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Topical Review

Molecular Analysis of a Secretory Organelle: Structure and Function of Synaptic Vesicle-Specific Proteins

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Introduction

The central element in brain function is the synapse. Synapses mediate the complex activities performed by the nervous system, including movement, sensation, cognition, and memory. A key feature of the majority of synapses is the calcium-dependent release of neurotransmitter which is subsequently detected by postsynaptic receptors. Synaptic vesicles play a key role in this process, and recent studies of long-term potentiation suggest they may be a primary substrate for synaptic modulation. Despite their importance for synaptic transmission, many aspects of synaptic vesicle function remain obscure, including the molecular basis for the calcium dependence of exocytosis, how synaptic vesicles are recycled and refilled with neurotransmitters following exocytosis, and their biogenesis during development.

Studies of synaptic vesicles have been facilitated by the ease of isolating large numbers of them in relatively pure form, from mammalian brain and from the elasmobranch electric organ. The resident proteins in synaptic vesicle membranes have been characterized by generating antibodies to purified vesicles, and in some cases, isolating cDNAs that encode these proteins. At least five integral membrane proteins have been described that are common to all synaptic vesicles: synaptophysin (p38), synaptotagmin (p65), synaptobrevin (VAMP), SV2, and p29 (Südhof & Jahn, 1991). These proteins are also found in endocrine cells, where synaptotagmin and SV2 are present both in large dense core vesicles as well as in a population of small clear vesicles that are considered the endocrine homologue of synaptic vesicles in these cells, while synaptophysin and synaptobrevin are found predominantly in small clear or synaptic vesicles (Navone et al., 1986). In addition to being found in all types of synaptic vesicles, these proteins are highly conserved throughout evolution. Synaptobrevin and synaptotagmin have been found in *Drosophila,* while synaptophysin and SV2 are present in synaptic vesicles from the marine rays, *Torpedo* and *Discopyge* (Johnston, Jahn & Südhof, 1989*b*, Südhof et al., 1989; Cowan, Linial & Scheller, 1990; Perin et al., 1991; Bindra, Knowles & Buckley, 1994). Through a variety of approaches, a number of laboratories have attempted to elucidate the roles of each of these proteins in synaptic vesicle formation, exocytosis, and recycling. In this review, I will summarize our current knowledge of potential functions for synaptotagmin, synaptophysin, synaptobrevin, and SV2 (no sequence information has been published yet for p29). I will also discuss the most recent models for synaptic vesicle biogenesis in neurons and endocrine cells, and point out important questions that remain unanswered.

Key words: Synaptic vesicle — Synaptophysin — SV2 — Synaptotagmin — Synaptobrevin — Exocytosis

Functional Studies of Synaptic Vesicle Proteins

Synaptophysin

Synaptophysin is the most abundant protein in synaptic vesicles, but appears in only small amounts, if at all,

in large dense core vesicles (Südhof & Jahn, 1991). Experiments from Heinrich Betz's laboratory suggest that synaptophysin forms a channel in black lipid membranes, and because of its structural homology to the connexins, it was hypothesized that oligomers of synaptophysin form a fusion pore during exocytosis (Thomas et al., 1988). More recent data suggest that synaptophysin's primary role may be in the endocytosis and formation of synaptic vesicles. Studies of synaptic vesicle formation in PC12 cells indicate that synaptic vesicles are formed from endosomes (Johnston et al., 1989*a*; Clift-O'Grady et al., 1990; Linstedt & Kelly, 1991; Régnier-Vigouroux, Tooze & Huttner, 1991). Interestingly, synaptophysin is the only synaptic vesicle membrane protein that is reproducibly targeted to endosomes both in transfected fibroblasts and in neuronal perikarya. We have shown that synaptophysin, but not SV2 or synaptotagmin, colocalizes with transferrin receptors, a marker for endosomes, in transfected CHO cells (Feany et al., 1993). Mundigl et al. (1993) examined the distribution of synaptic vesicle proteins in the perikarya and terminals of embryonic hippocampal neurons in culture. In the cell body of these neurons, as in CHO fibroblasts, synaptophysin is the only synaptic vesicle protein that colocalizes with the transferrin receptor. Other experiments have implicated synaptophysin in the release process itself. Alder and colleagues (1992) injected antibodies to synaptophysin into *Xenopus* embryos or directly into cultured spinal neurons, and observed a reduction both in the frequency of spontaneous synaptic currents and the amplitude of evoked currents. Preimmune sera had no effect on synaptic transmission. However, since synaptophysin is the most abundant membrane protein in synaptic vesicles, it is difficult to devise an adequate control for the possibility that release was affected by nonspecific effects of coating synaptic vesicles with antibodies.

