

Ca²⁺-regulated exocytosis and SNARE function

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Secretory vesicles formed at the *trans*-Golgi network of neuroendocrine and endocrine cells must undergo several steps, such as translocation, docking and priming, before they are ready to fuse with the plasma membrane and deliver their cargo into the extracellular space. This process is called regulated exocytosis and is controlled by Ca²⁺ (using synaptotagmin) and mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins. Recent studies from three leading laboratories reveal novel details about the mechanism by which Ca²⁺ and SNAREs regulate this complex process. These findings highlight the roles of both SNAP25 (synaptosome-associated protein of 25kD), one of the SNARE proteins, and CAPS (Ca²⁺-dependent activator protein for secretion), a Ca²⁺-sensor protein, in vesicle priming, depriming and fusion.

Membrane fusion physically merges the two lipid bilayers of previously separate compartments and enables transport of cargo from a donor to an acceptor compartment. The majority of these vesicular trafficking steps along the secretory and endocytotic pathways occur constitutively. The secretory pathway delivers newly synthesized lipids or proteins from the endoplasmic reticulum to the plasma membrane and several intracellular organelles. At the plasma membrane, transport can also occur constitutively or fusion is arrested, waiting for specific signals. Although the molecular mechanism responsible for this arrest has not been clarified, our understanding of the activation of fusion at the plasma membrane, called regulated exocytosis, has progressed significantly during the past four years [1,2].

Ca²⁺ and the sequential secretory vesicle pool model

There are two major types of regulated exocytotic events, one occurring in neuroendocrine, endocrine and mast cells, and the other at neuronal synapses [3]. The secretory vesicles in neuroendocrine and endocrine cells, also known as secretory granules or dense-core vesicles, show considerable size variation, have a diameter > 100 nm and frequently contain more than one cargo molecule [4]. The formation of these vesicles requires *de novo* synthesis and translocation of the mediators into the endoplasmic reticulum and transport to the Golgi and subsequently the *trans*-Golgi network, where secretory vesicles are formed. Once formed at the, the majority of secretory granules

accumulate in the cytosol, creating a large undocked vesicle pool, known as a reserve pool. Both classes of vesicles, synaptic and secretory, undergo the same series of events: recruitment of vesicles to the plasma membrane, specific tethering at the plasma membrane, their priming and then triggering membrane fusion [2] (Figure 1). A fraction of vesicles is docked at the plasma membrane but these vesicles are unprimed and are not ready for fusion. The priming of docked vesicles occurs in two stages, resulting in generation of two pools: a slow releasable pool and a rapidly releasable pool [1,2] (Figure 1).

The role of Ca²⁺ in the formation, priming and fusion of vesicles is complex and not completely understood. The resting [Ca²⁺] is sufficient to facilitate many constitutive membrane fusion reactions in the secretory pathway. The first priming step requires a moderate increase in [Ca²⁺], as well as the presence of MgATP, whereas a more substantial elevation in [Ca²⁺] is required for vesicle fusion, which can occur from either primed pools but at different rates. Release is mediated by complete fusion of secretory organelles with the plasma membrane or by the transient opening and closing of the fusion pore, also known as 'kiss-and-run'. Recent findings indicate that all transitional stages are reversible [2] (Figure 1). Dense-core vesicle exocytosis demands considerably longer latency than synaptic vesicles following the rise in [Ca²⁺], ranging from 30 ms to several seconds. In addition to Ca²⁺, several other intracellular messengers are involved in the control of exocytosis [1,2].

Docking and fusion mechanisms

It is believed that hormone release is mediated by SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and is regulated by synaptotagmin, a Ca²⁺-binding protein anchored to the membrane of secretory vesicles using a single transmembrane domain [4]. Syntaxin, SNAP25 (synaptosome-associated protein of 25kD), and synaptobrevin [vesicle-associated membrane protein (VAMP)] are collectively called SNARE proteins. VAMP anchored in the membrane of the secretory vesicles (known as vSNARE) pairs with plasma membrane (or other target membrane) proteins syntaxin and SNAP25 (known as tSNAREs) [5]. Their distribution in opposing membranes led to the hypothesis that tSNAREs form the acceptor site for vSNAREs [6]. Complex formation probably originates at the N-terminals of the SNAREs motifs. Their subsequent zippering into stable membrane-bridging complexes gradually leads into close associations and membrane fusion. The importance

