Exocytosis in excitable cells: a conserved molecular machinery from yeast to neuron

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Abstract

One of the basic cellular functions of nearly every cell type is the exocytotic release of synthesized molecules, stored and packaged into intracellular vesicles or granules. A variety of approaches has been used to identify and characterize the molecules that mediate vesicular trafficking along the secretory pathway. The findings obtained with these approaches suggest that common mechanisms may underlie a wide variety of vesicle-mediated transport steps.

This review presents some of the recent findings regarding the study of the cellular mechanisms which control neurotransmitter and hormone release from neurons and endocrine cells respectively, and focuses on regulation of these mechanisms. The similarities between these two cell types can be seen as evidence to support the hypothesis according to which the regulated exocytosis apparatus could have evolved from a constitutive fusion machinery to which some key modulators have been added. Insight into secretory vesicles will be relevant not only to the understanding of vesicular trafficking or cell polarity but also to the understanding of higher nervous functions resulting from synaptic plasticity.

European Journal of Endocrinology 137 1–9

Introduction

The membrane-bound compartments into which eukaryotic cells are divided allow specialized and segmented functions to occur in a series of distinct environments. Among these functions is the transport of macromolecules into and out of cells by endocytosis and secretion, as well as intracellular transport, which is required for the biogenesis of the compartments themselves. Movement between compartments is carried out by transport vesicles that bud from one compartment and fuse with another in a highly choreographed manner, allowing net movement of cargo in a unidirectional, prescribed pattern of flow. Membrane fusion must be a specific and carefully controlled process, otherwise the vesicle shuttle will be delivered to an incorrect acceptor compartment. Such a situation would destroy the highly differentiated, compartmentalized structure of the eukaryotic cell and disrupt the ordered, vectorial, sequential processing and trafficking of newly synthesized products. The means by which eukaryotic cells achieve such a degree of fidelity has recently been the topic of intense investigation which has highlighted the role of low molecular weight GTPases. Their function depends on their ability to alternate between inactive and active forms as well as on their localization. It has been proposed that, through their function, fidelity in the process of docking and/or fusion of vesicles with their correct acceptor compartment is ensured.

Membranes compartmentalize but also isolate cells from their immediate environment. The process of exocytosis is an important means of overcoming such isolation, since secretory products may be released into the external milieu through both constitutive and regulated secretion. Hormone release and synaptic transmission are indeed the currency of information exchange in the whole body. The key event in these information exchanges is exocytosis by membrane fusion of secretory granules or synaptic vesicles. A class of secretory vesicles contained in many cell types can fuse with the membrane only after cell activation. It is this regulated exocytosis that will be the subject of this review.

Models for the study of regulated secretory events

A significant contribution towards our knowledge of vesicular transport has come from genetic studies on the yeast Saccharomyces cerevisiae (1). However, since
this organism does not appear to possess a regulated exocytosis 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. An alternative approach which has been particularly fruitful in identifying the molecules that mediate vesicular trafficking along the regulated secretory pathway is the biochemical characterization of secretory granules and synaptic vesicles and their related proteins. Studies on a variety of cell types have shown that both constitutive and regulated exocytosis require cytosolic proteins and ATP to be optimal. However, constitutive exocytosis differs from regulated exocytosis in that it is unimpaired even if the concentration of cytosolic free calcium ([Ca^{2+}]) is reduced to well below resting levels. Many of the vesicular transport steps studied involve both formation and fusion of vesicles, but regulated exocytosis involves triggered fusion of already formed vesicles. This may explain why certain vesicular transport steps are markedly blocked as temperature is reduced to 20°C or below, whereas exocytic fusion does not show such an acute temperature discontinuity and continues down to 4°C.

**Secretory events studied in different cell types**

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 an internal signal to be transduced into activation of the dormant fusion machinery. This could involve removal of inhibition as well as direct activation. To understand fully the mechanism of regulated exocytosis it is necessary to identify those proteins that act as regulatory as well as essential components of the exocytic mechanism. 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 for regulated secretory cells has resulted in the evolution of a variety of distinct mechanisms for membrane fusion in constitutive exocytosis (2).

Even if there is still some debate, it has been generally accepted that vesicles are involved in the release of both neurotransmitters and hormonal products. Already, in the pioneering work of Douglas, it was suggested that hormonal release is an exocytotic process (3). When assaying the materials released from chromaffin cells with biogenic amines (norepinephrine and epinephrine), Douglas also found ATP, chromogranins and dopamine β-hydroxylase. Moreover, these constituents were present in the same molar ratios within the secretory vesicles isolated from the medulla by centrifugation.

The adenohypophysis is another endocrine tissue composed of different cells secreting a variety of hormones. These are stored within the cells in dense core granules and are released by appropriate stimuli. In addition to dense core granules, anterior pituitary cells contain small translucent vesicles, which are very similar to synaptic vesicles not only in their morphological appearance but also with regard to their protein constituents (4, 5). For example, chromogranins and secretogranins found in secretory granules and small vesicles of endocrine cells have also been detected in their neuronal counterparts (see 6 for a review). It is also noteworthy that membrane proteins from synaptic vesicles such as synaptotagmin, a calcium sensor protein, have been found associated with secretory granule membranes. All these findings indicate that neurons and neuroendocrine cells are very similar with respect to the composition of their characteristic small vesicles and large secretory granules besides having a variety of molecular, biochemical, and functional similarities (7). Such similarities in the molecular machinery for secretion suggest that processes responsible for secretion of neurotransmitters and hormones are likely to have evolved from the trafficking machinery that controls secretion in more simple cells such as in yeast (8).

**Packaging and storage in different secretory organelles**

Endocrine tissues share many properties with their neuronal counterparts. Secretory products from endocrine cells, including small molecules and expressed proteins, are – as in neurons – sequestered in two different cytosolic organelles: small vesicles and secretory granules. Vesicles (or synaptic vesicles in neurons) are small and very homogeneous in diameter (about 50 nm), and they may contain chemical neurotransmitters such as acetylcholine, γ-aminobutyric acid (GABA), glutamate, glycine and the biogenic amines, but they do not contain soluble proteins. Secretory granules (or large, dense core vesicles in neurons) are larger (70–400 nm) and have a characteristic dense core in electron micrographs due to their content of soluble proteins. Both classes of organelles release their contents by exocytosis, but the way by which they acquire this is different (for a review see 9). Although the current view is that these two populations of vesicles are distinct and independent of one another, the possibility that the smaller vesicles are derived from the larger ones has not been ruled out definitively. Vesicular stores constitute a large reserve of transmitter that is protected from intracellular catabolism. These vesicles may contain small-molecule transmitters or neuroactive peptides. Because the latter are synthesized as secretory products, it can be assumed that essentially all of the peptides within an endocrine cell or a neuron are packaged within vesicles. Unlike small-molecule transmitters, none of these peptides are synthesized in the cytosol and no mechanism for regulating their cytoplasmic concentration need exist. The absence of
specific enzymes for controlling the intracellular store of these messengers is an important feature.

The way by which secretory organelles acquire their contents is different in vesicles and granules. Packaging into synaptic vesicles is performed from cytoplasmic pools, hence synaptic vesicle membranes contain (i) electrogenic proton pumps making the inside positive with respect to the outside (10) and (ii) chloride channels which dissipate the membrane potential and allow the build-up of an acidic environment inside the vesicles (11). After releasing their contents, the secretory vesicles reform and fill with transmitter from the cytoplasmic pools: the electrochemical gradient generated by the proton pump drives the accumulation of transmitters via several classes of neurotransmitter transporters. On the other hand, since secretory granules contain proteins, they cannot refill in the cytoplasm after undergoing exocytosis. Hence, packaging into secretory granules occurs in the trans-Golgi network.

The exocytic machinery is regulated by calcium

The role of [Ca\(^{2+}\)]\(_i\) in secretion from endocrine cells has been studied in three ways: (i) the effect of elevated [Ca\(^{2+}\)]\(_i\) on secretion rate has been measured in permeabilized cells (12, 13); (ii) the average [Ca\(^{2+}\)]\(_i\) has been assessed during secretion triggered by depolarization or release of calcium from internal stores; (iii) the secretion stimulated by perfusion of cells with solutions of different calcium concentrations through a whole-cell patch pipette has been monitored. These studies have shown that there is a threshold level of peak [Ca\(^{2+}\)]\(_i\), for hormone release to occur. In addition, the release of hormones and neurotransmitters has been found to be linearly related to the time integral of [Ca\(^{2+}\)]\(_i\) elevation above this threshold (14).

