I. INTRODUCTION 1915

Secretory vesicles in the cytosol contain a variety of bioactive compounds such as neurotransmitters, hormones, enzymes, and cytokines and also carry membrane lipids and proteins (212). Fusion of the membrane of these secretory vesicles with the plasma membrane leads to the opening of the fusion pore, which connects the inside of the vesicle to the extracellular space (425). This releases the molecules contained in the vesicle into the extracellular space and induces lateral diffusion of molecules in the vesicle membranes into the plasma membrane, a process called exocytosis (476). Fusion of vesicles with the target membrane is generally well developed in eukaryotic cells and is used for trafficking various membrane organelles, including the endoplasmic reticulum, Golgi apparatus, lysosomes, and endosomes (283, 534). These membrane fusion events utilize a family of proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. There is a single vesicle SNARE (v-SNARE), called synaptobrevin or vesicle-associated membrane protein (VAMP), and two target plasma membrane SNAREs (t-SNAREs), called syntaxin and SNAP25, which are involved in synaptic exocytosis. When the three proteins are brought together, they form a stable α-helical ternary complex, a process that provides the energy for membrane fusion.

Exocytosis occurs either constitutively, without any external stimulus, or is triggered by extracellular signals (212). The extracellular signals for exocytosis are converted into intracellular signals via Ca\(^{2+}\), cAMP, or protein kinases. Ca\(^{2+}\)-dependent exocytosis plays a major role in the release of neurotransmitters from neurons and in the secretion of hormones and enzymes from endocrine and exocrine cells. However, in spite of similar underlying molecular mecha-
nisms, the kinetics of exocytosis triggered in secretory cells by increases in the concentration of cytosolic Ca$^{2+}$ ([Ca$^{2+}$]) are highly diverse (FIGURE 1). For example, exocytosis involved in neurotransmission in the brain occurs within 1 ms of the presynaptic action potential, whereas exocytosis involved in insulin secretion from the islets of Langerhans to control blood glucose levels can take up to 600 s. Importantly, the same synaptic terminals that show ultrafast exocytosis also show slow exocytosis, i.e., asynchronous and tonic exocytosis of synaptic vesicles and secretion of peptides contained in the large dense-core vesicles (FIGURE 1). Thus understanding exocytoses with diverse kinetic features is essential to synaptic physiology. There are a number of key bodily processes that involve slow exocytoses. For example, the diuretic action of vasopressin in the kidney duct cells is mediated by exocytotic insertion of vesicles that contain aquaporin into the apical plasma membrane, and the action of insulin causes exocytotic insertion of vesicles that contain GLUT4 into the plasma membranes of adipocytes and muscles (TABLE 1).

It is assumed that the ultrafast exocytosis of synaptic vesicles is preceded by the docking of vesicles to the plasma membrane followed by priming and triggering of exocytosis (22, 442, 479). This concept is primarily based on ultrafast (<1 ms) exocytosis (FIGURE 1) and is supported by a specific structure called the active zone. The same concept, however, may not apply to preparations that are devoid of the active zone. The key unresolved questions are as follows: 1) at which stages of SNARE assembly (initial SNARE configurations) do Ca$^{2+}$ and other triggers come into play, and 2) how does SNARE assembly proceed after the trigger? New methodologies have been developed to address these questions, and many mutant animals that lack the key proteins have been generated. Marked progress has been made since one of the authors of the present review wrote an article on the same topic in 1999 (301), and it would be beneficial to reassess the assumptions and terminology, and to interpret new data. We will review representative studies on a wide variety of cell types and present a general overview of diverse secretory phenomena, which are normally

<table>
<thead>
<tr>
<th>Time constants for fusion of single vesicles</th>
</tr>
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<tbody>
<tr>
<td><strong>Ultrafast</strong></td>
</tr>
<tr>
<td>Activation energy ($k_B T$)</td>
</tr>
<tr>
<td>Time constants</td>
</tr>
<tr>
<td>Synaptic vesicles</td>
</tr>
<tr>
<td>Ultrafast exocytosis</td>
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<tr>
<td>Asynchronous exocytosis</td>
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<tr>
<td>Tonic exocytosis</td>
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<tr>
<td>Spontaneous exocytosis</td>
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<tr>
<td>Large dense-core vesicles</td>
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<tr>
<td>Synapses</td>
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<tr>
<td>Chromaffin cells</td>
</tr>
<tr>
<td>Islet β cells</td>
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<tr>
<td>PC12 cells</td>
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<tr>
<td>Mast cells</td>
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<td>Exocrine cells</td>
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<tr>
<td>Transport vesicles</td>
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<td>Kidney duct cells</td>
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<tr>
<td>Gastric parietal cells</td>
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<tr>
<td>Adipocytes</td>
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<tr>
<td>Small vesicles</td>
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<tr>
<td>Chromaffin cells</td>
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<tr>
<td>Islet beta cells</td>
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<tr>
<td>PC12 cells</td>
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<tr>
<td>Mast cells</td>
</tr>
<tr>
<td>Proteoliposomes</td>
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<tr>
<td>SNAREs</td>
</tr>
<tr>
<td>SNAREs + Munc18</td>
</tr>
<tr>
<td>SNAREs in docked vesicles</td>
</tr>
</tbody>
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**FIGURE 1.** