidence indicate, however, that this was not the case. First, synaptophysin was packaged into constitutive secretory vesicles also under isotonic conditions as indicated by the efficient immunoisolation of these vesicles with an anti-synaptophysin antibody. Second, post-TGN vesicles containing synaptophysin labeled with [^{35}S]methionine without hypertonic pretreatment had a buoyant density similar to that of constitutive secretory vesicles, but distinct from immature secretory granules and SLMVs. Third, the exit of SgII from the TGN to immature secretory granules, which have been shown to contain clathrin on their cytoplasmic surface (Orci *et al.*, 1985; Tooze and Tooze, 1986), occurred with the same kinetics and efficiency under the present conditions as reported previously after [^{35}S]sulfate labeling without hypertonic pretreatment (Tooze and Huttner, 1990). Fourth, the inhibitory effect of hypertonic sucrose on receptor-mediated endocytosis has been shown to be completely reversed within 5 min in isotonic medium (Daukas and Zigmond, 1985). Since [^{35}S]sulfate labeling was carried out for 5 min in isotonic medium after the hypertonic pretreatment, and since the exit of [^{35}S]sulfate-labeled molecules from the TGN only starts after this labeling period (Tooze and Huttner, 1990; cf. Figure 2), i.e. at the beginning of the chase, the exit of [^{35}S]sulfate-labeled molecules actually took place in cells in which any effect of the hypertonic pretreatment should have been reversed.

At most ~70% of the total [^{35}S]sulfate-labeled synaptophysin was sensitive to cell surface biotinylation and cell surface endoglycosidase F digestion. This incomplete sensitivity does not contradict the conclusion that virtually all of the [^{35}S]sulfate-labeled synaptophysin left the TGN in constitutive secretory vesicles. Rather, an incomplete sensitivity of [^{35}S]sulfate-labeled synaptophysin to cell surface biotinylation and endoglycosidase F digestion would be expected if all newly synthesized synaptophysin is

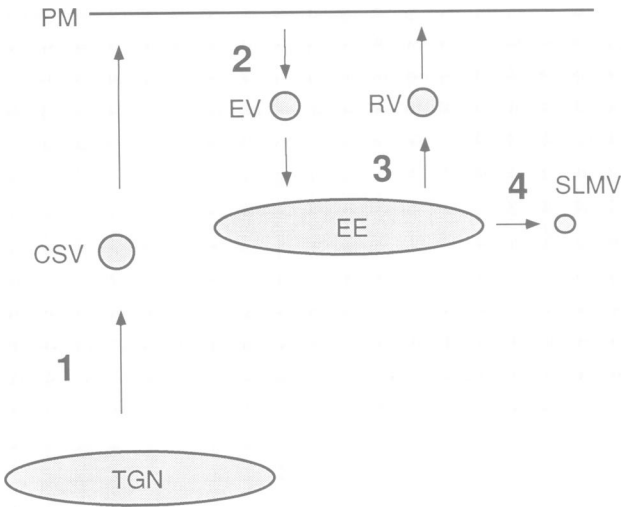

Fig. 9. Proposed biogenesis of SLMVs in neuroendocrine cells, as suggested from the present results and the work published by Johnston *et al.* (1989) and Clift-O'Grady *et al.* (1990). After synthesis in the rough endoplasmic reticulum and passage through the Golgi complex, integral membrane proteins destined to SLMVs first move from the TGN to the plasma membrane in constitutive secretory vesicles (1, CSV). They then cycle several times between the plasma membrane (PM) and the early endosome (EE), being transported in endocytotic vesicles (2, EV) and recycling vesicles (3, RV), respectively. Eventually, these proteins segregate from recycling proteins, concentrate in the membrane, and bud off from the early endosome in the form of SLMVs (4). In non-neuroendocrine cells, step 4 is lacking. In adult neurons, step 4 generates SSVs.

delivered to the plasma membrane but is rapidly internalized thereafter. Indeed, the results of cell surface biotinylation and endoglycosidase F digestion were consistent with internalization of [^{35}S]sulfate-labeled synaptophysin, the $t_{1/2}$ of internalization being apparently in the same range as the $t_{1/2}$ of arrival at the plasma membrane. This implies that after a 5 min pulse, some of the [^{35}S]sulfate-labeled synaptophysin molecules will already be internalized before all of them have been delivered to the cell surface. Hence, not all [^{35}S]sulfate-labeled synaptophysin can be sensitive to cell surface treatment at any moment in time although all of them pass through the plasma membrane.