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. The initial activation of exocytosis is believed to require a high cytoplasmic [Ca\(^{2+}\)], (10–100 μM) that is achieved locally at the presynaptic membrane following Ca\(^{2+}\) entry through plasma membrane channels (the resting value of [Ca\(^{2+}\)]\(_i\) is around 100 nM). These high values of Ca\(^{2+}\) will allow the release exerted by a ‘fusion-clamp’ molecule such as synapsin and synaptotagmin (see below). 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. In neuroendocrine cells there is an extensive actin network that acts as a barrier to regulated secretory vesicles in the cell periphery. The difference in organization in these cells compared with synapses may be that an actin
network would not be sufficient to impede smaller synaptic vesicles, leading to the requirement for synapsin and synaptotagmin for direct linkage of synaptic vesicles to the cytoskeleton.

In neuroendocrine cells, the rate of secretion achieved for a given measured \([\text{Ca}^{2+}]_i\) is substantially less than that observed in response to a brief depolarization. This indicates that depolarizing pulses open calcium channels so that \([\text{Ca}^{2+}]_i\), is locally elevated near release sites to levels substantially higher than the average \([\text{Ca}^{2+}]_i\), recorded with photometric techniques. Consistent with such an hypothesis, even at high cytoplasmic \([\text{Ca}^{2+}]_i\), (300–1000 nM), secretion rates during dialysis always remain clearly lower than those obtained during voltage pulses. Several other means of rapidly elevating \([\text{Ca}^{2+}]_i\), also yield higher secretion rates. Conversely, the total amount of secretion is always much larger with calcium dialysis than with rapid calcium elevation using caged calcium ions or depolarization-induced calcium entry. This observation may be related to the existence of multiple pools of hormone, i.e. a relatively small and rapidly releasable pool and a larger reserve pool. In this respect, both movement of vesicles between pools and secretion have been shown to be sensitive to the intracellular Ca\(^{2+}\) level (15, 16). In conclusion, exocytosis can be regulated at the level of secretory vesicle availability as well as by direct effects on membrane fusion.

**Implications of the SNARE hypothesis for secretory vesicle cycle**

Vesicle shuttles allow travel between a pair of membrane-bounded compartments. One member of the pair (the donor compartment) produces the transport vesicles and the other (the acceptor compartment) receives the vesicle and its cargo (Fig. 1). The set of all such shuttles constitutes the vesicle flow pattern of the cell. Individual proteins are transported within the pattern according to intrinsic signals that dictate their ability to enter or avoid the various shuttles. The core protein machinery that underlies vesicle transport includes coat proteins, which sculpt a vesicle out of a donor membrane; the vesicle- and target-specific identifiers y-SNAREs and t-SNAREs (for vesicle- and target-specific SNAP receptors respectively), which bind each other and thereby dock the vesicle to the acceptor membrane; NSF (for N-ethylmaleimide-sensitive fusion protein) and SNAP (for soluble NSF attachment protein) proteins, which bind to the SNARE complex and initiate fusion when NSF hydrolyzes adenosine triphosphate (for recent reviews see 17–19) (Fig. 1).

Although additional pieces of machinery and important mechanistic details probably remain to be uncovered, the SNARE hypothesis provides a general and complete mechanism for the specific docking and fusion of transport vesicles with their target membranes. According to this model, secretory vesicles dock and then proceed through a partial fusion reaction, priming, to make them competent for the final Ca\(^{2+}\)-triggered step. During priming, a complex called the core complex is assembled by three abundant synaptic proteins, two from the plasma membrane (syntaxin and SNAP-25) and one from secretory vesicles (synaptobrevin). The core complex forms the anchor for a cascade of protein–protein interactions required for exocytosis to occur. Once this trimeric core complex has assembled, it serves as a receptor for SNAPs and NSF. NSF is a trimeric ATPase with a universal function in membrane fusion. SNAPs are soluble proteins (there are three isoforms quoted. \(\alpha\), \(\beta\) and \(\gamma\)) required to recruit NSF from the cytosol to membranes in an ATP-dependent manner. Synaptobrevin, syntaxin and SNAP-25 were affinity purified on immobilized \(\alpha\)-SNAP but individually bind only weakly to \(\alpha\)-SNAP. Only their assembly onto the core complex results in the formation of a high-affinity binding site for \(\alpha\)-SNAP which in turn creates a receptor site for NSF. As a trimer, NSF then probably multi-merizes core complexes and disrupts them under ATP hydrolysis (see 17–21 for recent reviews). Thus an ordered sequence of protein–protein interactions leads to the assembly of a multimeric complex that is then disrupted by the enzymatic activity of NSF.

