REVIEW ARTICLE

Regulated exocytosis

Robert D. BURGOYNE and Alan MORGAN

The Physiological Laboratory, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

INTRODUCTION

Exocytosis is the final vesicular transport step in the secretory pathway. In many cell types a class of secretory vesicles is formed that can only fuse with the plasma membrane following cell activation (Burgoyne, 1990, 1991; Lindau and Gomperts, 1991; Plattner, 1989). It is this regulated exocytosis, necessary for the secretion of neurotransmitters, hormones and many other molecules, that will be the subject of this Review. In recent years, considerable progress has been made in the identification of proteins involved in the various vesicular transport steps of the secretory pathway (Pryer et al., 1992; Rothman and Orci, 1992). This has been due, in part, to the availability of 'cell-free' assays using permeabilized (or semi-intact) cells or isolated organelles that allow transport to be reconstituted and manipulated. Regulated exocytosis was the first intracellular transport step to be studied by cell permeabilization (Baker and Knight, 1978; Bennett et al., 1981). Despite this it is notable that in a recent review of vesicular transport (Pryer et al., 1992) regulated exocytosis was not mentioned. The likely reason behind this is that much of the work on regulated exocytosis has followed a pharmacological approach and it has only been relatively recently that some of the proteins likely to be involved have been identified following the spectacular success for earlier steps in the secretory pathway.

A second, important, difference between the study of regulated exocytosis and the earlier transport steps is that a significant contribution towards our knowledge of vesicular transport has come from genetic studies on the yeast *Saccharomyces cerevisiae* (Novick et al., 1981; Pryer et al., 1992). Since this organism does not appear to possess a regulated exocytotic pathway, this particular transport step has not been amenable to the type of genetic analysis that has been so informative for the constitutive secretory pathway.

The extensively used pharmacological approach to regulated exocytosis using a variety of inhibitors and activators of potentially important intracellular proteins has, with one or two exceptions, proven to be strikingly unsuccessful in the generation of reliable insights into the proteins involved. We will concentrate in this Review, therefore, on the more recent findings on proteins involved in regulated exocytosis.

BASIC REQUIREMENTS FOR EXOCYTOSIS

Studies on a variety of cell types have shown that both constitutive (Edwardson and Daniels-Holgate, 1992; Helms et al., 1990; Miller and Moore, 1991) and regulated exocytosis require cytosolic proteins (Ali et al., 1989; Koffer and Gomperts, 1989; Martin and Walent, 1989; Sarafian et al., 1987) and ATP (Dunn and Holz, 1983; Knight and Baker, 1982; Wilson and Kirshner, 1983) to be optimal. Regulated exocytosis in some cells such as myeloid cells appears to be exceptional. In mast cells, for example, ATP is not required and activation of GTP-binding proteins can

be sufficient to trigger exocytosis (Lindau and Gomperts, 1991). Constitutive exocytosis differs from regulated exocytosis in that it is unimpaired even if the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) is reduced to well below resting levels (Edwardson and Daniels-Holgate, 1992; Helms et al., 1990; Miller and Moore, 1991; Turner et al., 1992a,b) and it is blocked by activation of GTP-binding proteins by the non-hydrolysable GTP analogue GTP γ S (Edwardson and Daniels-Holgate, 1992; Helms et al., 1990; Miller and Moore, 1991). In contrast, regulated exocytosis in both animal and plant (Zorec and Tester, 1992) cells is activated by a rise in $[Ca^{2+}]_i$ and GTP analogues have complex but often stimulatory effects (Gomperts, 1990; Lindau and Gomperts, 1991). The requirement for ATP and cytosolic proteins is common to all vesicular transport steps (Pryer et al., 1992). Many of the vesicular transport steps studied involve both formation and fusion of vesicles, whereas regulated exocytosis involves triggered fusion of already formed vesicles. This may be a partial explanation for the finding that certain vesicular transport steps are markedly blocked as temperature is reduced to 20 °C or below (Saraste and Kuismanen, 1984) whereas exocytotic fusion does not show such an acute temperature discontinuity and continues down to 4 °C (Oberhauser et al., 1992b).

One difficulty in the study of regulated exocytosis is that, in addition to essential components involved in the fusion machinery, there must be proteins involved in the regulation of exocytosis by second messengers. Regulated secretory vesicles must normally be prevented from fusing with the plasma membrane since in the same cells constitutive secretory vesicles do so readily. A mechanism must exist that allows a Ca^{2+} signal, for example, to be transduced into activation of the dormant fusion machinery. This could involve removal of inhibition as well as direct activation. Added complexity is due to variation between cell types in the regulation of exocytosis and in specialized features of the process. For example, in certain synapses neurotransmitter release has to be extremely rapid and it is likely that exocytosis is triggered and fusion complete within 100 μ s or so of Ca^{2+} channels being opened to allow Ca^{2+} entry and may result from exocytosis of only synaptic vesicles docked to presynaptic Ca^{2+} channels (Almers, 1990; Augustine et al., 1991; Llinas et al., 1992). In contrast, exocytosis in neuroendocrine cells is triggered after a lag period of 3–50 ms (Chow et al., 1992; Neher and Zucker, 1993; Thomas et al., 1993) and in mast cells after a lag period of a minute or so (Fernandez et al., 1984). It is possible that in neuroendocrine cells, such as pituitary cells or adrenal chromaffin cells and in mast cells, there would be sufficient time for a protein fusion complex to assemble and trigger exocytosis. In contrast, in fast neurotransmitter release, a minimal conformational change in a protein at the site of exocytosis is likely to be the only event for which there is sufficient time. This would mean that the fusion complex is already assembled on docked synaptic vesicles.

Figure 1 Exocytosis at the synapse

In the resting state some synaptic vesicles are closely associated with the presynaptic membrane and it is these that undergo exocytosis following depolarization and Ca^{2+} entry. Other vesicles are cross-linked by synapsin I to each other or to fodrin and are only released following phosphorylation of synapsin I. These vesicles can then move to the plasma membrane ready for the next depolarization.

To fully understand the mechanism of regulated exocytosis it is necessary to identify those proteins that act as regulatory as well as essential components of the exocytotic mechanism in addition to 'house-keeping' proteins that prepare the components for fusion. A comparison of the various cell types in which regulated exocytosis occurs should reveal whether there are universal protein components of the exocytotic machinery or whether the specialization required of regulated secretory cells has resulted in the evolution of a variety of distinct mechanisms for membrane fusion in regulated exocytosis.