Synaptotagmin (p65)

Synaptotagmin (p65) was the first integral membrane protein of synaptic vesicles to be identified (Matthew, Tsavaler & Reichardt, 1981). In contrast to synaptophysin, it appears to be equally abundant in large dense core vesicles and synaptic vesicles. The amino acid sequence indicates that synaptotagmin contains a single transmembrane domain, and the cytoplasmic portion of the protein consists of two highly homologous repeats that are equally homologous to the regulatory domains of protein kinase C (Perin et al., 1990). This region of the protein binds calcium, and is capable of specific interaction with negatively charged phospholipids (Perin et al., 1990; Brose et al., 1992). In addition, interactions have been observed between synaptotagmin and several nerve terminal membrane proteins: the ω-conotoxin receptor (calcium channel), α-latrotoxin receptor (neurexin), and syntaxin (Petrenko et al., 1991; Bennett, Calakos & Scheller, 1992*b*; Yoshida et al., 1992). These properties led to the hypotheses that synaptotagmin either acts as a calcium-dependent fusion protein or plays a role in the docking of synaptic vesicles. Injection of anti-synaptotagmin antibodies or peptides that mimic the C2 domain interfere with secretion of catecholamines from PC12 cells and block synaptic transmission at the squid giant synapse (Bommert et al., 1993; Elferink, Peterson & Scheller, 1993). In contrast to these results, a mutant PC12 cell line that completely lacks synaptotagmin shows no apparent deficit in calcium-dependent release of catecholamines (Shoji-Kasai et al., 1992). Synaptotagmin mutants have also been described in *Caenorhabditis elegans* and *Drosophila.* Nonet et al. (1993) have characterized the phenotype of a *C. elegans* mutant, called *ric*-2, that can be rescued by a 17 kb synaptotagmin genomic clone. Animals with this mutation are small, slow-growing, uncoordinated, and contract less than wild type, but retain some capabilities of normal motor movement, which suggests that synaptotagmin is not the fusion protein itself, but instead plays a modulatory role in neurotransmitter release. Synaptotagmin mutations in *Drosophila* produce a similar phenotype (DiAntonio, Parfitt & Schwarz, 1993; Littleton et al., 1993). Most embryos that lack synaptotagmin fail to hatch, and show reduced, uncoordinated muscle contractions. Surprisingly, although calcium-dependent release of neurotransmitter is severely reduced, the frequency of spontaneous release of neurotransmitter, which is not calcium dependent, is increased by twofold in these mutants (Littleton et al., 1993).

Taken together, the results of these studies suggest that exocytosis can occur in the absence of synaptotagmin, but that calcium-dependent fusion of synaptic vesicles is not as efficient. One hypothesis that reconciles seemingly contradictory data is that synaptotagmin normally *inhibits* exocytosis of the docked synaptic vesicles, perhaps by interaction with a receptor protein in the plasma membrane such as syntaxin, neurexin, or the calcium channel. The influx of calcium that accompanies depolarization of the nerve terminal could cause a conformational change in the structure of either synaptotagmin or its receptor that disrupts the interaction and frees the vesicle for exocytosis. This hypothesis is particularly appealing since we think of regulated secretory vesicles as being constrained from exocytosis, as compared to constitutive secretory vesicles. Consistent with this model, Bommert et al. (1993) observed a twofold increase in the number of "docked" synaptic vesicles (synaptic vesicles adjacent to the presynaptic membrane) in squid synapses in which synaptic transmission was blocked by injection of synaptotagmin peptides.