Synaptophysin traffic—from plasma membrane to early endosomes

While the arrival of [^{35}S]sulfate-labeled synaptophysin at the plasma membrane was a relatively rapid process, the arrival at its final destination, the SLMVs, took significantly longer, leveling off after 3 h of chase. We believe that in the period between its appearance at the plasma membrane and its arrival in SLMVs, synaptophysin cycled for several times between the early endosome and the plasma membrane (Figure 9, steps 2 and 3), for the following reasons. The sensitivity of [^{35}S]sulfate-labeled synaptophysin to cell surface biotinylation and endoglycosidase F digestion was consistent with a cycling between the plasma membrane and some intracellular compartment(s). The latter compartment was neither the TGN nor late endosomes since [^{35}S]sulfate-labeled synaptophysin was not chased to the corresponding positions on the velocity gradient. Rather, the bulk of the [^{35}S]sulfate-labeled synaptophysin was found over the entire 60 min chase period studied in fractions which also contained the peak of horseradish peroxidase activity after a 7 min uptake period. We conclude that newly synthesized synaptophysin cycles between the plasma membrane and early endosomes prior to its incorporation into SLMVs. This conclusion is consistent with the observations that at steady state, some of the synaptophysin in PC12 cells is present in endosomal structures (Johnston *et al.*, 1989; Clift-O'Grady *et al.*, 1990) which have very recently been identified as early endosomes and the vesicles shuttling between them and the plasma membrane (Cameron *et al.*, 1991). In light of these reports, we find it likely that the larger membranes containing [^{35}S]sulfate-labeled synaptophysin after prolonged chase times, which were pelleted with the P2 fraction upon differential centrifugation, included early endosomes.

What may be the molecular mechanism underlying the cycling of synaptophysin between the plasma membrane and early endosomes prior to SLMV formation? As far as we are aware, the 'signal' in the synaptophysin molecule that is responsible for its endocytosis has not been identified. In light of what is known about endocytotic signals in other proteins (Davis *et al.*, 1986; Collawn *et al.*, 1990), it is tempting to speculate that some of the tyrosines present in the cytoplasmic tail of synaptophysin (Buckley *et al.*, 1987; Leube *et al.*, 1987; Südhof *et al.*, 1987) may play a key role in its endocytosis. Once internalized into early endosomes, synaptophysin can be expected to be transported back to the plasma membrane by default unless sorted to another compartment.

Synaptophysin traffic—from early endosomes to SLMVs

Characterization of SLMVs from PC12 cells, as determined by synaptophysin immunoreactivity at steady state, revealed that SLMVs were lighter and smaller than any other post-TGN organelle studied; they migrated as a distinct peak upon equilibrium sucrose gradient centrifugation and were included in the column upon CPG chromatography. This ability to separate SLMVs from essentially all other post-TGN organelles has allowed us to investigate the transport of newly synthesized synaptophysin into SLMVs. As mentioned above, even after prolonged chase times, only a portion of the [^{35}S]sulfate-labeled synaptophysin (at most 40%) was recovered in the P3 fraction and found to be present in SLMVs upon equilibrium sucrose gradient analysis. Since most, if not all, of the newly synthesized [^{35}S]sulfate-labeled synaptophysin was initially delivered to the plasma membrane, the portion of [^{35}S]sulfate-labeled synaptophysin recovered in SLMVs at later chase times must have been derived from the plasma membrane. Direct evidence in support of this conclusion comes from the observation that 90% of the newly synthesized [^{35}S]sulfate-labeled synaptophysin chased into SLMVs had been accessible to cell surface biotinylation earlier in the chase.

In light of the cycling of synaptophysin between the plasma membrane and early endosomes discussed above, we find it likely that the transport of newly synthesized synaptophysin from the plasma membrane to SLMVs occurred via early endosomes (Figure 9, step 4), although we do not have direct evidence for this assumption. Transport via the early endosome would imply a sorting process which segregates synaptophysin from proteins recycling to the plasma membrane. It is tempting to speculate that this sorting process

is lacking in non-neuroendocrine cells and rate-limiting for SLMV biogenesis in neuroendocrine cells such as the PC12 cells studied here. The traffic route of newly synthesized SLMV membrane proteins proposed in the present study is consistent with the subcellular distribution of synaptophysin expressed in fibroblasts and with the hypothesis that SLMVs represent an adaptation of an early endosome-derived pathway common to all cells (Johnston *et al.*, 1989; Jahn and De Camilli, 1991). Our model may also offer an explanation to reconcile the data obtained after synaptophysin expression in fibroblasts (Johnston *et al.*, 1989) with the results obtained after synaptophysin expression in another non-neuroendocrine cell line, the hepatoma-derived PLC cells (Leube *et al.*, 1989). Hepatocytes are known to transcytose from the basolateral to the apical surface and this capacity may still exist in PLC cells. One should therefore consider the possibility that at least some of the synaptophysin-positive vesicles seen after transfection of PLC cells were related to transcytotic vesicles arising from early endosomes.

Implications for the biogenesis of SSVs in neurons

Since the present study was performed with undifferentiated PC12 cells, we do not know whether the transport of synaptophysin from the TGN via constitutive secretory vesicles and the plasma membrane to its final destination is also true for neurons. However, several observations are consistent with the possibility that the biogenesis of SSVs, at least in terms of the traffic of the membrane proteins destined to these organelles, is similar to the biogenesis of SLMVs in that the principal traffic route is the same. These observations include: (i) the lack of accumulation of the typical 50 nm SSVs proximal to an axonal transport block (Tsukita and Ishikawa, 1980); and (ii) the differences in subcellular localization (Kagotani *et al.*, 1991) and ontogeny (A.Tixier-Vidal, A.Barret, A.Faivre-Bauman, W.B.Huttner and B.Wiedenmann, submitted for publication) between synaptophysin and SgII in neurons. If the biogenesis of SSVs occurs in axon terminals, the site of biogenesis would equal the site of regeneration if the exocytotic–endocytotic cycle of SSVs would involve early endosomes. Developing neurons, like certain epithelial cells, deliver plasma membrane proteins to either the axonal or dendritic domain via two distinct constitutive secretory pathways (Dotti and Simons, 1990). Neuronal growth cones contain early endosomes as indicated by the presence of tubular-vesicular structures labeled after short exposure to horseradish peroxidase (e.g. Bunge, 1977). Thus, neurons possess all the organelles and membrane traffic pathways to deliver SSV components to axon terminals in a manner as described here for undifferentiated PC12 cells, and to recruit these components into functional SSVs upon formation of a synapse.

Materials and methods

Cell culture and metabolic labeling

PC12 cells were grown as previously described (Tooze and Huttner, 1990). Labeling of cells with carrier-free [^{35}S]sulfate (Amersham) and L-[^{35}S]methionine (Amersham) was performed as follows.

Long-term [^{35}S]sulfate labeling. PC12 cells (150 mm dishes) were incubated at 37°C in 10% CO_2 either for 6 h in sulfate-free DMEM (DMEM lacking sulfate with 1% of the normal methionine and cysteine concentration) supplemented with 1% dialysed horse serum and 0.5% dialysed fetal calf serum and containing 0.2 mCi/ml [^{35}S]sulfate, or for 30 h in sulfate-free DMEM supplemented with 5% dialysed horse serum and 2.5% dialysed fetal calf serum and containing 0.2 mCi/ml [^{35}S]sulfate.

Isotonic preincubation and [^{35}S]sulfate pulse-labeling. PC12 cells (150 mm dishes) were incubated on a rocker at 37°C in 10% CO_2 for 30 min in 5 ml of sulfate-free DMEM supplemented with 1% dialysed horse serum and 0.5% dialysed fetal calf serum followed by the addition of 4 mCi of [^{35}S]sulfate and further incubation for 5 min.