STEPS IN REGULATED EXOCYTOSIS

As noted above, neurotransmitter release can be so fast that exocytosis must involve fusion of pre-docked synaptic vesicles tightly associated with the presynaptic release sites (Figure 1). Since the other synaptic vesicles are cross-linked within a cytoskeletal network (Hirokawa et al., 1989) by the extrinsic vesicle protein synapsin I (Sudhof et al., 1989), further events must occur subsequently in the synapse to allow recruitment of new vesicles to the plasma membrane in preparation for the next stimulus. These are believed to include phosphorylation of synapsin I leading to its dissociation from the vesicles and release of vesicles, bound by the cytoskeleton, that can then move to the presynaptic membrane. Synapsin I would then be rapidly dephosphorylated (DeCamilli and Greengard, 1986; Valtorta et al., 1992; Sudhof and Jahn, 1991). The initial activation of

exocytosis is believed to require a high ($10\text{--}100\text{ }\mu\text{M}$) $[Ca^{2+}]_i$ that is achieved locally at the presynaptic membrane following Ca^{2+} entry through plasma membrane channels (Augustine et al., 1991; Llinas et al., 1992). Synapsin I phosphorylation will be triggered by a lower rise in $[Ca^{2+}]_i$ due to activation of calmodulin-dependent kinase II (Valtorta et al., 1992). This kinase is also a synaptic vesicle protein that acts in the attachment of synapsin I to the vesicle (Benfenati et al., 1992). These ideas about the role of synapsin I in the regulation of synaptic vesicle availability have been supported by the findings that injected dephosphorylated synapsin I reduces neurotransmitter release from the squid giant synapse (Llinas et al., 1985) and rat brain synaptosomes (Nichols et al., 1992) and introduced calmodulin kinase II increases neurotransmitter release (Llinas et al., 1985; Nichols et al., 1990). Synapsin I is specific to nerve terminals and so this suggested mechanism does not occur in other cell types.

In other regulatory secretory cells, where exocytosis is triggered more slowly and can continue for prolonged periods, the situation is more complex and activation of the cells results in the sequential recruitment of multiple pools of secretory vesicles (Neher and Zucker, 1993; Thomas et al., 1993). In adrenal chromaffin cells (Aunis and Bader, 1988; Burgoyne and Cheek, 1987; Cheek and Burgoyne, 1986, 1987, 1992; Vitale et al., 1991), parotid salivary gland cells (Perrin et al., 1992), mast cells (Koffer et al., 1990) and various other cell types (reviewed in Cheek and Burgoyne, 1992; Trifaro and Vitale, 1993) there is an extensive actin network in the cell cortex that acts as a barrier to regulated secretory vesicles

Figure 2 Discrete stages in exocytosis triggering in non-neuronal cells

Secretory vesicles or granules can be present at various stages in the resting state. In most non-neuronal cells, few vesicles would be in a pre-fusion state. Other docked vesicles could fuse with relatively short lag times. Disassembly of the cortical actin network would allow further vesicles to move to the plasma membrane for exocytosis at later times.

in the cell periphery (Figure 2). The difference in organization in these cells compared to synapses may be that such an actin network would not be sufficient to impede smaller synaptic vesicles, leading to the requirement for synapsin I for direct linkage of synaptic vesicles to the cytoskeleton. From electron microscopical observations on adrenal chromaffin cells, around 450 secretory granules are present in the cortical zone but the majority are excluded (Burgoyne et al., 1982; Burgoyne, 1991). In response to stimulation, many thousand granule fusions can be triggered in chromaffin cells and disassembly or reorganization of the actin network occurs reversibly after stimulation to allow secretory granules to move to the plasma membrane (Cheek and Burgoyne, 1986; Rodriguez Del Castillo et al., 1990; Vitale et al., 1991; Wu et al., 1992). Kinetic studies on digitonin-permeabilized chromaffin cells (Bittner and Holz, 1992a,b) and the use of patch-clamp analysis of exocytosis in chromaffin cells (Neher and Zucker, 1993) or pituitary cells (Thomas et al., 1993) have revealed that exocytosis can occur with several distinct kinetic components. The initial phases of release are ATP-independent but may already have been primed by ATP (Bittner and Holz, 1992a; Holz et al., 1989). ATP-dependent priming may involve vesicle binding through filamentous connections to the plasma membrane (Morimoto et al., 1990). The most likely interpretation of these kinetic data is that, in these cells, exocytotic fusion can be activated not only for secretory granules near the plasma membrane but also for other granules deeper within the cell following reorganization of the cytoskeleton. The first phase of release is fast and complete within 50 ms while later phases occur at slower rates (Neher and Zucker, 1993; Thomas et al., 1993). This fast burst of release involves an estimated 200 (Neher and

Zucker, 1993) or 450 (Thomas et al., 1993) vesicles from capacitance measurements or 1.3% of total catecholamine (around 390 secretory granules) from biochemical measurement (Bittner and Holz, 1992a). These values are similar to the estimated 450 granules near the plasma membrane in chromaffin cells (Burgoyne, 1991). Exocytosis can, therefore, be regulated at the level of secretory vesicle availability as well as by direct effects on membrane fusion. This again complicates the analysis of the components involved in the late steps leading to fusion.

IDENTIFICATION OF PROTEINS IN EXOCYTOSIS

Recent work has begun to identify cytosolic and membrane proteins involved in exocytosis. These proteins could be involved either in a regulatory function, or as essential components of the fusion machinery, but specific functions can not yet be assigned to the identified proteins. Amongst the proteins required for exocytosis must be at least one that acts as a Ca^{2+} -binding protein. It was originally believed that such a protein would be a high-affinity Ca^{2+} -binding protein with an affinity for Ca^{2+} in the range of 1–10 μM . The finding that fast exocytosis is triggered by high Ca^{2+} concentrations at the plasma membrane (Augustine et al., 1991; Augustine and Neher, 1992; Llinas et al., 1992; Neher and Zucker, 1993; O'Sullivan et al., 1989; Thomas et al., 1993) has led to the realization that a Ca^{2+} -binding protein with affinity in the range 10–100 μM may be part of the mechanism. The various kinetic steps in exocytosis appear to have different Ca^{2+} -dependencies (Bittner and Holz, 1992a; Neher and Zucker, 1993), suggesting that exocytosis could be triggered or controlled by multiple Ca^{2+} -binding proteins with varying Ca^{2+} affinities. In

the vesicular transport steps from endoplasmic reticulum to Golgi and between Golgi elements, multiple proteins are required including cytosolic, extrinsic and intrinsic membrane proteins (Pryer et al., 1992; Rothman and Orci, 1992). Even in constitutive exocytosis in yeast, 10 genes are known to be required and several of the gene products appear to assemble to form a cytosolic protein complex (Bowser et al., 1992). Therefore, we can expect to find a similar requirement for several different proteins in regulated exocytosis. Studies on mutants of *Paramecium* in which regulated exocytosis of trichocysts is impaired have already revealed a requirement for at least 13 distinct genes (Bonnemain et al., 1992).