Synaptobrevin (VAMP)

Synaptobrevin/VAMP (vesicle-associated membrane protein) is a relatively small protein (M_r 18,000 daltons) with a single transmembrane domain; the N-terminal and the bulk of the protein is cytoplasmic (Trimble et al., 1988; Baumert et al., 1989; Elferink, Trimble & Scheller, 1989; Südhof et al., 1989). Synaptobrevin/VAMP has a number of interesting characteristics that point to a direct role in the fusion event: (i) Botulinum and tetanus toxin, two neurotoxins that block neurotransmitter release, appear to act by specifically cleaving synaptobrevin (Link et al., 1992; Schiavo et al., 1992; Schiavo et al., 1993). (ii) Synaptobrevin interacts with syntaxin 1, a neuron-specific plasma membrane protein (Bennett et al., 1992*b*; Bennett et al., 1993; Bennett & Scheller, 1993). (iii) Synaptobrevin and syntaxin have been shown to have a specific interaction with the cytosolic proteins NSF and SNAP, two components of a protein complex used for membrane fusion by all cells (Söllner et al., 1993*a*; Söllner et al., 1993*b*). The NSF/SNAP complex is thought to link vesicles to their target membranes by virtue of its interaction with specific receptors, called SNAREs, which are membrane proteins in each compartment. Rothman and colleagues suggest that the interaction between synaptobrevin, the synaptic vesicle or v-SNARE, and syntaxin, the target membrane or t-SNARE, is mediated by the soluble complex NSF/SNAP. This interaction between pairs of membrane receptors ensures specificity in the interaction between synaptic vesicles and the nerve terminal membrane. If this is a general mechanism for membrane fusion shared by all cells, similar proteins to syntaxin should be located in other target membranes, such as Golgi, and proteins similar to synaptobrevin should be found in the appropriate transport vesicle. Consistent with this hypothesis, McMahon et al. (1993) have identified a non-neuronal homologue of synaptobrevin, called cellubrevin, that is uniformly expressed in all cells and tissues examined, and Bennett et al. (1993) have identified a family of syntaxin isoforms that are expressed outside the nervous system.

