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Synaptin/synaptophysin, p65 and SV2: their presence in adrenal chromaffin granules and sympathetic large dense core vesicles

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The subcellular distribution of three proteins of synaptic vesicles (synaptin/synaptophysin, p65 and SV2) was determined in bovine adrenal medulla and sympathetic nerve axons. In adrenals most p65 and SV2 is confined to chromaffin granules. Part of synaptin/synaptophysin is apparently also present in these organelles, but a considerable portion is found in a light vesicle which does not contain significant concentrations of typical markers of chromaffin granules (cytochrome *b*-561, dopamine β-hydroxylase or the amine carrier). An analogous finding was obtained for sympathetic axons. The large dense core vesicles contain most p65 and also SV2 but only a smaller portion of synaptin/synaptophysin. A lighter vesicle containing this latter antigen and some SV2 has also been found. These results establish that in adrenal medulla and sympathetic axons three typical antigens of synaptic vesicles are not restricted to light vesicles. Apparently, a varying part of these antigens is found in chromaffin granules and large dense core vesicles. On the other hand, the light vesicles do not contain significant concentrations of functional antigens of chromaffin granules. Thus, the biogenesis of small presynaptic vesicles which contain all three antigens as well as functional components like the amine carrier is likely to involve considerable membrane sorting.

Introduction

Early studies had strongly indicated that several proteins identified in brain synaptic vesicles (synaptin, SV2 and p65) were also found in adrenal chromaffin granules [1–3]. These results were obtained either by immunoassays of density gradient fractions (synaptin: [1]; SV2: [2]) or by electron immunocytochemistry (p65: [3]). In 1985 synaptophysin (syn) was characterized in brain synaptic vesicles [4,5], but later it was recognized that this protein was identical with synaptin which had been discovered previously [6]. Some studies have suggested that syn is only present in small synaptic vesicles but absent from neuronal large dense core vesicles (Ref. 7, 8, see Ref. 9). Furthermore, in endocrine tissues syn was localized by immunocytochemistry at the ultrastructural level to a small electronlucent vesicle different from the hormone storing organelles [7]. On the other hand, several subcellular fractionation and immunoadsorption studies have indicated that syn is also present in chromaffin granules and large dense core vesicles [10–17].

For posterior pituitary, Navone et al. [18], using both immunohistochemistry and density gradient centrifugation, claimed that p65 and syn are confined to the small vesicle population and are not present in the large dense core neurosecretory vesicles. This is in obvious contrast to findings of Fournier and Trifaro [19], who detected p65 also in the latter type of organelle. Furthermore, p65 has been found in adrenal chromaffin granules by Western immunoblotting [16] and in large, substance-P-containing synaptic vesicles from rat brain by immunoprecipitation [20].

Thus, the question of whether the major membrane proteins of brain synaptic vesicles are also present in large dense core vesicles and chromaffin granules is a

Abbreviation: syn, synaptin/synaptophysin.

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rather controversial topic. This is somewhat unfortunate, since the question of similarities in the make-up of these two types of vesicle is of obvious relevance for understanding the mechanism of their biogenesis and function (see Ref. 21, 9). We therefore attempted to settle this question by defining the subcellular localization of three vesicle antigens together and in two representative tissues, i.e., the bovine adrenal medulla and the bovine splenic nerve, which contains mainly sympathetic axons.

Materials and Methods

Subcellular fractionation

Bovine splenic nerves were collected 20 min post mortem and immediately chilled. Usually, 5 to 8 g of nerves were cleared from surrounding tissue, minced with scissors and homogenized (10 s, 4°C) in 0.3 M sucrose solution with an Ultra Turrax. Bovine adrenal medullae were also minced with scissors and suspended in 0.3 M sucrose. Suspensions of the two tissues were then homogenized with a Potter-Elvejhem homogenizer (Teflon pestle, clearance 0.08 cm). Homogenates were centrifuged at $800 \times g$ for 20 min. The supernatants (supernatant I) were centrifuged directly on sucrose gradients, or at $12000 \times g$ for 20 min to sediment a large granule fraction or at $225000 \times g$ for 90 min to sediment all cell particles together. The resulting pellets were resuspended and layered [22] on top of a sucrose gradient (prepared 6–8 h before use by layering sucrose solutions above each other starting with a cushion of 1 ml of 2.5 M sucrose followed by 1 ml each of 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.55, 0.5, 0.45 and 0.4 M sucrose solutions). After centrifugation of the gradient tubes (for conditions see legends) a needle was pierced through the bottom of the centrifuge tube and 12 fractions were collected. The four fractions from the top of the gradient were diluted with two volumes of 0.3 M sucrose solution; the remaining fractions were diluted with only half the volume. The diluted fractions were centrifuged for 90–120 min at $130000 \times g$ to collect pellets for further analyses.