Hypertonic preincubation, pulse-labeling and chase (standard protocol). We found that synaptophysin could be efficiently [^{35}S]sulfate-labeled if PC12 cells were first subjected as follows to a hypertonic pretreatment. PC12 cells (150 mm dishes) were first incubated on a rocker at 37°C in 10% CO_2 in 5 ml of sulfate-free Ringer's solution (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM HEPES–NaOH pH 7.2, 10 mM glucose) containing 0.5% bovine serum albumin (BSA) for a total period of 15 min (3 × 5 min) and further incubated for 15 min in 5 ml of sulfate-free Hank's balanced salt solution (HBSS; 138 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM $NaHPO_4$, 4.2 mM $NaCO_3$, pH 7.4, 5.6 mM glucose) containing 0.45 M sucrose. Cells were washed 3 times with sulfate-free HBSS in order to remove the sucrose and then [^{35}S]sulfate-labeled for 5 min in 5 ml of sulfate-free HBSS containing 4 mCi of [^{35}S]sulfate.

At the end of the various protocols of [^{35}S]sulfate labeling, the labeling medium was removed and cells were either cooled on ice for further manipulation, or chased, by the addition of normal growth medium (DMEM, 10% horse serum, 5% fetal calf serum) supplemented with twice the normal concentration of sulfate, without rocking at 37°C in 10% CO_2 for various times and then cooled.

[^{35}S]methionine pulse-labeling and chase. PC12 cells (150 mm dishes) were pre-incubated in sulfate-free Ringer's solution followed by sulfate-free HBSS and labeled for 5 min in sulfate-free HBSS as described above for the hypertonic condition, except that the 0.45 M sucrose was omitted and 300 μCi [^{35}S]methionine was used instead of [^{35}S]sulfate. The labeling medium was removed and cells chased for various times by the addition of HBSS supplemented with 0.4 mM methionine. The cells were then cooled on ice for further manipulation.

For subcellular fractionation experiments, cells were incubated for 30 min on a rocker in 5 ml of methionine-free DMEM (Sigma) supplemented with 1% dialysed horse serum, 0.5% dialysed fetal calf serum and 0.02 mM methionine, followed by addition of 500 μCi of [^{35}S]methionine and further incubation for 5 min. The labeling medium was removed and the cells were chased by the addition of normal growth medium containing 0.4 mM methionine.

Immunoisolation of synaptophysin-positive vesicles

Immunoisolation was performed on fractions enriched in either constitutive secretory vesicles or immature secretory granules prepared as previously described (see Tooze and Huttner, 1990). Briefly, a post-nuclear supernatant from PC12 cells [^{35}S]sulfate-labeled for 5 min and chased for 15 min was subjected to velocity sucrose gradient centrifugation (Tooze and Huttner, 1990). Fractions 1–4 which contained the bulk of the [^{35}S]sulfate-labeled hsPG and SgII were pooled and subjected to equilibrium sucrose gradient centrifugation (Tooze and Huttner, 1990). From this gradient, fractions 5 and 6, containing the bulk of the [^{35}S]sulfate-labeled hsPG, were pooled and are referred to as 'constitutive secretory vesicles'; fractions 8 and 9, containing the bulk of the [^{35}S]sulfate-labeled SgII, were pooled and are referred to as 'immature secretory granules'. For both pools, the sucrose concentration was adjusted to 0.5 M by dilution with 10 mM HEPES, pH 7.2 and 1/3 of each pool was either kept as a starting material or subjected to immunoisolation with or without the monoclonal SY38 anti-synaptophysin antibody (kindly given by Dr B.Wiedenmann; also purchased from Boehringer-Mannheim (Mannheim, FRG) using magnetic beads coated with sheep anti-mouse IgG1 antibody, as previously described (Leube *et al.*, 1989). Two cycles of immunization were performed on the pool containing constitutive secretory vesicles. The starting material as well as 1/3 of the unbound material and the entire immunoisolated material were analyzed by SDS–PAGE and fluorography.

Immunoisolation was also performed on constitutive secretory vesicles and immature secretory granules obtained by sequential velocity and equilibrium centrifugation from PC12 cells preincubated in the hypertonic HBSS, [^{35}S]sulfate-labeled for 5 min in HBSS and chased for 7 min, with horseradish peroxidase (see below) being present during the chase in some of these experiments. In this case, immunoisolation with the monoclonal

SY38 anti-synaptophysin antibody was performed according to Gruenberg and Howell (1985) using polyacrylamide beads.

