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Biogenesis of synaptic vesicles

R. B. Kelly, Frank Bonzelius, Ann Cleves, Lois Clift-O'Grady, Eric Grote and Gary Herman

Department of Biochemistry and Biophysics and the Hormone Research Institute, University of California, San Francisco, USA

SUMMARY

The basic endosomal recycling pathway can be modified to generate transcytotic vesicles, storage vesicles and synaptic vesicles. Sorting into synaptic vesicles requires specialized sorting information not present in the transcytotic and storage vesicle proteins. Using mutagenesis we have distinguished the signals for rapid endocytosis and SV targeting in synaptobrevin. Finally, we have evidence that synaptic vesicles can be generated from an endosomal compartment in vitro.

Key words: synaptic vesicles, endosome, synaptobrevin, VAMP, PC12 cells

SPECIALIZATION OF THE ENDOSOMAL PATHWAY

Two factors have helped make the biogenesis of synaptic vesicles an attractive area for study. The first is that synaptic vesicles can be found in cultured endocrine cell lines, particularly the pheochromocytoma cell line, PC12. The second factor is that synaptic vesicle generation in PC12 cells and in neurons is by endocytosis. Endocytosis is probably one of the best understood aspects of cell biology.

Although it is well established that synaptic vesicles arise by endocytosis, it is still not certain whether an endosome is involved. Favoring an endosomal origin are the data that synaptic vesicle membrane proteins co-localize with endosomal markers in neurons, in PC12 cells and in transfected fibroblasts (Cameron et al., 1991; Linstedt and Kelly, 1991). Furthermore, using chimeric proteins, we have been able to show that a synaptic vesicle membrane protein, synaptophysin, has all the sorting information necessary to target it to the LDL receptor-containing endosomes and to recycle back to the cell surface with approximately the same kinetics as the LDL receptor itself (Kaneda, F. Bonzelius, G. Herman and R. B. Kelly, unpublished). It is generally assumed therefore that synaptophysin, the transferrin receptor and the LDL receptor are co-targeted in transfected fibroblasts and PC12 cells to endosomes. In PC12 cells, however, synaptic vesicles arise from the endosome by sorting proteins such as synaptophysin from other endosomal proteins such as the LDL receptor.

The synaptic vesicle, then, is a specialized endocytotic compartment. Other cells have specialized endocytotic compartments that also exclude recycling endosome markers such as the LDL receptor. In epithelial cells, for example, the transferrin and LDL receptors go only to the basolateral endosomes. The apical membrane of epithelial cells gives rise to specialized endocytotic vesicles, for example those that store plasma membrane proteins such as water channels, the chloride channels or the proton pumps in an intracellular pool (Kaplan, this volume; Brown, this volume). A second specialized endocytotic vesicle of epithelial cells is the transcytotic vesicle, which carries, for example, the polyIg receptor from basolateral to apical surfaces. Because of the parallels that it is suggested occur between neurons and epithelial cells (Dotti, this volume), PC12 cells were transfected with DNA encoding the polyIg receptor to discover whether there is any similarity between transcytotic and synaptic vesicles. We found that the polyIg receptor was excluded from synaptic vesicles when we used either the wild-type polyIg receptor, or the Asp664 mutant form, which is targeted to transcytotic vesicles better than wild-type receptor. Even when the PC12 cells were exposed to NGF or the immunoglobulin ligand for the polyIg receptor, synaptic vesicles were free of the polyIg receptor.

Storage endocytotic vesicles are used to provide an intracellular pool of surface proteins in cells other than epithelial cells. In adipose and muscle cells, one form of the glucose transporter (GLUT4) is internalized by endocytosis, but sorted from the endosome into a small vesicle or tubulovesicular structure of approximately the dimensions of the synaptic vesicle. These vesicles accumulate in the cytoplasm in the vicinity of the Golgi complex (Slot et al., 1991). They are believed to contain an analogue of VAMP (Cain et al., 1992) and also a form of the Rab3 protein (Baldini et al., 1992). Like synaptic vesicles, these storage endocytotic vesicles undergo regulated exocytosis. When adipose and muscle cells are exposed to insulin, the rate of fusion of these vesicles with the plasma membrane is markedly increased (Jhun et al., 1992; Yang and Holman, 1993). To test the possible similarities between the two endosomal pathways we transfected GLUT4 into PC12

cells. Once again however we found that GLUT4 was completely excluded from synaptic vesicles. We conclude that the formation of synaptic vesicles involves a unique modification of the endosomal recycling pathway, using sorting signals different from those used in either the generation of transcytotic vesicles or the GLUT4-containing storage endocytotic vesicles.