The role of the NSF/SNAP-dependent pathway in both constitutively ‘on’ fusion events, such as intracellular transport, and regulated fusion events, which are kept ‘off’ in the absence of a special signal, such as triggered exocytosis, raises the question of how the general fusion program can be kept inactive until a signal for exocytosis is received. One possibility is that one or a series of ‘fusion clamps’ stop progress along the general pathway at critical junctures. These clamps would be removed in response to second messengers inducing exocytosis. Such clamps would generally be membrane proteins so as not to stop other fusion processes (which must remain constitutive in most cells). Synaptotagmin plays the primary role in triggering exocytosis by binding Ca\(^{2+}\) and then changing conformation when Ca\(^{2+}\) is elevated following an action potential, although other triggering mechanisms also exist. Synaptotagmin also competes with \(\alpha\)-SNAP for binding the SNARE complex and could, therefore, prevent fusion by blocking assembly of the general fusion apparatus. Upon entry of Ca\(^{2+}\), synaptotagmin could dissociate from the SNAREs, allowing \(\alpha\)-SNAP to bind and fusion to proceed (for recent reviews, see 17–21).

**Membrane capacitance measurements reveal exocytotic and endocytotic activities**

**The patch-clamp technique**

The whole-cell recording configuration of the patch-clamp technique offers a number of tools to study the signal pathways involved in regulated secretion. First, it
allows the recording of whole-cell currents that are involved in the control of \([\text{Ca}^{2+}]_{i}\). Secondly, in favorable cases it provides an assay for the secretory process itself through membrane capacitance measurements by providing direct measurement of surface area. Indeed, when a secretory granule fuses with the plasma membrane during exocytosis, the surface area of the secreting cell increases. Conversely, the surface area of a cell decreases when membrane is retrieved from the plasma membrane by endocytosis. These changes in the cell surface can be followed by monitoring the electrical capacitance of the cell. The technique has such high-quality resolution that fusion events of even single vesicles can be recorded. Thirdly, it is readily combined with the measurement of \([\text{Ca}^{2+}]_{i}\), by calcium indicator dyes (i.e. Fura-2, Indo-1, Fluo-3). Finally, it allows introduction of solutions of known composition into the cell interior, since there is rapid diffusional exchange between a patch pipette milieu and a small cell compartment, in the tight-seal whole-cell recording configuration.

As early as 1776, long before the nature of electricity was understood, Cavendish wondered whether the thin membranes of the cells in the electric organ of *Torpedo* (the electric ray) might not function like the glass walls of Leiden jars, which were used at that time to store electricity. Today, we know that because cell membranes are able to maintain a difference of electrical potential, they exhibit electrical properties such as membrane resistance and capacitance. The basic rules for measuring voltages and currents in a circuit containing electrical elements such as those found in a cell are: (i) voltages must be measured from one end of the elements to the other; and (ii) currents must be measured in the path containing the element. Making such measurements in cells poses several problems; one side of the membrane faces the inside of the cell, neither side is facing metallic electrical conductors, and cells are small. Electrical access to the inside face of the membrane can be obtained with fine, membrane-penetrating glass microelectrodes or with a ‘patch’ electrode isolating a ruptured patch of membrane. Depending on the desired resolution, various methods derived from the patch-clamp recording technique can be used to assess membrane capacitance. The methods and details for their application have been described elsewhere (22–26).

In the resting state, secretory vesicles are docked and primed for fusion by a combination of plasma membrane, vesicle and cytoplasmic proteins that form a docking/fusion complex. At this stage, the capacitance of the vesicle membrane is not electrically visible because the vesicle interior has no electrical continuity with the extracellular space. Upon depolarization, exocytosis is initiated as calcium ions enter via open calcium channels and bind to a sensor molecule(s) that could be part of the fusion complex. The initial stage of exocytosis in non-neural secretory cells is the formation of a fusion pore which establishes an electrical and diffusional connection between the vesicle interior and the extracellular space. Various lines of evidence suggest that after the fusion pore opens, the vesicle usually collapses completely and incorporates fully into the plasma membrane. The pore itself is thought to be a protein, similar to an ion channel, which expands after its initial opening and promotes lipid–lipid fusion between the vesicle and plasma membranes. Alternatively, the opening of the fusion pore might be a reversible process.