SV2

The synaptic vesicle protein SV2, originally characterized as a transmembrane glycoprotein, has recently been demonstrated to be the principal proteoglycan in synaptic vesicles (Buckley & Kelly, 1985; Scranton, Iwata & Carlson, 1993). The glycosaminoglycan consists of keratan sulfate that may be N-linked to the core protein. We and other laboratories have recently shown that the amino acid sequence of SV2 suggests that it is a novel type of transmembrane transporter (Bajjalieh et al., 1992; Feany et al., 1992; Gingrich et al., 1992). The amino acid sequence contains twelve hydrophobic domains, and the protein has significant sequence homology with a family of proteins that transport sugar across cell membranes. Because of this homology, it has been suggested that SV2 mediates the transport of neurotransmitters, ions, or other small molecules across the synaptic vesicle membrane, but neither the direction of transport nor potential substrates have been identified. Parsons and colleagues suggested that SV2 is the acetylcholine transporter in synaptic vesicles purified from the *Torpedo* electric organ. They found that a series of acetylcholine analogues can be used to photoaffinity-label SV2 purified from synaptic vesicles (Bahr et al., 1992; Bahr & Parsons, 1992). It seems unlikely, however, that SV2 acts as the vesicular acetylcholine transporter. The mRNA for SV2 is present in many types of neurons that are not cholinergic, and although two isoforms have been identified (Bajjalieh et al., 1993), neither one is preferentially localized to neurons of a particular transmitter phenotype (Bajjalieh et al., 1993; Marazzi & Buckley, 1993). In addition, a *C. elegans* mutation has been identified that probably encodes the vesicular acetylcholine transporter, and its sequence is not homologous to SV2 (Alfonso et al., 1993). Moreover, a vesicular amine transporter has also been cloned (Erickson, Eiden & Hoffman, 1992; Liu et al., 1992) as well as a putative vesicular GABA transporter (McIntire, Jorgensen & Horvitz, 1993); the identification of these different vesicular neurotransmitter transporters makes it unlikely that SV2 acts as a relatively nonspecific neurotransmitter transporter in synaptic vesicles. What other substances are in synaptic vesicles? Many types of synaptic vesicles contain ATP, calcium and chloride, and the transporters or channels involved in the movement of these molecules into synaptic vesicles have not been identified. SV2 might also move substances out of synaptic vesicles. For example, after exocytosis and recycling of synaptic vesicle membrane, presumably newly formed vesicles contain high concentrations of sodium and bicarbonate, and whatever proteins or osmolites that are present in the extracellular fluid. In order to maintain osmotic balance, these molecules must be pumped out of the vesicle when neurotransmitters are pumped in. SV2 may mediate this efflux. Finally, it has been proposed that SV2 may function as a fusion protein, presumably because of its multiple transmembrane domains (Bennett & Scheller, 1993). Whatever function is mediated by SV2 must be critical for synaptic vesicles, as this protein is present in all synaptic vesicles and is highly conserved throughout evolution. Elucidation of SV2 function may reveal a previously unsuspected but clearly essential function of synaptic vesicles.

Biogenesis of Synaptic Vesicles

The biogenesis of synaptic vesicles can be considered to occur in two distinct stages. One is the *de novo* assembly of synaptic vesicles from newly synthesized proteins that occurs during development and in mature neurons to replenish the supply of synaptic vesicles at the nerve terminal. The other is the assembly of recycled synaptic vesicles from synaptic vesicle proteins that have been inserted into the plasma membrane as the result of exocytosis of pre-existing synaptic vesicles. Recent studies of synaptic vesicle formation suggest that there may be some similarities between these two processes. Surprisingly, for such an abundant organelle, the origin of synaptic vesicles during development has long been mysterious. Vesicles of the size and shape of synaptic vesicles can be observed in growth cones and in the earliest synaptic contacts, but it was not known whether these vesicles arise in the perikarya of neurons and are transported to the terminal, or if they are created by budding from larger membranes at the nerve terminal. Two different experiments suggest that newly synthesized synaptic vesicles are formed at the nerve terminal. First, the ligation of axons leads to the accumulation of synaptic vesicle *proteins* on both sides of the ligature (Dahlstrom, Czernick & Li, 1992), but the *organelles* that accumulate on the proximal side are much larger than synaptic vesicles (Tsukita & Ishikawa, 1980). Second, recent experiments by Mundigl et al. (1993) suggest that in the perikarya of embryonic hippocampal neurons, the synaptic vesicle proteins synaptophysin, synaptotagmin, and SV2 are not in the same subcellular compartment. Neurons were treated with the drug brefeldin A, a fungal metabolite that disrupts the normal morphology of intracellular compartments. After brefeldin treatment, synaptophysin colocalizes with the endosomal marker, transferrin receptor, whereas the other synaptic vesicle proteins do not. In contrast, at the nerve terminal, the distributions of synaptophysin, synaptotagmin and SV2 are completely overlapping. This difference between the distribution of the synaptic vesicle proteins in the cell body and the nerve terminal suggests that the synaptic vesicle proteins are not assembled into mature synaptic vesicles until they reach the nerve terminal.