Cytosolic and extrinsic membrane proteins

A requirement for soluble ('cytosolic') proteins in Ca^{2+} -dependent exocytosis has been shown for adrenal chromaffin cells (Ali et al., 1989; Morgan and Burgoyne, 1992a; Sarafian et al., 1987; Wu and Wagner, 1991), GH_3 cells (Martin and Walent, 1989) PC12 cells (Lomneth et al., 1991), mast cells (Howell and Gomperts, 1987; Koffer and Gomperts, 1989) and brain synaptosomes (Kish and Ueda, 1991). Two of the defective proteins in the *Paramecium* mutants mentioned above are cytosolic proteins (Bonnemain et al., 1992). Earlier pharmacological experiments using phorbol esters have suggested that one cytosolic protein, protein kinase C (PKC), is a regulator of Ca^{2+} -dependent exocytosis (Knight and Baker, 1983; Pocotte et al., 1985) and this has been confirmed by introduction of purified PKC into permeabilized chromaffin cells (Morgan and Burgoyne, 1992b), pituitary (Naor et al., 1989) and PC12 cells (Ben-Shlomo et al., 1991; Nishizaki et al., 1992). The role of soluble proteins has become apparent from the run-down of secretory responsiveness of permeabilized cells as they leak such proteins. This has formed the basis of an assay for the identification of certain essential or regulatory cytosolic and extrinsic membrane proteins.

Using reintroduction of proteins into digitonin-permeabilized chromaffin cells, several stimulatory proteins have been identified. The first to be identified in this way was annexin II (Ali et al., 1989) a member of the annexin family of Ca^{2+} - and phospholipid-binding proteins (Burgoyne and Geisow, 1989; Creutz, 1992). Annexin II is found on the inner surface of the plasma membrane of intact chromaffin cells (Nakata et al., 1990) and so is not normally a cytosolic protein but behaves as an extrinsic membrane protein at resting Ca^{2+} concentrations. It is extracted from the plasma membrane following permeabilization in the presence of EGTA (Burgoyne and Morgan, 1990). Annexin II has been suggested to be involved in exocytosis due to its location on the plasma membrane and the possibility that it forms filamentous cross-links between granules and plasma membranes (Nakata et al., 1990). Furthermore, it has the ability to aggregate isolated secretory granules in the presence of micromolar Ca^{2+} and to fuse them after addition of arachidonic acid (Drust and Creutz, 1988). Incubation with exogenous, purified annexin II resulted in an increase in Ca^{2+} - and ATP-dependent exocytosis in permeabilized chromaffin cells (Ali et al., 1989; Ali and Burgoyne, 1990). The stimulatory effect of annexin II was suggested to be dependent upon PKC-mediated phosphorylation of the protein (Sarafian et al., 1991) though other data do not agree with this conclusion (Ali and Burgoyne, 1990). The extreme N-terminus of annexin II, which contains sites for phosphorylation and for binding of the subunit p11, is critical for its ability to stimulate exocytosis (Ali and Burgoyne, 1990; Burgoyne and Morgan, 1990). Endogenous annexin II seems likely to be involved in exocytosis in chromaffin cells since exocytosis assayed without

added annexin II was partially inhibited by a synthetic peptide corresponding to the most conserved annexin domain (Ali et al., 1989). In contrast, a synthetic peptide corresponding to the N-terminal 15 residues of annexin II was without effect on exocytosis (Ali and Burgoyne, 1990).

As run-down is allowed to proceed for longer periods, the ability of annexin II to stimulate exocytosis is lost (Burgoyne and Morgan, 1990; Sarafian et al., 1991), suggesting that other required proteins leak from the permeabilized cells. In order to attempt to detect additional cytosolic proteins that regulate exocytosis, adrenal medullary and brain cytosols were fractionated and tested in a run-down/reconstitution assay. Three activities were detected, one was inhibitory and the other two (Exo1 and Exo2) were stimulatory (Morgan and Burgoyne, 1992a). Purified Exo1 consists of a family of proteins belonging to the 14-3-3 gene family (Morgan and Burgoyne, 1992a, Morgan et al., 1993b). These proteins were independently purified from adrenal medulla using a similar approach by another laboratory (Wu et al., 1992) and the Exo1/14-3-3 proteins were found to leak from permeabilized chromaffin cells (Morgan et al., 1993; Wu et al., 1992). Wu et al. (1992) used an immunodepletion approach to show that the 14-3-3 proteins were major stimulatory components of cytosol. Little is known about Exo2 except that it apparently behaves as a single 44 kDa protein. The ability of Exo1 to stimulate exocytosis is Ca^{2+} - and ATP-dependent, is blocked by tetanus toxin and is potentiated by activation of PKC using phorbol esters or co-introduction of purified rat brain PKC (Morgan and Burgoyne, 1992a,b; Morgan et al., 1993a). It did not, however, appear to be a good substrate itself for PKC. 14-3-3 proteins have been cloned from mammalian tissues and from *Xenopus*, *Drosophila*, plants and yeast. The proteins all show a high degree of sequence similarity with one another (Aitken et al., 1992; Isobe et al., 1992; Martens et al., 1992) but it is not clear whether all of the mammalian proteins are able to stimulate exocytosis. One of the domains conserved in the entire range of 14-3-3 proteins is homologous to the C-terminus of the annexins and is most similar to annexin II (Aitken et al., 1990). It seems possible that this domain could be necessary for interaction with a common target protein required for exocytosis stimulated by both classes of protein. The synaptic vesicle protein synaptotagmin (see below) and PKC have been shown to bind to target proteins known as RACK (receptor for activated C-kinase) proteins and this binding is inhibited by a synthetic peptide similar to this conserved sequence (Mochly-Rosen et al., 1992). Little is known about the RACK proteins but they may include certain annexins (Mochly-Rosen et al., 1991). Direct evidence that the common annexin/14-3-3 protein domain is important in exocytosis was shown by the finding that a 16-residue synthetic peptide, but not truncated peptides, based on the C-terminus of annexin II partially inhibited Ca^{2+} -dependent exocytosis in permeabilized chromaffin cells (Roth et al., 1993). It has been suggested that the 14-3-3 proteins have Ca^{2+} -dependent phospholipase A_2 (PLA_2) activity (Zupan et al., 1992). This could be significant since, as noted above, secretory granules cross-linked by annexin II fuse when arachidonic acid is added. The work of Zupan et al. (1992) did not actually demonstrate PLA_2 activity but only substrate binding and we and other laboratories were unable to detect PLA_2 activity in purified brain Exo1 using 1-palmitoyl-2-arachidonoylphosphocholine as substrate (Morgan et al., 1993b). Arachidonic acid generation and exocytosis in permeabilized chromaffin cells had previously been dissociated, suggesting that Ca^{2+} -dependent arachidonic acid production is not required for the activation of exocytosis (Morgan and Burgoyne, 1990).