Measurements of cell membrane capacitance (\(C_m\)) have been applied to the investigation of the influence of \([\text{Ca}^{2+}]_{i}\) on exocytosis of neurons and neuroendocrine cells (for reviews see 26–29). Intracellular \([\text{Ca}^{2+}]\) can be modulated by either dialyzing the cytosol with different calcium-containing pipette solutions or changing the membrane potential. High calcium resulted in a large \(C_m\) increase, demonstrating the role for \([\text{Ca}^{2+}]_{i}\) in stimulus-secretion coupling as previously suggested in pituitary cells (30). Interestingly, in some pituitary cells such as lactotropes (prolactin secreting cells), a tonic calcium influx through voltage-gated calcium channels measured at resting membrane potentials was found to play a major role in secretory activity monitored by \(C_m\) (for a review see 27). A number of other signaling pathways have also been suggested to modulate hormonal release from pituitary cells. In gonadotropes, breakdown of polyphosphoinositides induces calcium oscillations which trigger rhythmic exocytosis (31). In lactotropes, cAMP was found to increase the magnitude and rate of calcium-induced exocytosis. In contrast, cAMP had no detectable effect on \(C_m\) when intracellular calcium was low (32). Therefore, it can be concluded that cAMP facilitates calcium-induced secretion by acting directly on the secretory apparatus of anterior pituitary cells. The role of GTP-binding proteins on \(\text{Ca}^{2+}\)-induced exocytosis has also been investigated in lactotropes using non-hydrolyzable GTP analogs (GTP-\(\gamma\)-S and GMP-PNP) to irreversibly activate all GTPases. In this way, two distinct effects of G-protein activation could be distinguished: the maximum \(C_m\) increase due to intracellular calcium injection was diminished, while the rate of \(C_m\) increase (\(\Delta C_m/\Delta t\)) was facilitated, revealing a converse stimulatory role of G-proteins in the translocation of secretory granules to the fusion sites (33, see below).

**Spatial and temporal constraints for exocytosis**

Exocytosis in neurons and neuroendocrine cells is triggered by an increase in the cytosolic concentration of calcium, and is followed by endocytotic membrane retrieval. These excitable cells have several systems which regulate \([\text{Ca}^{2+}]_{i}\) levels. Calcium influx into the cytoplasm is largely mediated by voltage-sensitive and ligand-gated plasma membrane channels (34) as well as
by release from internal organelle compartments. On the other hand, mechanisms reducing $[\text{Ca}^{2+}]$ levels include plasma membrane extrusion systems, such as the $\text{Ca}^{2+}$-ATPase, the $\text{Na}^{+}$-$\text{Ca}^{2+}$ exchanger, organelle sequestration and $\text{Ca}^{2+}$-binding proteins. All of these processes control both the resting $[\text{Ca}^{2+}]$, levels and the regulation of $[\text{Ca}^{2+}]$, transients occurring as a result of electrical activity and/or other environmental chemical signals such as hormones and neurotransmitters.

It is of interest that as one moves through the yeast secretory pathway towards the plasma membrane, the vesicular trafficking components become more homologous with the nerve terminal components. For example, synaptobrevin is most like SNC, Rab3 is most like SEC4, and syntaxin is most like SSO (SNC, SEC 4 and SSO are all membrane-bound proteins first characterized in yeast). Therefore, the current model for exocytosis in excitable cells (see Fig. 1) has counterparts in the yeast secretory pathway as well as potential regulatory elements likely to be specific to hormone and neurotransmitter release. This suggests the interesting possibility that the machinery responsible for secretion of neurotransmitters is likely to have evolved directly from the trafficking machinery that mediates delivery of vesicles from the Golgi to the plasma membrane. Added diversity into the molecular machinery of exocytosis is due to variation between cell types in the regulation of exocytosis and in specialized features of the process. Synaptic transmission requires a localized and rapid signal that can be repeated at high frequency and up- or downregulated with time. 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 $\mu$s or so of $\text{Ca}^{2+}$ channels being opened to allow $\text{Ca}^{2+}$ entry; it may result exclusively from exocytosis of synaptic vesicles docked to presynaptic $\text{Ca}^{2+}$ channels (35, 36). It should also be added that the stimulation of primed synaptic vesicles is poorly efficient: only one of many docked synaptic vesicles fuses, and only one in every three to ten action potentials leads to exocytosis. In contrast, exocytosis in neuroendocrine cells is triggered after a lag period of 3–50 ms (15). Part of this delay might be a result of the recruitment of undocked secretory granules and the assembly of the fusion machinery, since secretory granules that are docked tightly as synaptic vesicles in synapses are not usually observed in resting neuroendocrine cells. In nerve terminals, dense-core granules are also not docked on the plasma membrane, require a higher frequency of stimulation for exocytosis and are likely to show a slower time-course of release (see 37 for a recent review). Exocytosis of neuroendocrine secretory granules might, therefore, resemble exocytosis of neuronal dense-core granules more closely than that of synaptic vesicles. It is possible that in neuroendocrine cells, such as pituitary cells or adrenal chromaffin 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 suggests that the machinery responsible for synaptic vesicle fusion may be preassembled or ‘primed’ in a prefusion complex.