Immunoprecipitation of synaptophysin

All steps were performed at 4°C. [^{35}S]methionine- or [^{35}S]sulfate-labeled PC12 cells were washed with PBS and lysed for 60 min in lysis buffer (50 mM Tris−HCl pH 7.8, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.2 mM PMSF; 2 ml per 150 mm dish). Lysates were centrifuged for 10 min at 13 000 r.p.m. in an Eppendorf microfuge in order to remove nuclei and cell debris, and 500 μl of the resulting supernatant (cleared lysate) were incubated overnight with the monoclonal SY38 anti-synaptophysin antibody (10 μg/ml final) on a rotating wheel. The antigen−antibody complexes were incubated for 2 h on a rotating wheel with a rabbit anti-mouse antibody (Sigma) which had been bound to protein A−Sepharose CL-4B (Pharmacia). The bound complexes were washed once with lysis buffer, twice with buffer A (10 mM Tris−HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), twice with buffer B (10 mM Tris−HCl pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40) and once with 10 mM Tris−HCl pH 7.4. The immunoprecipitated material was eluted from the beads by boiling in Laemmli sample buffer and analyzed by SDS−PAGE and fluorography.

Endoglycosidase H digestion

[^{35}S]methionine-labeled synaptophysin was immunoprecipitated and washed as described above. After two further washes in 10 mM Tris−HCl pH 7.4, samples were divided into two equal aliquots and incubated overnight at 37°C in 100 mM sodium citrate pH 5.5, 0.2 mM PMSF, 10 μg/ml aprotinin with or without 10 mU/ml endoglycosidase H (Boehringer-Mannheim). The beads were then washed twice with 10 mM Tris−HCl pH 7.4, and the bound material was eluted from the beads by boiling Laemmli sample buffer and analyzed by SDS−PAGE and fluorography.

Cell surface biotinylation

All steps were performed at 4°C. PC12 cells (60 mm dishes) [^{35}S]sulfate-labeled for 5 min (standard protocol) and chased for various times were washed three times for 5 min on a rocker with PBS$^+$ (Dulbecco's PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1.6 mM Na_2SO_4). Cells were incubated twice with NHS-LC-biotin (1 mg/ml in PBS$^+$; Pierce) for 30 min each, quenched for 15 min with PBS$^+$ containing 50 mM glycine and extensively washed with PBS$^+$ to remove free biotin. The biotinylated cells were lysed for 60 min in lysis buffer (500 μl per dish), and cleared lysates were obtained as above. The cleared lysates were incubated for 60 min on a rotating wheel with 30 μl of a suspension of streptavidin−agarose beads (Sigma), which had been washed three times with lysis buffer and resuspended to the original volume. The beads were centrifuged at 13 000 r.p.m. in an Eppendorf microfuge and the resulting supernatant (unbound material) was kept. The beads were washed once with the lysis buffer, twice with buffer A, twice with buffer B and once with 10 mM Tris−HCl pH 7.4. The material eluted from the beads with Laemmli sample buffer and the unbound material were analyzed by SDS−PAGE and fluorography.

Cell surface endoglycosidase F treatment

Cell surface endoglycosidase F treatment was carried out at 4°C as previously described (Johnston *et al.*, 1989). PC12 cells (60 mm dishes) [^{35}S]sulfate-labeled for 5 min (standard protocol) and chased for various times were washed three times with PBS$^+$ and incubated with 200 mU/ml endoglycosidase F (Boehringer-Mannheim) in PBS$^+$ (final volume 800 μl per dish) for 6 h on a rocker. Cells were then lysed with lysis buffer (250 μl per dish) for 60 min, and cleared lysates were prepared as above and directly analysed by SDS−PAGE and fluorography.

Horseradish peroxidase experiments

Experiments were performed essentially as described (Gorvel *et al.*, 1991). PC12 cells (150 mm dishes) [^{35}S]sulfate-labeled for 5 min were incubated for 7 min on a rocker at 37°C in 10% CO_2 with 1.5 mg/ml horseradish peroxidase (Sigma) in MEM containing 10 mM HEPES pH 7.4, 5 mM glucose, 0.2% BSA and supplemented with twice the normal concentration of sulfate. Dishes were either cooled on ice or chased for 53 min in chase medium (see above) and then cooled on ice. The cells were carefully washed at 4°C three times for 15 min each with PBS$^+$ containing 0.5% BSA. Post-nuclear supernatants were then subjected to velocity and equilibrium sucrose gradient centrifugation as decribed below. Peroxidase activity was assayed in each gradient fraction as follows. 100 μl of each fraction were mixed with 1 ml of 0.5 M NaH_2PO_4 pH 5.0, 0.1% Triton X-100, 13 mg/ml *o*-dianisidine (Sigma) and 0.003% H_2O_2, and incubated at room temperature in the dark for 3 h. The reaction was stopped with 0.002% NaN_3 and the optical density determined at 455 nm.