Immunological studies

The following antisera were used: anti bovine dopamine β-hydroxylase [23], anti cytochrome *b*-561 (kindly provided by D.K. Apps, Edinburgh), anti rat SV2 (monoclonal antibody 10H3 [2], anti rat syn (p38; kindly provided by R. Jahn, Munich) and anti rat p65 (monoclonal antibody 48 [3]). Quantitative immunoblotting was performed as described previously [24,25].

Assays

[^{3}H]Dihydrotetrabenazine binding to the monoamine carrier was measured at 2 nM as already described [26].

Results

Subcellular fractionation of adrenal medulla

Adrenal medullae were homogenized and the supernatant, after removal of cell debris and nuclei, was applied to a sucrose density gradient ranging from 0.4 M to 1.4 M (see Fig. 1). Two different centrifugation conditions (Fig. 1) were used. With high centrifugal force the chromaffin granules (markers: cytochrome *b*-561 and dopamine β-hydroxylase) sediment to the densest fractions. The distribution of the amine carrier matches exactly. Two of the brain synaptic vesicle antigens (SV2 and p65) are found in a distribution analogous to that of chromaffin granules. On the other hand, syn exhibits a clearly different pattern. In addition to the peak in the densest fraction, there is a

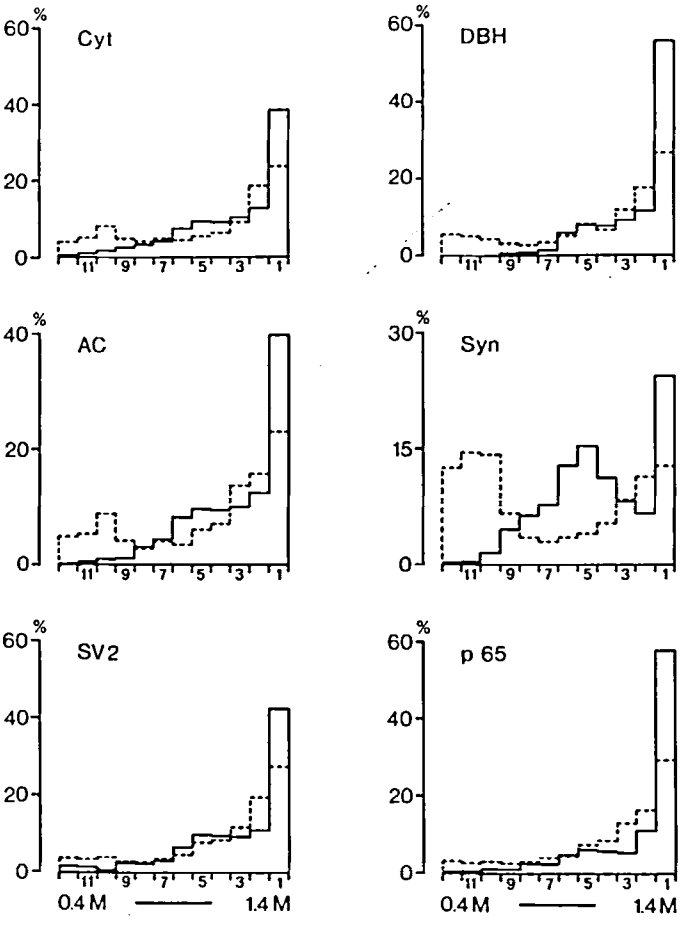

Fig. 1. Subcellular fractionation of bovine adrenal medulla. A supernatant I was centrifuged over a sucrose (0.4–1.4 M) density gradient either for 35 min at $70000 \times g$ (broken line) or for 3 h at $150000 \times g$ (solid line). After centrifugation, fractions were analysed for the various antigens. The results are presented in histograms where the fractions from left to right correspond to the fractions from the top to the bottom of the centrifuge tube. The ordinates give the percentage per fraction of the total amount recovered from the gradient. For each centrifugation time, the results of two closely corresponding experiments were combined. Cyt: cytochrome *b*-561; DBH: dopamine β-hydroxylase; AC: amine carrier; Syn: synaptin/synaptophysin.