Although GLUT4 is not in synaptic vesicles in PC12 cells, it is almost exclusively found in a population of vesicles that sediment only slightly faster than synaptic vesicles. The GLU-4-enriched vesicles exclude the polyIg receptor, the synaptic vesicle protein SV2 and the LDL receptor. In PC12 cells, therefore, there may be two independent modifications of the standard endosomal recycling pathway, one which generates the GLUT4 vesicle, and the other which generates the synaptic vesicle.

SORTING INTO SYNAPTIC VESICLES

To be targeted to synaptic vesicles, synaptic vesicle membrane proteins must have sorting information that is absent from other endocytosed proteins such as the LDL receptor, the polyIg receptor or GLUT4. To identify such sorting domains we have begun an analysis of one of the synaptic vesicle proteins, synaptobrevin or VAMP. VAMP is a type II membrane protein which has only two amino acids of its carboxy terminus in its lumenal domain, located inside the synaptic vesicle (Trimble et al., 1991). To follow the targeting of VAMP inside transfected cells, we modified that intralumenal carboxy-terminal region of VAMP by the addition of a T-antigen epitope tag and transfected DNA encoding the construct (VAMP-Tag) into CHO and PC12 cells.

We were concerned that the addition of a large epitope and spacer to the lumenal domain would alter the ability of the T antigen to insert into plasma membranes. Our worry was unfounded. The modified epitope-tagged VAMP reaches the plasma membrane and is endocytosed. The addition of antibodies against the epitope tag to the outside of transfected PC12 cells and transfected fibroblasts has shown us that the endocytosis rates of the VAMP-Tag are comparable to those of other endocytosed markers. Finally, a fraction of the endocytosed VAMP can be recovered inside synaptic vesicles isolated by velocity sedimentation. Since the extension of VAMP-Tag was driven by a cytomegalovirus promoter, we were able to elevate the expression levels of the constructs by adding butyrate to the transfected cells. In this way we could achieve expression levels of the epitope-tagged VAMP that were 5-10 times greater than that of the endogenous VAMP. Despite this overexpression, endogenous VAMP could still be recovered in synaptic vesicles, implying that we had not saturated the sorting system.

To make a preliminary identification of endocytosis and synaptic vesicle sorting domains, constructs were prepared that expressed variants of epitope-tagged VAMP that lacked 30 amino acids from the cytoplasmic amino-terminal domain (del 2-30), that lacked 60 amino acids (del 2-60) or with the transmembrane domain of VAMP replaced by that of the transferrin receptor (TfR-TM). A fourth construction in which the cytoplasmic domain of VAMP-Tag was replaced with that of the transferrin receptor failed to reach the cell surface. When endocytosis of surface-labeled VAMP-Tag was measured, the extent of endocytosis was higher than wild type for two of the variants (del 2-30 and TfR-TM) and about half that of normal VAMP for the third (del 2-60). We conclude that the amino-terminal domain has no affect on endocytosis, and amino acids between 30 and 60, a small effect.

We examined targeting of the constructs into synaptic vesicles by two techniques, looking at the distribution by western blotting across a velocity gradient and by looking at the targeting of anti-epitope antibodies to the synaptic vesicles from the cell surface. Both techniques gave the same result, namely that a region between amino acids 30 and 60 is necessary to allow targeting to synaptic vesicles. However, while the endocytosis rates were faster in those proteins containing the 30-60 amino acid sequence, targeting to synaptic vesicles was 5-20% of normal.

The endocytosis targeting signal is a robust one and is relatively unaffected by the mutations made in the VAMP molecule. However, in contrast to the endocytosis signal, the signals which target to synaptic vesicles are much more labile. To get the maximum efficiency of targeting to synaptic vesicles, the transmembrane region and the variable amino terminal region are both required.

BIOGENESIS OF SYNAPTIC VESICLES IN VITRO

To identify cytosolic components that might be interacting with sorting domains on synaptic vesicle membrane proteins, we have examined synaptic vesicle biogenesis in vitro. We began our reconstitution with a membrane fraction that contained endosomes that had been washed free of synaptic vesicles. When this fraction was incubated with ATP and cytosol, it generated a population of small vesicles that could be readily identified by velocity sedimentation. Generation of these vesicles was blocked by *N*-ethylmaleimide.

To determine if the synaptic vesicle-sized vesicles generated in our in vitro system were arising from endosomes, we labeled the endosomal compartment. To do this, PC12 cells were biotinylated on their surface in the cold. The cells were washed and incubated for 20 minutes at 20°C, conditions which do not allow the biotin label to get to synaptic vesicles but which readily labeled endosomal compartments. Surface label was then stripped from the cells. When biotinylated membranes were incubated with ATP in cytosol, small vesicles of the size of synaptic vesicles were generated which contained the biotin label. The biotinylated proteins that were in the budded vesicle compartment were not identical to those in the starting material. One of them appeared to be synaptophysin.

We conclude, therefore, that we can observe the generation in vitro of vesicles that co-migrate with synaptic vesicles and which are derived from endosomes.

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