Subcellular fractionation

Velocity and equilibrium sucrose gradient centrifugation. Post-nuclear supernatants were prepared from [^{35}S]methionine-labeled or [^{35}S]sulfate-labeled (standard protocol) PC12 cells (two 150 mm dishes) and subjected to velocity and equilibrium sucrose gradient centrifugation as described (Tooze and Huttner, 1990), with some minor modifications. Velocity gradient fractions 8−10 from cells [^{35}S]sulfate-labeled for 5 min without subsequent chase, and velocity gradient fractions 2−4 from cells either [^{35}S]sulfate- or [^{35}S]methionine-labeled and chased for various times, were pooled and 2 ml were loaded onto a linear sucrose gradient prepared from 0.6 M (5 ml) to 1.6 M (5 ml) in 10 mM HEPES-KOH pH 7.2. The gradient was centrifuged to equilibrium at 25 000 r.p.m. for 15 h in a Beckman SW40 rotor. Aliquots (600 μl) of the 1 ml fractions collected from both velocity and equilibrium gradients were used for CPG chromatography; 300 μl aliquots of the remaining material of each fraction were mixed with 450 μl of PBS containing 10 μg of hemoglobin and then with 3 ml of acetone (−20°C), and precipitated proteins were collected by centrifugation and analyzed by SDS−PAGE and fluorography.

Differential and equilibrium centrifugation. Post-nuclear supernatants were prepared from [^{35}S]sulfate-labeled PC12 cells (four 150 mm dishes) as described (Tooze and Huttner, 1990) and subjected to differential and equilibrium centrifugation according to Huttner *et al.* (1983), with some modifications. The post-nuclear supernatant was centrifuged at 25 000 *g* for 20 min in the SS34 rotor. The pellet, referred to as 'P2 fraction', was discarded, and the supernatant (corresponding to LS1; Huttner *et al.*, 1983) was collected and centrifuged at 165 000 *g* in a Beckman 75Ti rotor for 2 h. The supernatant (corresponding to LS2) was discarded, and the pellet, referred to as 'P3 fraction' (corresponding to LP2; Huttner *et al.*, 1983), was resuspended in 2 ml of 0.5 M sucrose in 10 mM HEPES−KOH pH 7.2. To augment the resuspension process, the suspension was subjected to 10 up-and-down strokes in a glass-Teflon homogenizer at 1200 r.p.m. followed by passing the suspension once back and forth through a 25-gauge needle. The suspension was loaded onto the 0.6 M−1.6 M sucrose gradient and centrifuged at 25 000 r.p.m. for 15 h in a Beckman SW40 rotor. Aliquots of the 1 ml fractions collected from the gradient were either used for CPG chromatography, or subjected to acetone precipitation followed by SDS−PAGE, fluorography and immunoblotting with the monoclonal SY38 anti-synaptophysin antibody.

Permeation chromatography on controlled-pore glass

Permeation chromatography on controlled-pore glass (CPG 10 Polyol, 3000 Å, 120−200 mesh, 75-125 μm from Serva) was performed as previously described (Huttner *et al.*, 1983). A CPG column (1 cm i.d. × 50 cm) was prepared and calibrated with a preparation of rat brain SSVs (SG-V pool, Huttner *et al.*, 1983). The SG-V pool (2 ml), which contained a trace of phenol red, was chromatographed in buffered glycine (300 mM glycine, 5 mM HEPES-NaOH pH 7.2) at a flow rate of 25 ml/h. Fractions of 1 ml were collected and analyzed for their absorbance at 280 nm. The fraction containing the peak of the void volume material was arbitrarily numbered as fraction 5.