prominent peak in the middle of the gradient (fraction 5). After a lower centrifugal force there is a slight change in the position of chromaffin granules (cytochrome *b*-561, dopamine β-hydroxylase and the amine carrier) which are now found more evenly spread through the denser fractions 1–4. An analogous change takes place for SV2 and p65 and for the syn peak in the dense part of the gradient. However, the second peak of syn is now present in the three top fractions of the gradient. Apparently, the large and dense chromaffin granules have nearly reached equilibrium density at the low centrifugal force, whereas the light syn-rich vesicles sediment much more slowly. In the fractions 10–12 where these light vesicles equilibrate, the other markers are not concentrated in an analogous and defined peak. The presence of small amounts of these markers in these fractions is probably due to membranes of chromaffin granules damaged during homogenization [27]. The gradient (0.4 to 1.4 M) described above was chosen to obtain a good separation in the low-density regions. It should be emphasized that experiments were also performed with the more usual gradient ranging from 1.0 to 2.0 M sucrose solution [22]. In such gradients, chromaffin granule markers were found in a peak at 1.8 M sucrose. The synaptic vesicle markers (SV2, syn and p65) also exhibited a peak in this region, but only syn was present in an additional peak at low density (results not shown; for syn see Ref. 11).

Subcellular fractionation of bovine splenic nerve

After gradient centrifugation of a low-speed supernatant from bovine splenic nerve a marker for large dense core vesicles, cytochrome *b*-561, showed a peak in the denser fractions of the gradient (Fig. 2). Other

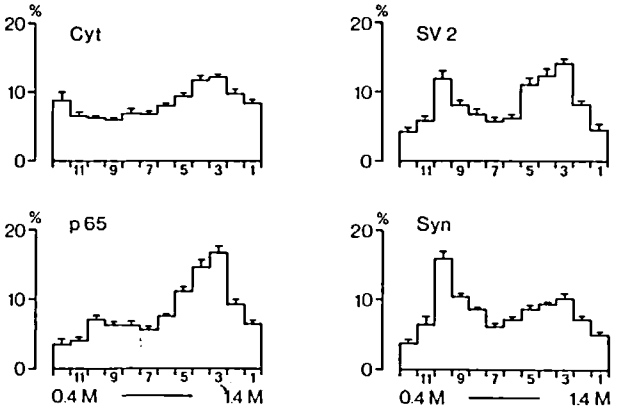

Fig. 2. Subcellular fractionation of bovine splenic nerve. A supernatant I from a homogenate of bovine splenic nerve was centrifuged to sediment all particles. The resuspended pellet was subjected to sucrose (0.4–1.4 M) density gradient centrifugation (70000× *g* for 3 h). The results (mean value ± S.E.) are expressed as in Fig. 1 and represent two experiments (six analyses). Cyt: cytochrome *b*-561; Syn: synaptin/synaptophysin.

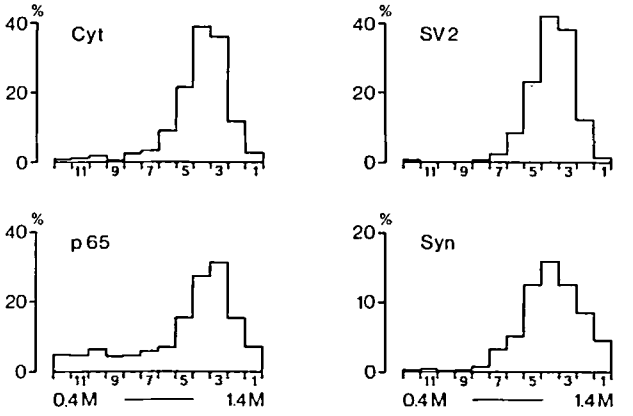

Fig. 3. Subcellular fractionation of bovine splenic nerve. A large granule fraction (12000× *g* for 20 min) was obtained after centrifugation of a supernatant I. The resuspended large granules were subjected to sucrose (0.4–1.4 M) density gradient centrifugation (150000× *g* for 3 h). The results (mean values of two closely corresponding experiments) are expressed as in Fig. 1. Cyt: cytochrome *b*-561; Syn: Synaptin/synaptophysin.