It has become apparent that the diffusion of $\text{Ca}^{2+}$ in the cytoplasm is severely limited by immobile $\text{Ca}^{2+}$ buffers. The diffusion constant for $\text{Ca}^{2+}$ in the cytosol has been determined to be approximately 10–13 $\mu$m$^2$ s$^{-1}$ so that $\text{Ca}^{2+}$ would diffuse no more than 10 nm from the mouth of the $\text{Ca}^{2+}$ channel within the 200 $\mu$s that is required for fast neurotransmission, supporting the idea that synaptic vesicles must be associated closely with $\text{Ca}^{2+}$ channels. The recent report showing that calcium channels interact with the synaptic core complex supports this idea (38). Data obtained from imaging of the distribution of $\text{Ca}^{2+}$ and theoretical considerations have suggested that exocytosis in synapses is controlled by a $\text{Ca}^{2+}$-binding protein(s) with a low affinity (saturating at >100 $\mu$M free $\text{Ca}^{2+}$), but that exocytosis in neuroendocrine cells is regulated by a $\text{Ca}^{2+}$-binding protein(s) with higher affinity (see 37 for a recent review).

Data obtained from capacitance measurements and those provided by other techniques such as fluorescent membrane markers indicate a good start towards establishing an accurate picture of synaptic vesicle and secretory granule dynamics. Technical advances to improve the detection of exocytosis of small numbers of vesicles combined with judicious choice of biological preparations may expand this emerging picture of presynaptic processes.

**Modulation of regulated exocytosis involves G-proteins**

Vesicle docking at the plasma membrane involves secretory vesicle proteins which act as key elements of the fusion mechanism subject to regulation. Over the past few years, extensive work has been carried out in an attempt to characterize secretory vesicle proteins, and considerable information is now available on the membrane proteins of secretory granules and synaptic vesicles (for recent reviews see 19, 21, 39). Nearly all secretory granule and synaptic vesicle proteins so far identified are members of small gene families that are specific to neural or endocrine tissues. For example, the superfamily of Ras-related, small GTP-binding proteins currently comprises over 50 members, which have been found to regulate a large spectrum of elementary cellular processes.

Many vesicular steps are regulated by GTP-binding proteins (or G-proteins; for recent reviews see 21, 39). The first identified G-protein involved in vesicular transport was the monomeric G-protein, Sec4, which is required for constitutive exocytosis in yeast (40). A search for the mammalian counterparts of Sec4 has led
to the identification of a large number of Ras-related proteins termed Rab proteins for ‘Ras-like proteins from rat brain’. The proteins of the Rab gene subfamily have common not only their structural features, but also their ability to regulate intracellular vesicle traffic and sorting at the plasma membrane (41, 42). By analogy with other G-proteins, the Rab proteins are considered inactive in their GDP-bound form. This form is stabilized by guanine nucleotide dissociation inhibitors (GDI).

Upon stimulation by a guanine nucleotide releasing protein (GNRP), the GDP is exchanged for GTP, and the Rab proteins switch to their active, GTP-bound form. This state is quasi-irreversible until the Rab proteins hydrolyze the bound GTP to GDP, this effect being stimulated by a GTPase activating protein (GAP). Thus, the proteins become inactivated, allowing the process to be repeated. Hence, as with other G-proteins, Rab proteins behave as molecular switches cycling from an active (GTP-bound) to an inactive (GDP-bound) conformation (43). Since the general role of G-proteins is to control the specificity and the temporal coherence of intermolecular recognition processes, it is very likely that Rab proteins act as regulators of vesicular targeting from an upstream to a downstream compartment. It is now clear that each Rab family member has distinct subcellular locations within cells and that most of the organelles involved in exocytosis or endocytosis possess at least one of these distinct members (5, 44).