Using an alternative cell permeabilization technique, known as

cell-cracking, in which cells are sheared using a ball homogenizer and cytosol completely removed by washing, other cytosolic proteins have been identified which stimulate exocytosis in GH₃ and PC12 cells (Hay and Martin, 1992; Martin and Walent, 1989; Nishizaki et al., 1992; Walent et al., 1992). A protein in brain cytosol which stimulated exocytosis in GH₃ cells was partially characterized initially and then a 145 kDa protein which stimulated exocytosis in PC12 cells was purified from brain cytosol. The activity of this protein was Ca²⁺- and ATP-dependent and it was found to be present in a range of tissues showing regulated secretion (Walent et al., 1992). Antiserum against this protein produced a marked inhibition of the ability of cytosol to stimulate exocytosis, indicating that it is a major contributor to the activity of crude cytosol. The protein did not appear to be related to any known proteins. The ability of p145 to stimulate exocytosis was increased by PKC-mediated phosphorylation and p145 is a substrate for the kinase (Nishizaki et al., 1992). The lack of detection of a requirement for this protein in the digitonin-permeabilized chromaffin cell system may be due to the fact that the native state of p145 is that of a dimer and it would be likely to leak slowly from digitonin-permeabilized cells, which have limited permeability, and thus p145 would not become rate-limiting.

Hay and Martin (1992) have recently developed a protocol that can resolve two stages in exocytosis in permeabilized PC12 cells similar to those previously detected in chromaffin cells (Bittner and Holz, 1992a; Holz et al., 1989). These are an ATP-dependent priming stage and a Ca²⁺-dependent triggering stage. Each stage was stimulated by distinct protein fractions derived from brain or adrenal medullary cytosols and the 145 kDa protein isolated by this laboratory was functional in only the Ca²⁺-triggering stage whereas a 20 kDa factor was active in priming.

These studies on permeabilized adrenal chromaffin and PC12 cells have revealed multiple cytosolic (or membrane-associated in the case of annexin II) proteins that stimulate Ca²⁺-dependent exocytosis and that might function to accelerate distinct stages in the exocytotic process. The different permeabilization methods and protocols used to assay exocytosis would almost certainly result in different steps (or proteins) being rate-limiting in each cell type studied and there is no reason to believe that the fact that different cytosolic proteins were revealed is in any way contradictory. On the contrary, it merely reflects the complexity of regulated exocytosis. The interactions between the cytosolic proteins identified so far in chromaffin and PC12 cells and their exact function in exocytosis remains to be established.

Triggered exocytosis in *Paramecium* is accompanied by the rapid dephosphorylation of a protein, pp63, that behaves as a soluble cytosolic protein but is also localized within the cell cortex (Gilligan and Satir, 1982; Ziesness and Plattner, 1985; Momayezi et al., 1987; Hohne-Zell et al., 1992). This dephosphorylation event does not occur at the non-permissive temperature in various non-discharge mutants (Gilligan and Satir, 1982; Ziesness and Plattner, 1985) and microinjected anti-pp63 antibodies inhibit trichocyst discharge (Stecher et al., 1987), suggesting that pp63 is involved in exocytosis. A related protein has been detected in various species and mammalian tissues (Satir et al., 1989) but pp63 has not yet been characterized in any detail and no sequence data is available.

Secretory vesicle proteins

Vesicle docking at the plasma membrane must surely involve secretory vesicle proteins and these could act as key elements of the fusion mechanism. Over the past few years, extensive work

has been carried out in attempts to characterize secretory vesicle proteins and considerable information is now available on the membrane proteins of the synaptic vesicle (Sudhof and Jahn, 1991). Because the synaptic vesicle is so small (around 50 nm in diameter) and can contain limited amounts of protein (Bennett et al., 1992a), the protein composition of this vesicle is close to being fully characterized. Once transport or biosynthetic proteins are excluded, relatively few synaptic vesicle proteins remain as potential players in the exocytotic mechanism. One or more of these must be a key element in rapid exocytotic fusion at the synapse. Amongst the transmembrane vesicle proteins characterized so far, three have stood out as prime candidates—synaptophysin, synaptotagmin (p65) and synaptobrevin.

Synaptophysin is the major integral synaptic vesicle protein (Navone et al., 1986) and the first to be sequenced (Sudhof et al., 1987). It is believed to span the vesicle membrane four times and in some respects has the form of a channel protein. The reconstituted protein was shown to have channel activity in planar lipid bilayers (Thomas et al., 1988) and it was thought to be a Ca²⁺-binding protein (Rehm et al., 1986). It was, therefore, suggested to be involved in initial pore formation (see the section on fusion pore below) prior to exocytosis (Thomas et al., 1988). It has been pointed out (Sudhof and Jahn, 1991), however, that the voltage-dependency of the synaptophysin channel would result in channel closure during exocytosis and the ability of synaptophysin to bind Ca²⁺ has been disputed (Brose et al., 1992). Studies on *Xenopus* oocytes support the idea that synaptophysin is required for exocytosis (Alder et al., 1992a). Total mRNA from brain was injected into oocytes and protein synthesis allowed to proceed. The oocytes developed the ability to release the neurotransmitter glutamate in response to a Ca²⁺ ionophore (Alder et al., 1992a) presumably due to the synthesis *de novo* of functional synaptic vesicles. Neurotransmitter release was inhibited by an antisense oligonucleotide designed to disrupt synaptophysin expression and also by anti-synaptophysin antibodies. These results suggest that synaptophysin may be necessary for synaptic vesicle exocytosis but do not distinguish between a purely structural role for this highly abundant vesicle protein and an essential mediatory role in exocytosis. The same comment applies to the demonstration that anti-synaptophysin antibodies inhibit neurotransmitter release at the neuromuscular junction (Alder et al., 1992b). In addition, all antibody experiments of this type can be criticized due to the possibility of a non-specific block of exocytosis by large antibody molecules decorating the synaptic vesicle.

Synaptotagmin was first discovered by chance as a widespread synaptic vesicle protein known as p65 (Matthew et al., 1981) and was found to be present in several secretory granules from endocrine tissues (Trifaro et al., 1989). Most of the protein projects into the cytoplasm (Perin et al., 1991; Tugal et al., 1991), it possesses two C₂-like domains (Figure 3) related to those of PKC (Perin et al., 1990) and it binds Ca²⁺ and phospholipid (Brose et al., 1992; Perin et al., 1990). Synaptotagmin has been found to bind to the receptor for α -latrotoxin, a spider venom component that acts extracellularly to activate exocytosis (Petrenko et al., 1991). The α -latrotoxin receptor belongs to a family of synaptic membrane proteins, the neuexins, and synaptotagmin binds in a Ca²⁺-independent manner to the cytoplasmic C-termini of these proteins (Hata et al., 1993). Synaptotagmin has also been found associated with N-type Ca²⁺ channels (Bennett et al., 1992b; Leveque et al., 1992) which suggests an intriguing scenario in which synaptotagmin is responsible for docking of synaptic vesicles by binding to Ca²⁺ channels, thus holding the vesicle precisely at the site of Ca²⁺ entry for rapid exocytosis in synapses (Figure 4). Synaptotagmin

Figure 3 Schematic diagram of the domain structure of synaptotagmin (p65)

The figure is based on sequence data described by Perin et al. (1990).

is present on many synaptic vesicle and endocrine secretory granules (but not exocrine granules), supporting a widespread role in exocytosis in many but not all cell types. Recent work on PC12 cells has, however, argued against such a role. Shoji-Kasai et al. (1992) selected synaptotagmin-deficient PC12 cell lines. These cell lines not only secreted perfectly well but the extent of secretion was greater than that in the parent cell line. The exocytosis assayed would have involved dense core granules and so one possibility is that synaptotagmin is required for fast exocytosis of small synaptic vesicles but is not essential for slower, dense-core granule exocytosis. It is also possible that the role of synaptotagmin is not as a Ca^{2+} sensor in the essential fusion machinery but is simply in the docking of synaptic vesicles at exocytotic sites. Synthetic peptides based on the synaptotagmin sequence inhibit exocytosis in nerve terminals (Bonnert et al., 1993) and a partial reduction in the extent of exocytosis, mainly in a subpopulation of highly-secreting cells, was observed in a study in which antibodies against synaptotagmin or certain fragments of the cytoplasmic domain of synaptotagmin were microinjected into PC12 cells (Elferink et al., 1993). None of the studies have so far examined the kinetics of exocytosis in cells in which synaptotagmin function has been disrupted; if its role is in vesicle docking then such disruption might be manifest as a reduction in the initial rate of exocytosis or a loss of the first rapid phase of exocytosis.

Synaptobrevin is an evolutionarily conserved synaptic vesicle protein that exists in two isoforms in mammalian brain which are also known as VAMP 1 and 2 (Archer et al., 1990; Baumert et

al., 1989; Elferink et al., 1989). Little had been known about synaptobrevin but now evidence has been obtained suggesting that the synaptobrevins are essential for neurotransmitter release. The neurotoxins tetanus toxin and the botulinum toxins potentially produce long lasting blocks of neurotransmitter release. If the active fragments of these toxins are able to enter the cytosol of neurons or neuroendocrine cells, a specific inhibition of regulated exocytosis occurs. The toxins have been extensively studied since the view has been taken, almost certainly correctly, that the substrates for the toxin must be key components of the machinery for neurotransmitter exocytosis. It has now been shown that tetanus and botulinum B toxins have zinc-dependent protease activity (Schiavo et al., 1992a,b; Link et al., 1992) and in synaptic vesicles specifically cleave one of the synaptobrevin isoforms at a sequence motif apparently not found in any other known proteins (Figure 5). Botulinum toxins type A and E do not appear to act by precisely the same mechanism as type B and tetanus toxin and their mode of action is unknown. Unfortunately, no data is available on whether the tetanus and botulinum B toxins cleave any cytosolic, cytoskeletal or plasma membrane proteins nor on the exact substrate sequence motif that is sufficient to allow proteolysis. This limitation of the published data make it difficult to decide whether synaptobrevin 2 really is the sole substrate. If it is, then it is clearly essential for neurotransmitter release and it is important that more information on this point is now obtained. Tetanus and botulinum toxins inhibit exocytosis in neuroendocrine cells (Holz et al., 1992) but not exocrine secretory cells (Stecher et al., 1992); it is

Figure 4 Organization of synaptic vesicles and calcium channels at the presynaptic membrane

In order to account for fast neurotransmitter release it is likely that synaptic vesicles are docked at the presynaptic membrane close to the Ca^{2+} channels, so that exocytosis is rapidly triggered by the cloud of Ca^{2+} at the mouth of the channel.