large dense core vesicle markers, such as dopamine β-hydroxylase or chromogranin A, are found in a similar position (see Ref. 28). The distributions of the three synaptic vesicle antigens are different. Most p65 is present in the denser fractions of the gradient where the large dense core vesicles are found. This is also true for SV2, although a smaller peak is also present in lighter fractions (around fraction 10). Syn has its highest concentration in this light fraction but a second peak is found in the position where the large dense core vesicles equilibrate.

When a large granule fraction of splenic nerve is subjected to density gradient centrifugation, the results obtained are quite different. All four antigens are concentrated in the dense fractions of the gradient (see Fig. 3). The second peak in the light fraction (compare Figs. 2 and 3) is now absent. This demonstrates that the lighter syn-containing vesicles are not sedimented by the centrifugal force (12000 × *g* for 20 min) used to sediment the large dense core vesicles.

Discussion

The results described in this paper should settle the controversy on the localization of synaptic vesicle proteins in adrenal chromaffin granules and in large dense core vesicles. In adrenal medulla, by far the largest part of p65 (see also Ref. 16) and SV2 is confined to chromaffin granules. Syn, in agreement with our previous study (Ref. 11, see also Ref. 16), has a bimodal distribution. A significant part apparently cosediments with chromaffin granules, whereas the rest is present in a special lighter vesicle. Analogous results were obtained with different sucrose gradients and centrifugation times. The lighter syn-containing vesicles [13] (for

PC12 cells see Ref. 29) sediment much more slowly during density gradient centrifugation than the heavier and denser chromaffin granules.

Can this clear-cut result be attributed to a possible artefact? In a recent study [30] it was claimed that syn cosedimenting with chromaffin granules could be preferentially removed by immunobeads. The authors implied therefore that syn found in chromaffin granules was not a true constituent of these organelles. However, our present results exclude the possibility that SV2, p65 and syn are confined to a single synaptic vesicle-like compartment in chromaffin cells, part of which somehow cosediments with chromaffin granules, e.g., after nonspecific adsorption. If this were the case, the distribution of all three antigens should be practically identical in the gradient, which was clearly not observed. Fischer v. Mollard et al. [30] also claimed that the special syn vesicles contain some cytochrome *b*-561, which they suggested could be expected from an 'equivalent' of synaptic vesicles. Our results make it unlikely that the syn vesicle contains significant amounts of "functional adrenergic" constituents such as cytochrome *b*-561, dopamine β-hydroxylase (see also Ref. 11) or the amine carrier. A recent paper on PC12 cells also suggested that syn vesicles can not accumulate catecholamines and are therefore likely to be devoid of the amine carrier [29].

Thus, we are left with the fact that the adrenal medulla contains syn vesicles which may also contain synaptobrevin [31], but contain only little, if any, of the typical synaptic vesicle constituents like SV2 and p65 and also little if any of the adrenergic functional proteins. On the other hand, most of p65 and SV2 is confined to chromaffin granules. Thus, the simple concept that the chromaffin cell contains two vesicle types – (i) a microvesicle equivalent to synaptic vesicles containing syn, SV2 and p65 [30] and (ii) chromaffin granules devoid of major antigens of synaptic vesicles – has to be abandoned. For syn, one should be careful in drawing the firm conclusion that it is a typical constituent of chromaffin granules, being evenly spread in their membrane like other antigens (e.g., cytochrome *b*-561 [32]). Our results do not rule out the possibility that syn-rich vesicles can fuse with chromaffin granule membranes in vivo or in vitro and therefore cosediment with chromaffin granules. Baumert et al. [33] have described in electron micrographs syn-labelled evaginations of secretory granules which might be consistent with such a concept. In any case, one of the important outcomes of the present study is that in adrenal medulla neither of the vesicle antigens syn, p65 or SV2 can be considered as a reliable marker of just one cellular compartment.