Aliquots (700 μl) of fractions 5 and 6 of equilibrium gradients, performed after differential centrifugation of post-nuclear supernatants from [^{35}S]sulfate-labeled PC12 cells chased for various times, were pooled and subjected to CPG chromatography as above. These fractions contained the bulk of the [^{35}S]sulfate-labeled hsPG and synaptophysin (see Figure 7). Alternatively, aliquots (600 μl) of fractions 2−4 of velocity gradients, prepared from [^{35}S]sulfate-labeled PC12 cells chased for various times, which contained the bulk of the post-TGN hsPG, SgII and synaptophysin (see Figure 3C,E), were pooled, diluted to 3 ml with homogenization buffer (Tooze and Huttner, 1990), and 2 ml were subjected to CPG chromatography.

After CPG chromatography of either rat brain- or PC12 cell-derived vesicles, fractions received $MgCl_2$ (10 mM final), and the vesicles present in the fractions were pelleted by centrifugation at 235 000 *g* for 60 min in a Beckman 75Ti rotor. The supernatant was discarded; the pellet was resuspended in Laemmli sample buffer and analyzed by SDS−PAGE followed by fluorography or immunoblotting with the monoclonal SY38 anti-synaptophysin antibody.

SDS−PAGE and immunoblotting

SDS−PAGE was performed and gels were processed as described (Lee and Huttner, 1983). Immunoblotting was performed as previously described (Rosa *et al.*, 1989) except that blocking of the nitrocellulose sheets was in PBS containing 10% low fat milk powder for 2 h at room temperature. The monoclonal SY38 anti-synaptophysin antibody was used at 0.2 μg/ml final. Immunoreactive synaptophysin was revealed either by sequential

incubations with 1 μg/ml of rabbit anti-mouse IgG followed by [^{125}I]protein A (0.12 μCi/ml final; NEN), or by incubation with peroxidase conjugated to goat anti-mouse IgG (1:1000 dilution; Jackson Immunoresearch Laboratory).

[^{35}S]sulfate- and [^{35}S]methionine-labeled hsPG, SgII and synaptophysin were quantified using methods described previously (Tooze and Huttner, 1990). Immunoreactive synaptophysin was quantified by either γ-counting of the relevant pieces of the ^{125}I-labeled nitrocellulose sheet, densitometric scanning of the autoradiogram derived therefrom, or densitometric scanning of the wet, peroxidase-labeled nitrocellulose sheet.

Tyrosine sulfate analysis

[^{35}S]sulfate-labeled synaptophysin present in fixed polyacrylamide gels was analyzed for the presence of tyrosine [^{35}S]sulfate by standard methods (Huttner, 1984).