Figure 5 Schematic diagram of the domain structure of rat brain synaptobrevins

This is based on the data in Archer et al. (1990). Tetanus and botulinum B toxins (Schiavo et al., 1992b) specifically cleave only synaptobrevin 2 between Q and F in the amino acid sequence shown. The sequences are those of the rat synaptobrevin isoforms.

not known whether exocrine cells express synaptobrevin. The details of the tissue distribution of the synaptobrevins are sparse but clearly more information on this point is now essential in order to determine whether synaptobrevin is present on the secretory vesicles of all cell types sensitive to tetanus and botulinum B toxins and to enable assessment of whether synaptobrevin is a general or a synapse-specific component of the exocytotic machinery. In favour of a more general distribution is the discovery of synaptobrevin- (VAMP)-related proteins in adipocytes in secretory vesicles that are responsible for the insertion of the GLUT4 glucose transporter into the plasma membrane in a regulated fashion following exposure to insulin (Cain et al., 1992).

GTP-binding proteins

Many vesicular transport steps are regulated by GTP-binding proteins (Pryer et al., 1992; Rothman and Orci, 1992). The first GTP-binding protein to be identified that is involved in vesicular transport was the monomeric GTP-binding protein, Sec4, which is required for constitutive exocytosis in yeast (Salminen and Novick, 1987). A family of mammalian proteins related to Sec4, known as the rab proteins, have subsequently been found to be localized at distinct sites within the cell (Chavrier et al., 1990) and may each be involved in specific vesicular transport steps (Pfeffer, 1992). Additional GTP-binding proteins, including the monomeric ARF proteins (Taylor et al., 1992) and heterotrimeric G proteins (Barr et al., 1992) have been shown to control vesicle budding and transport. Indications of the importance of GTP-binding proteins has come from the finding that the non-hydrolysable GTP analogue GTP γ S blocks many vesicular transport steps. This inhibition may be at the level of transport vesicle formation rather than fusion with the acceptor membrane (Barr et al., 1992).

In the case of regulated exocytosis, GTP γ S and other GTP analogues were found to stimulate exocytosis, in some cases in the absence of Ca²⁺ (Barrowman et al., 1986; Gomperts, 1990; Lindau and Gomperts, 1991). In some cell types an additional effect can be demonstrated which is an inhibition of Ca²⁺-dependent exocytosis if the permeabilized cells are first pre-incubated with GTP γ S (Ahnert-Hilger et al., 1992; Knight and Baker, 1985; DeMatteis et al., 1991; Davidson et al., 1991; Smolen et al., 1991; Turner et al., 1992b). Synaptic vesicles and secretory granules possess both monomeric (Burgoyne and Morgan, 1989; Darchen et al., 1990; Fischer von Mollard et al.,

1991) and trimeric (Toutant et al., 1987) GTP-binding proteins. It is not clear, however, which type of GTP-binding protein is involved in the stimulatory or inhibitory effects of GTP γ S. Antibody inhibition experiments on permeabilized chromaffin have suggested that G_o normally exerts an inhibitory effect on Ca²⁺-dependent exocytosis (Ohara-Imaizumi et al., 1992). In the nerve terminal of the squid giant axon, injected GTP γ S and GDP β S produced a slow block of exocytosis of the docked synaptic vesicles (Hess et al., 1993). The data would be consistent with a role for a monomeric GTP-binding protein in vesicle docking and GTP hydrolysis having to occur before Ca²⁺ could trigger exocytosis.

One approach to investigating the function of the rab proteins has been to use synthetic peptides based on the postulated 'effector domain' of these proteins. One such 16-residue peptide known as rab3AL inhibited endoplasmic reticulum-to-Golgi and intra-Golgi transport (Plutner et al., 1990) and the interpretation of this finding was that the peptide bound to the downstream effector protein for the rab protein to antagonize the action of the GTP-bound form of the native rab protein. Surprisingly, the rab3AL peptide acts as an activator of exocytosis in pancreatic acinar cells (Padfield et al., 1992) and mast cells (Oberhauser et al., 1992a) in the absence of any other stimuli. It is not known whether rab3 is expressed in these cell types. Chromaffin cells do express rab3 (Darchen et al., 1990) but in digitonin-permeabilized chromaffin cells the rab3AL peptide produced only a small enhancement of Ca²⁺-dependent exocytosis (Senyshyn et al., 1992). In the work on mast cells, patch clamp measurement demonstrated directly that the effect of the peptide was to stimulate exocytotic membrane fusion. Even more surprising was that a five-residue peptide corresponding to residues 33–37 of rab3 was sufficient to activate complete degranulation of the mast cells (Oberhauser et al., 1992a). The rab peptides required an extensive lag period of 10 min or so. The reason for this and the mechanism of action of the peptides and which rab proteins are expressed by mast cells are unknown. An earlier study has shown that injection of oncogenic ras protein resulted in mast cell degranulation after a prolonged lag period (Bar-Sagi and Gomperts, 1988). Much further work will be needed to determine the involvement of the rab proteins and exactly which GTP-binding proteins control regulated exocytosis.

Plasma membrane proteins

Little information is available on plasma membrane proteins in

Figure 6 Models for the structure of the fusion pore in exocytosis

The two models shown are modified from Almers (1990) in (a) or based on the model of Monck and Fernandez (1992) as shown in (b). In the Almers model the fusion pore involved the formation of an oligomeric protein structure involving vesicle and plasma membrane proteins (1). This structure opens during stimulation to form the pore (2) and disassembly of the oligomeric subunits within the bilayer allows full exocytotic fusion to occur (3). In the Monck and Fernandez model proteins act to pull the bilayers into close apposition (1) and inward dimpling of the plasma membrane bilayer (2) is followed by formation of a lipid pore (3).

regulated exocytosis. Binding partners for the synaptic vesicle proteins synaptophysin (physophilin; Thomas and Betz, 1990) and synaptotagmin have been identified but their significance is unclear. Synaptotagmin was found to associate with the α -latrotoxin receptor (Petrenko et al., 1991) which forms part of a family of synaptic plasma membrane proteins known as the neurexins (Hata et al., 1993; Ushkaryov et al., 1992). The role of the neurexins in exocytosis is unclear since their structure is related to that of proteins involved in cell adhesion. One worrying possibility about the numerous interactions of synaptotagmin with other proteins that have been detected (Bennett et al., 1992a,b; Leveque et al., 1992; Petrenko et al., 1991) is that they simply represent non-specific interactions of a 'sticky' protein.