If we keep in mind that chromaffin cells and sympathetic neurons have a strong developmental relationship, analogies in the properties of their subcellular structures are likely. In fact, the close similarity of large dense core vesicles of adrenergic neurons to adrenal chromaffin granules has already been clearly established [23,34]. It was therefore reassuring that subcellular fractionation of sympathetic axons yielded results resembling closely those obtained with chromaffin cells. The similarity of the results in the two tissues makes it unlikely that in the splenic nerve vesicles derived from non-adrenergic axons do significantly contribute to the patterns of subcellular distribution of the various antigens. However, this possibility can not be completely excluded. In any case, in splenic nerves most of the antigen p65 and also a large part of SV2 cosedimented with the large dense core vesicles, whereas syn (and partly SV2) was again found in a bimodal distribution. Such a bimodal distribution for syn was found by us previously [28]; however, in this earlier study a different gradient was used and therefore the separation of the two peaks was less clear-cut. Our present results, in analogy to the adrenal medulla, exclude the possibility that there is a single type of synaptic-vesicle-like membrane artifactually cosedimenting with the large dense core vesicles. Large dense core vesicles in sympathetic axons contain most of the p65, a large part of the SV2 but also a considerable portion of syn. This finding is in contrast to claims that in brain nerve terminals (see Ref. 9) syn is confined to small synaptic vesicles and that in terminals of posterior pituitary neither syn nor p65 is found in large dense core vesicles [18]. However, Fournier et al. [16] found p65 in these latter vesicles and we have recently confirmed their findings (Kapelari, S. and Winkler, H., unpublished data). Furthermore, subcellular fractionation studies of cholinergically innervated tissues also suggest that cholinergic large dense core vesicles contain syn [14,15] and a very recent study demonstrated that in optic nerve axons syn is present in vesicles containing neuropeptides [17]. Finally, axonal large dense core vesicles of bovine splenic nerve have also been reported to contain synapsin [35], which in terminals is thought to be bound only to synaptic vesicles (see Ref. 9). In addition to anterograde transport, vesicle membranes are also likely to be transported retrogradely. Apparently subcellular fractionation does not reveal a defined vesicle population involved in this latter transport. It seems likely that retrogradely transported membranes are heterogeneous and therefore are spread throughout the gradient which might, for example, explain the presence of cytochrome-containing membrane in the upper part of the gradient (see Fig. 2).

Our results impose significant new conditions on any model of the biogenesis of the two types of vesicle in nerve. It has been suggested [9] that large dense-core vesicles and small synaptic vesicles are separate entities formed and transported axonally as independent popu-

lations. For adrenergic nerves we can apparently exclude this: the axons contain and presumably transport both large dense core vesicles and a light vesicle population which is obviously different from synaptic vesicles found in the terminals, since it is rich in syn, but poor in SV2 and with little if any p65. Furthermore, as already shown previously [28], these light vesicles do not contain significant amounts of the amine carrier or catecholamines. Therefore they do not represent vesicles identical to those synaptic vesicles present in nerve terminals which are characterized not only by the presence of all three antigens, syn, p65 and SV2 [36] but which are obviously functional, and therefore the adrenergic variety of it must also contain the amine carrier. A second hypothesis on biogenesis of neuronal vesicles [37,38] proposes that in sympathetic terminals small dense core vesicles (the adrenergic synaptic vesicles) are formed from large dense core vesicles after exocytosis. Since the membranes of large dense core vesicles apparently contain all antigens found in small ones, our results are consistent with this hypothesis. However, there are apparent differences between the membranes of large dense core vesicles and nerve terminal small synaptic vesicles (e.g., higher content of syn in synaptic versus large dense core vesicles and differences in synapsin-binding [9,39]). Furthermore, at least in PC12 cells, there is evidence from pulse label experiments that large dense core vesicles and syn rich vesicles are synthesized independently [40]. One might therefore suggest that in neurons membranes of large dense core vesicles and those of the axonally transported syn-rich vesicles reach the terminal by axonal transport and may be there subjected to membrane sorting after the large dense core vesicle membranes have been retrieved after exocytosis. Proteins of both membranes may mix to form the final and functional terminal synaptic vesicle. Our present results add further weight to this recently proposed [21] hypothesis. This concept is also supported by data on cholinergic terminals. A careful immunohistochemical analysis revealed that SV2 in terminals is found in synaptic vesicles, but in the axon is also present in electron dense granules and vesiculotubular structures [41].

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