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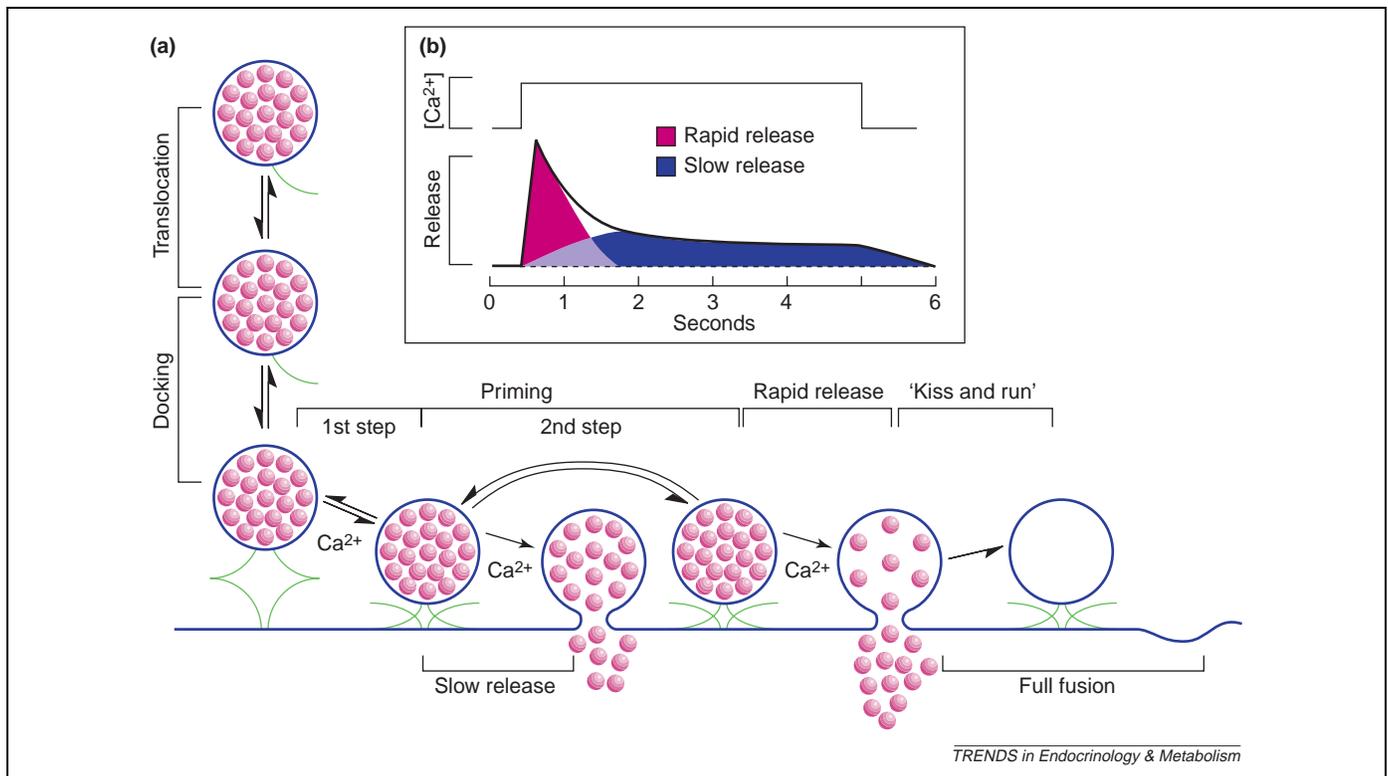


Figure 1. Sequential pool model for dense core vesicle exocytosis. **(a)** After formation at the trans-Golgi network, cytoplasmic secretory vesicles are translocated toward the plasma membrane. A fraction of secretory vesicles near the plasma membrane (10–30%) undergoes docking, followed by priming, which occurs at least in two steps. MgATP and a modest increase in $[Ca^{2+}]$ are required for the first step, resulting in the formation of a slowly releasable pool of secretory vesicles (~2–5%). This is followed by completion of priming, generating the rapidly releasable pool of vesicles (2–5%). An increase in Ca^{2+} in micromolar concentrations triggers the fusion of vesicles from both primed pools. Release is mediated by 'kiss and run' or complete fusion of secretory vesicles with the plasma membrane. **(b)** At the single cell level, the participation of two primed pools in response to a square-wave type increase in $[Ca^{2+}]$ could be separated (schematic representation).

of intact SNARE complex formation for Ca^{2+} -dependent exocytosis was confirmed in experiments with tetanus toxin and different botulinum neurotoxins, which specifically cleave members of the SNARE complex [7]. It is also believed that synaptotagmin is the other key molecular component of the fusion process because it detects elevations in $[Ca^{2+}]$ that probably provide the final force to overcome the energy barrier of lipid rearrangement during the fusion process. Acceleration of SNARE-mediated fusion probably occurs by incorporation of synaptotagmin into VAMP-containing secretory vesicles upon the rise in $[Ca^{2+}]$ [4].

Vesicle priming and depriming

Initiation of the SNARE complex is probably the key molecular event underlying the priming process. Functional SNARE proteins are absent before a Ca^{2+} signal and undergo assembly into complexes following a rise in $[Ca^{2+}]$. Furthermore, formation of the tSNARE complex precedes vSNARE–tSNARE assembly into *trans*-SNAREs. The details of tSNARE complex formation *in vivo* have not been characterized and *in vitro* only partial SNARE complexes can form. However, it is not clear from these studies which of these complexes represent the core complex precursor *in vivo*. An and Almers [8] describe the development of an elegant method for monitoring the formation of SNARE complexes in intact PC12 (immortalized neuroendocrine) cells, using a fluorescent SNAP25 that reports entry into SNARE complexes

by FRET (intramolecular fluorescence resonance energy transfer). This provides evidence for the assembly of a high affinity syntaxin–SNAP25 complex that requires only the N-terminal SNARE motif of SNAP25. Furthermore, this complex assembles reversibly during transient elevations in $[Ca^{2+}]$ induced by depolarization of cells and tolerates a mutation that blocks formation of other syntaxin–SNAP25 complexes. Thus, this complex might function as the plasma membrane 'receptor' to recognize VAMP when vesicles dock, followed by *trans*-SNARE formation during a Ca^{2+} -dependent priming step of exocytosis.

Syntaxin has to be in an 'open' rather than 'closed' conformation to initiate SNARE complex formation. This indicates that any molecule that could perform such function would be a good candidate as the priming factor. Recent studies have assigned such a role to the protein Munc13 in some synapses [9]. Because this protein has several putative Ca^{2+} -binding domains, it might also be responsible for the observed role of elevated $[Ca^{2+}]$ in the priming process. The other potential candidate is CAPS (Ca^{2+} -dependent activator protein for secretion), which exhibits sequence homology with the Munc13 proteins [10]. Recently Grishanin *et al.* [11] suggested that the priming of secretory vesicles occurs in two steps (in contrast to the one step shown in Figure 1). The first priming step is dependent on MgATP and involves synthesis of phosphatidylinositol (4,5)-bisphosphate ($PtdIns(4,5)P_2$). According to this model, the second priming step is

relatively slow and requires Ca^{2+} and CAPS, which binds $\text{PtdIns}(4,5)\text{P}_2$. The exact role of $\text{PtdIns}(4,5)\text{P}_2$ binding in CAPS function is still not known, and the functionally important $\text{PtdIns}(4,5)\text{P}_2$ binding site on CAPS remains to be identified. However, such Ca^{2+} - and CAPS-dependent priming only occurs if MgATP-dependent priming has been completed. This model accommodates the desensitizing effects of sustained elevation in $[\text{Ca}^{2+}]$ on tonic secretion in neuroendocrine cells, which could have a role in depriming secretory vesicles.

Nagy *et al.* [12] also explored the molecular mechanisms of vesicle priming and depriming, focusing on the role of SNAP25 in this process. They used phosphorylation mutants of SNAP25 to show that protein kinase A (PKA)-dependent phosphorylation of SNAP25 increases the size of slowly and rapidly releasable secretory vesicle pools without affecting the kinetics of vesicle fusion, whereas calcineurin activity has the opposite effect. However, protein kinase C (PKC) activity and its phosphorylation of SNAP25 regulate the refilling of pools after they are emptied. The authors further show the role of an additional and still uncharacterized target of PKA that participates in the control of priming. Their data also indicate that the rates of vesicle priming are not affected by SNAP25 and other target-protein phosphorylation, which implies that the PKA signaling pathway affects the rate of vesicle depriming. They also describe the potential role of differential splicing of SNAP25 (which exists in two forms) in control of the vesicle-depriming rate. It appears that the baseline size of the releasable pools is determined independently by the particular splice variant of SNAP25, whereas phosphorylation by PKA regulates the relative size of the exocytotic burst for both variants [13].

Summary

The overall message from these studies is that the intermolecular changes during the formation of the core tSNARE and *trans*-SNARE complexes occur in steps that require a complex intracellular messenger milieu. The first step in this process is the formation of a syntaxin-SNAP25 complex, which probably functions as a precursor

to the core complex formed during the Ca^{2+} -dependent priming step of exocytosis. It appears that cells have two ways of regulating SNAP25 functional contribution in this process: alternative splicing and phosphorylation of this molecule. In addition to SNAP25, CAPS also contributes to the priming of secretory vesicles. SNAP25 and CAPS not only represent the qualitative elements of priming but also influence the size of releasable vesicle pools, reflecting the pre-translation and post-translation modification of SNAP25 and regulation of $\text{PtdIns}(4,5)\text{P}_2$, respectively.

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Everything you want to know about androgens

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The third edition of the encyclopedic book *Testosterone: Action, Deficiency, Substitution* covers almost all aspects of the general biology of androgens and their clinical applications. The authors are primarily from Europe but

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