A rather specific interaction has been discovered involving synaptic plasma membrane proteins, the synaptic vesicle protein synaptobrevin and a set of proteins believed to be required for vesicle targeting in early stages of the secretory pathway (Sollner et al., 1993). From reconstitution studies on vesicular transport between Golgi cisternae, the protein NSF and the SNAP proteins (α , β and γ ; Whiteheart et al., 1993) have been identified as essential components that interact in a 20 S particle (Rothman and Orci, 1992; Clary and Rothman, 1990; Wilson et al., 1992). In an attempt to identify integral membrane receptors for the 20 S particle an affinity chromatography approach was used with a

detergent-solubilized extract of total brain membranes. Only four polypeptides were found to interact specifically, that is in an ATP-dependent manner, with the 20 S particle. Peptide sequencing revealed these to be synaptobrevin 2, syntaxin A and B and SNAP-25 (Sollner et al., 1993). Syntaxin A and B were originally identified as synaptic membrane proteins that co-immunoprecipitated with synaptotagmin (Bennett et al., 1992) but no synaptotagmin was detected associated with the 20 S particle. SNAP-25 has been little studied but is a previously identified synapse-specific protein (Oyler et al., 1989). No direct functional data is available on the role of the NSF/SNAP proteins in neurotransmitter release, but from what is known of their function in intra-Golgi transport it has been suggested that the interaction between the 20 S particle and the synaptic proteins could be a mechanism for vesicle targeting and docking on the presynaptic membrane. So far there is no evidence that NSF or the SNAPs actually act as membrane fusion proteins during vesicular transport or that these proteins interact with membrane lipids in any way and so it is not clear if they have any further function after docking on the target membrane. It is also important to note that NSF and SNAPs only function in Golgi transport in the presence of several additional cytosolic proteins (Clary and Rothman, 1990; Waters et al., 1992) and so it seems likely that additional cytosolic proteins would also be required

for regulated exocytosis as discussed above. It is not known whether NSF and SNAPs are present in nerve terminals and this important point needs to be addressed to allow assessment of this recent intriguing data.

GAP-43 (B-50) is an extrinsic plasma membrane protein found predominantly in neurons. Antibodies against this protein inhibited Ca^{2+} -dependent exocytosis in permeabilized brain synaptosomes (Dekker et al., 1989) but the exact function of GAP-43, which is also highly expressed in developing neurons, is not known. Expression of an antisense construct that reduced GAP-43 levels in PC12 cells lead to an increase in basal secretion and a concomitant decrease in evoked secretion (Ivins, 1993). These results suggest that GAP-43 may exert an inhibitory influence to prevent exocytosis at resting Ca^{2+} concentration. A plasma membrane protein from adrenal chromaffin cells that binds to secretory granules has been identified. Antibodies against this 51 kDa protein inhibited exocytosis when introduced into chromaffin cells via a patch pipette (Schweizer et al., 1989) but relatively little is known about this protein.

THE FUSION PORE

One of the most powerful methods for the study of regulated exocytosis is the patch-clamp capacitance recording technique, since it allows direct measurement of membrane fusion of single secretory vesicles with high time resolution as well as single cell biochemistry (for more details see Monck and Fernandez, 1992). The method is based on the fact that addition of secretory vesicle membrane into the plasma membrane as fusion occurs will result in a step-wise increase in plasma membrane capacitance that can be detected electrophysiologically (Neher and Marty, 1982). The most important insights from capacitance measurement have come from studies of either normal or mutant mast cells from the beige mouse. The secretory vesicles of these cells are amongst the largest known, 0.8 μm or 1–5 μm in diameter for wild type and beige mice respectively, and exocytosis in mast cells is slow. For these reasons, not only can irreversible fusion events be measured but transient events, originally called capacitance flicker (Fernandez et al., 1984) can be detected. These appear to represent the initial events in exocytosis, the reversible formation of the 'fusion pore'. The fusion pore has the characteristics of a channel 1–2 nm in diameter with a fixed conductance (Breckenridge and Almers, 1987a) that can either close again or explosively open in a full exocytotic event (Breckenridge and Almers, 1987a,b; Monck et al., 1990; Zimmerberg et al., 1987). The lifetime of the fusion pore in mast cells is considerable, lasting up to several seconds. This is at least 10^4 times longer than the entire exocytotic process in a fast synapse. Therefore, there may be some very odd aspects of the mast cell that allow a fusion pore to open, remain stable in the fused lipid bilayers for such a prolonged period and then re-close. Two extreme models (Figure 6) have been suggested for the structure of the fusion pore as well as a third intermediate version (Zimmerberg et al., 1991). The first suggests that the pore is proteinaceous and must involve proteins in the secretory vesicle and plasma membrane that can initially act like a gap-junction channel (Almers, 1990). Synaptophysin was suggested to be able to act in this way in synapses by Thomas et al. (1988). This oligomeric channel would then have to spontaneously fall apart (Figure 6a), a scenario that seems thermodynamically improbable since considerable energy would need to be expended to split apart polypeptides that needed to be closely associated in the lipid bilayer in the first phase of the mechanism. The second extreme model, based on a lipid pore (Figure 6b), has been devised by Monck and Fernandez (1992). Previous work from this laboratory had suggested that lipid flow

could occur through the fusion pore while it was reversibly open (Monck et al., 1990). Secondly, a pore with all the characteristics of the exocytotic fusion pore could be induced in a pure lipid membrane and theoretical modelling demonstrated that closure or explosive opening of a fusion pore could be determined by the nature of the lipids flowing into the pore (Nanavati et al., 1992). In the lipidic fusion pore model of Monck and Fernandez the role of proteins would be to pull the plasma membrane and secretory vesicle membranes together. This idea is consistent with ultrastructural data on *Limulus* amoebocytes, *Paramecium* and mast cells, in which late stages in exocytosis could be visualized after rapid freezing (Chandler and Heuser, 1980; Knoll et al., 1991; Ornberg and Reese, 1981). In these studies widening fusion pores were seen but the most significant observation was that prior to pore formation the plasma membrane and vesicle membranes were linked by short filaments and the plasma membrane was pulled down on to the vesicle membrane at a focal point. This could then become the site of fusion pore formation (Monck and Fernandez, 1992). It is possible that this would require the presence, or the generation, of membrane-destabilizing lipids in this region. In *Paramecium* the site of fusion pore formation on the plasma membrane is within an ordered array of membrane particles (proteins) known as the rosette (Knoll et al., 1991) that could be important for fusion pore formation. The fusion pore that forms during haemagglutinin-mediated influenza virus fusion with the cell surface is preceded by the formation of an ordered array of haemagglutinin proteins within which the fusion pore is believed to form (White, 1992). Candidates for the proteins involved in cross-linking the bilayers and lipid reorganization during exocytosis are the annexins (discussed above, and see Creutz, 1992; Pollard et al., 1991). Growing evidence shows that the annexins can form multimeric structures on lipid bilayers (Zaks and Creutz, 1991) and interact with the bilayer sufficiently to act as Ca^{2+} channels (Pollard et al., 1992).