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References

Baeuerle,P.A. and Huttner,W.B. (1987) *J. Cell Biol.*, **105**, 2655–2664.
Buckley,K.M., Floor,E. and Kelly,R.B. (1987) *J. Cell Biol.*, **105**, 2447–2456.
Bunge,M.B. (1977) *J. Neurocytol.*, **6**, 407–439.
Burgess,T.L. and Kelly,R.B. (1987) *Annu. Rev. Cell Biol.*, **3**, 243–293.
Cameron,P.L., Südhof,T.C., Jahn,R. and De Camilli,P. (1991) *J. Cell Biol.*, in press.
Clift-O'Grady,L., Linstedt,A.D., Lowe,A.W., Grote,E. and Kelly,R.B. (1990) *J. Cell Biol.*, **110**, 1693–1703.
Collawn,J.F., Stangel,M., Kuhn,L.A., Esekogwu,V., Jing,S., Trowbridge,I.S. and Tainer,J.A. (1990) *Cell*, **63**, 1061–1072.
Cutler,D.F. and Cramer,L.P. (1990) *J. Cell Biol.*, **110**, 721–730.
Daukas,G. and Zigmond,S.H. (1985) *J. Cell Biol.*, **101**, 1673–1679.
Davis,C.G., Lehrman,M.A., Russel,D.W., Anderson,R.G.W., Brown,M.S. and Goldstein,J.L. (1986) *Cell*, **45**, 15–24.
De Camilli,P. and Jahn,R. (1990) *Annu. Rev. Physiol.*, **52**, 625–645.
Dotti,C.G. and Simons,K. (1990) *Cell*, **62**, 63–72.
Friederich,E., Fritz,H.-J. and Huttner,W.B. (1988) *J. Cell Biol.*, **107**, 1655–1667.
Gorvel,J.-P., Chavrier,P., Zerial,M. and Gruenberg,J. (1991) *Cell*, **64**, 915–925.
Griffiths,G. and Simons,K. (1986) *Science*, **234**, 438–443.
Gruenberg,J. and Howell,K.E. (1985) *Eur. J. Cell Biol.*, **38**, 312–321.
Heuser,J.E. and Anderson,R.G.W. (1989) *J. Cell Biol.*, **108**, 389–400.
Huttner,W.B., Schiebler,W., Greengard,P. and De Camilli,P. (1983) *J. Cell Biol.*, **96**, 1374–1388.
Huttner,W.B. (1984) *Methods Enzymol.*, **107**, 200–223.
Jahn,R., Schiebler,W., Ouimet,C. and Greengard,P. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4137–4141.
Jahn,R. and De Camilli,P. (1991) In Gratzl,M. and Langley,K. (eds), *Markers for Neural and Endocrine Cells*. VCH, Weinheim, pp. 25–92.
Janetzko,A., Zimmermann,H. and Volknandt,W. (1989) *Neuroscience*, **32**, 65–77.
Johnston,P.A., Cameron,P.L., Stukenbrok,H., Jahn,R., De Camilli,P. and Südhof,T.C. (1989) *EMBO J.*, **8**, 2863–2872.
Kagotani,Y., Picart,R., Barret,A., Wiedenmann,B., Huttner,W.B. and Tixier-Vidal,A. (1991) *J. Histochem. Cytochem*, in press.
Kelly,R.B. (1988) *Neuron*, **1**, 431–438 .
Kimura,J.H., Lohmander,L.S. and Hascall,V.C. (1984) *J. Cell Biol.*, **26**, 261–278.
Lee,R.W.H. and Huttner,W.B. (1983) *J. Biol. Chem.*, **258**, 11326–11334.
Leube,R.E., Kaiser,P., Seiter,A., Zimbelmann,R., Franke,W.W., Rehm,H., Knaus,P., Prior,P., Betz,H., Reinke,H., Reyreuther,K. and Wiedenmann,B. (1987) *EMBO J.*, **6**, 3261–3268.
Leube,R.E., Wiedenmann,B. and Franke,W.W. (1989) *Cell*, **59**, 433–446.
Lowe,A.W., Madeddu,L. and Kelly,R.B. (1988) *J. Cell Biol.*, **106**, 51–59.
Miller,S.G. and Moore,H.-P.H. (1991) *J. Cell Biol.*, **112**, 39–54.
Nagy,A., Baker,R.R., Morris,S.J. and Whittaker,V.P. (1976) *Brain Res.*, **109**, 285–309.
Navone,F., Di Gioia,G., Jahn,R., Browning,M., Greengard,P. and De Camilli,P. (1986) *J. Cell Biol.*, **103**, 2511–2527.
Orci,L., Ravazzola,M., Amherdt,M., Louvard,D. and Perrelet,A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5385–5389.
Rosa,P., Weiss,U., Pepperkok,R., Ansorge,W., Niehrs,C., Stelzer,E.H. and Huttner,W.B. (1989) *J. Cell Biol.* **109**, 17–34.
Südhof,T.C., Lottspeich,F., Greengard,P., Mehl,E. and Jahn,R. (1987) *Science*, **238**, 1142–1144.
Südhof,T.C. and Jahn,R. (1991) *Neuron*, **6**, 665–677.
Tixier-Vidal,A., Faivre-Baumann,A., Picart,R. and Wiedenmann,B. (1988) *Neuroscience*, **26**, 847–861.
Tooze,J. and Tooze,S.A. (1986) *J. Cell Biol.*, **103**, 839–850.
Tooze,S.A. and Huttner,W.B. (1990) *Cell*, **60**, 837–847.
Tooze,S.A. (1991) *Federation of European Biochemical Societies*, **285**, in press.
Trimble,W., Linial,M. and Scheller,R. (1991) *Annu. Rev. Neurosci.*, **14**, 93–122.
Tsukita,S. and Ishikawa,H. (1980) *J. Cell Biol.*, **84**, 513–530.
Wiedenmann,B. and Franke,W.W. (1985) *Cell*, **41**, 1017–1028.
Wiedenmann,B. and Huttner,W.B. (1989) *Virchows Archiv B Cell Pathol.*, **58**, 95–121.

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