How does this assembly occur? Some clues to the steps involved in vesicle biogenesis have been obtained by studying the formation of synaptic vesicles in the endocrine cell line PC12. PC12 cells contain two types of regulated secretory vesicles: large dense core vesicles that contain catecholamines and a population of small vesicles, with the biochemical and biophysical properties of synaptic vesicles, that contains acetylcholine (Bauerfeind et al., 1993). Large dense core vesicles are assembled in the trans Golgi network (TGN) and undergo additional maturation after budding from the TGN (Tooze et al., 1991). At least a portion of the synaptic vesicles in PC12 cells can be labeled with horseradish peroxidase (HRP), indicating that, at some point, they arise by endocytosis from the plasma membrane. Since the vesicles that contain HRP may be mature vesicles that are recycling, Régnier-Vigouroux (1991) asked how these vesicles arise *de novo* by following the fate of newly synthesized synaptophysin from the trans-Golgi network. Cutler and Cramer (1990) had previously demonstrated that less than 5% of the total synaptophysin in PC12 cells is contained in large dense core vesicles, and that synaptophysin is not delivered to the plasma membrane by these vesicles. Régnier-Vigoroux discovered that synaptophysin reaches the cell surface via a constitutive secretory pathway in PC12 cells. From there, it is internalized and delivered to endosomes, and finally recovered in synaptic vesicles. The synaptic vesicle protein synaptotagmin also appears to arrive at the plasma membrane in constitutive vesicles in embryonic neurons (Matteoli et al., 1992). The involvement of an endosomal intermediate in the *de novo* biogenesis of synaptic vesicles in neurons, as well as in PC12 cells, is supported by the observation that synaptophysin colocalizes with transferrin receptor (i.e., endosomes) in perikarya of embryonic neurons, whereas at the terminal, it is found in the same vesicles as the other synaptic vesicle proteins (Mundigl et al., 1993).

Expression of synaptic vesicle proteins in non-neuronal cells has provided a useful model to study the biogenesis of synaptic vesicles. We have expressed the synaptic vesicle proteins synaptophysin, synaptotagmin, SV2, and synaptobrevin in CHO cells (Feany et al., 1993). When expressed alone, synaptophysin colocalizes with endosomes, SV2 is found in an intracellular compartment that does not contain the transferrin receptor, synaptotagmin is targeted to the plasma membrane, and synaptobrevin appears to be colocalized with clathrin-coated vesicles. When three of these proteins (synaptophysin, synaptotagmin, and SV2) are coexpressed in a single cell line, their characteristic distributions are unchanged. These data, and the previously described observations of Mundigl et al. (1993), argue against a model for *de novo* vesicle formation in which the synaptic vesicle proteins associate with one another to form complexes that are targeted to synaptic vesicles. However, such a mechanism may operate during recycling of mature synaptic vesicles. Bennett et al. (1992*a*) analyzed the interactions between synaptic vesicle proteins by detergent solubilization of purified rat brain synaptic vesicles, followed by size fractionation or immunoprecipitation. They observed a variety of different complexes depending on the detergent used and suggested that such stable interactions between multiple synaptic vesicle proteins might be important for recycling.

No data are available on the route taken by proteins

other than synaptophysin to synaptic vesicles in PC12 cells. Since synaptotagmin and SV2 are contained in large dense core vesicles as well as synaptic vesicles, they may reach the plasma membrane by exocytosis of large dense core vesicles, and subsequently arrive at endosomes and synaptic vesicles. Alternatively, they may be delivered to the plasma membrane by constitutive vesicles, as is synaptophysin, as well as large dense core vesicles.

In summary, it is clear from these studies that tremendous progress has been made in defining the components of synaptic vesicles, in particular, the proteins that are likely to be involved in the fusion complex. Future avenues of research include the assignment of specific functions to each of the synaptic vesicle-specific proteins, and the elucidation of the mechanism of synaptic vesicle biogenesis, during development and during recycling.

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