The Monck and Fernandez model is fully consistent with all available data and seems intuitively reasonable. The correctness of this model will only become apparent when the essential exocytotic proteins are identified, but at the moment it provides an important pointer to the idea that these proteins do not necessarily need to be transmembrane vesicle or plasma membrane proteins as long as they can, alone or with other proteins, cross-link lipid bilayers in response to a rise in $[\text{Ca}^{2+}]_i$ (or the activation of GTP-binding proteins) and produce focal changes in lipid structure or organization.

The influence of lipid composition on membrane fusion in biological systems still remains to be fully explored, as does potential lipid changes during exocytosis. It had been suggested that lysolipids could promote membrane fusion (Poole et al., 1970) but more recent findings have clearly shown the opposite, that lysolipids inhibit biological membrane fusion (Chernomordik, 1993). Further work is needed in this area to define the role of lipids in the formation of the fusion pore.

SUMMARY AND FUTURE PROSPECTS

After many years of work that had given only limited insights into the proteins involved in regulated exocytosis, several soluble and membrane proteins that are essential or regulatory components of the exocytotic machinery have been identified recently. The picture that is emerging suggests that several proteins are likely to act in discrete steps in exocytosis or act together to form some kind of fusion machine. Further components are likely to be identified in the near future and a major area for investigation will be the nature of the interactions between these proteins.

Work on enveloped viruses has identified proteins with specific fusion peptide sequences that mediate viral fusion with the plasma membrane (White, 1992). These proteins act in binding the virus to the membrane as well as in membrane fusion. The fusion peptides from various viruses do not possess any marked sequence similarity, but all of them can potentially fold as a helix with a hydrophobic domain on one side. A protein that mediates sperm fusion with the egg has structural similarities to these viral proteins (Blobel et al., 1992). So far, no protein has been identified as being involved in exocytosis that has the properties of these fusion peptides. It will be interesting to see if intracellular bilayer fusion turns out to involve mechanisms related to or distinct from that in extracellular fusion, although one important point in these considerations will be the different lipid compositions of the inner and outer leaflets of the plasma membrane bilayer. It is interesting that viruses have evolved quite distinct fusion peptide sequences to solve the same biological problem and so it is conceivable that exocytosis could involve quite different proteins.

One important consideration is the relationship between constitutive and regulated exocytosis and between exocytosis and other intracellular transport vesicle steps. As described above, certain proteins known to be required for various intracellular vesicular transport steps (NSF and SNAPs) have been implicated in neurotransmitter release (Sollner et al., 1993) and in constitutive exocytosis (NSF; Sztul et al., 1993). In addition, annexins have been implicated in various membrane fusion events in the endocytic pathway (Emans et al., 1993; Lin et al., 1992). It is not yet clear to what extent the same or similar proteins are involved in membrane fusion of both constitutive and regulated secretory vesicles, but homology has been noted between nerve terminal proteins involved in Golgi-to-plasma-membrane transport in yeast (Bennett and Scheller, 1993). Since constitutive secretory vesicles can readily fuse with the plasma membrane immediately after their formation this suggests that fusion of regulated vesicles must be inhibited at resting $[Ca^{2+}]_i$. One possibility is that Ca^{2+} acts primarily to disinhibit the fusion mechanism. The inhibition could be due to the cytoskeleton acting as a cortical barrier, or holding regulated vesicles in a network (e.g. via synapsin I) to prevent exocytosis, or to additional mechanisms. Recent work on the biogenesis of small synaptic-like microvesicles in PC12 cells has suggested that synaptic vesicle proteins may be exported from the trans-Golgi network to the plasma membrane in constitutive secretory vesicles, recycled via the endosome pathway and only then become segregated to the regulated secretory vesicle (Cutler and Cramer, 1990; Linstedt and Kelly, 1991; Regnier-Vigouroux et al., 1991). The implications of this is that the synaptic vesicle proteins begin their life in vesicles (constitutive and recycling endocytic) that can spontaneously fuse with the plasma membrane but then become restricted to vesicles that can only fuse in response to a $[Ca^{2+}]_i$ rise. Does this mean that an inhibitory control becomes associated with the synaptic vesicle at a late stage in its biogenesis? This question may be significant for models in which synaptic vesicle membrane proteins are key elements in the fusion machinery.

Further progress towards solving the details of regulated exocytosis will require continued functional analysis, particularly of membrane proteins. An *in vitro* fusion system would be particularly useful for such studies and the *Xenopus* oocyte promises to provide an important experimental system. As we described above, synaptic vesicles can be assembled in oocytes from injected brain mRNA and the roles of particular vesicle proteins in neurotransmitter release assessed (Alder et al., 1992a). In addition, the oocytes possess a regulated secretory pathway

and Ca^{2+} -dependent exocytosis of chromaffin granules has been observed after their injection into *Xenopus* oocytes (Scheuner et al., 1992). This system could allow systematic manipulation of granule membrane proteins prior to the injection of the granules and thus provide functional data regarding these proteins.

Finally, future work on mutants of regulated exocytosis in *Tetrahymena* (Turkewitz et al., 1991) or *Paramecium* (Bonnemai et al., 1992) is likely to provide key insights into the nature of the proteins essential for exocytosis in the same way that the study of *Saccharomyces cerevisiae* has illuminated the constitutive secretory pathway. *Paramecium* may be a particularly useful organism for such studies, despite the difficulty of molecular genetics on this organism, because the biochemistry and morphology of exocytosis in *Paramecium* has been studied in detail (Hohne-Zell et al., 1992; Knoll et al., 1991; Momayez et al., 1987; Stecher et al., 1987). The progress of biochemical studies on regulated exocytosis in the past year or so has been considerable, and the combination of this approach with a genetic approach should mean that within the next few years regulated exocytosis will be as well understood as other vesicular transport steps in the secretory pathway.

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