Depending essentially upon the sequence of their C-terminus, each member of the Rab protein family seems to be associated with a particular vesicular compartment (see 42 for a review). In addition, a posttranslational isoprenylation of cysteine residues at the C-terminus of the Rab proteins is required for their functional association with the vesicular membrane (45). Although most of the Rab family members are uniformly distributed in mammalian cells (46), they could also be involved in more differentiated secretory or compartmentalized processes specific to neurons, endocrine or epithelial cells. In this respect, it has been reported that Rab3A is specifically expressed in neurons (47, 48), where it could control the recruitment of synaptic vesicles and their fusion with the plasma membrane during exocytosis (49, 50). Other members of the Rab3 subfamily, termed Rab3B and Rab3C (51–53) are thought to be associated with the membrane of secretory vesicles or granules, although this has not yet been demonstrated. Whether the Rab3 protein isoforms are functionally different proteins expressed in different cell types, or proteins associated with different kinds of secretory vesicles, awaits further investigation.

Additional G-proteins, including the monomeric ARF proteins (GTPases of the Ras family) and heterotrimeric G-proteins, have also been shown to control vesicle budding and transport. The α-subunits of the heterotrimeric G-proteins were first identified by their function in the transduction of hormonal or neurotransmitter signals arriving at the plasma membrane. Several reports indicate that the function of α-subunits is more complicated than originally anticipated: transport may involve several G-proteins regulating each step in opposing directions (for a review see 54). Hence, monomeric and heterotrimeric G-proteins have been suggested as candidate address tags; the switching mechanisms involved in recruiting, activating and inactivating the vesicle address tags are likely to involve Rab, ARF and heterotrimeric G-proteins.

**Overall view**

Recently, after many years of work that had given only limited insights into the identity of proteins involved in regulated exocytosis, several soluble and membrane proteins that are essential or regulatory components of the exocytotic machinery have been identified (for a review see 55). 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. 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 hormonal and neurotransmitter release as well as in constitutive exocytosis. It is not yet clear to what extent the same or similar proteins are involved in membrane fusion of both constitutive and regulated secretory pathways, but it is noteworthy that homology has been noted between nerve terminal proteins involved at synaptic transmission and those found at the Golgi-to-plasma-membrane transport in yeast (8).

Membrane trafficking along the exocytic and the endocytotic pathways is mediated by a number of vesicular intermediates. This vesicular transport is vectorial since each step is mediated by specific targeting, docking and fusion events. Among these processes, calcium and GTP regulated exocytosis lead to the final stage of the secretory pathway. The combination of yeast genetics, biochemistry of synaptic proteins and in vitro reconstitution of vesicular transport has provided convincing evidence for a common mechanism which involves elements conserved throughout evolution and indicates that regulated exocytosis is a modification of constitutive secretion. Each of the proteins described in the secretory pathways (i.e. synaptobrevins, syntaxins and small G-proteins) or in the putative docking complex (i.e. NSF and SNAPs) has homologs in yeast. The yeast forms of these proteins with the greatest similarity to nerve terminal proteins are those involved in the Golgi-to-plasma-membrane stage of secretion. It is, therefore, not surprising that neurons and endocrine cells are very similar with respect to the composition of their characteristic small vesicles and large secretory granules, besides having a variety of functional similarities. However, more recent
morphological and biochemical observations indicate that synaptic transmission differs in many respects from glandular release. Release by endocrine cells mainly involves the large dense-cored granules that contain high concentrations of hormonal products; these large vesicles interact slowly with the plasma membrane of the gland cell. In nerve endings, the small vesicles contain little, if any, core proteins whilst the large vesicles contain low molecular weight amine transmitters, core proteins, and peptides. Since all the neuropeptides are found primarily, or even perhaps exclusively, in larger vesicles of neurons, nerve terminals would seem to maintain the more 'primitive' mechanism of release used by gland cells.

Acknowledgements

The author would like to thank Prof. J-D Vincent for illuminating the path followed and Dr P Vernier for his many inspirational conversations and collaborations. This work has been supported over the years by several grants from the CNRS, UFR Kremlin Bicêtre and Fondation pour la Recherche Medicale.

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Received 22 January 1997
Accepted 8 April 1997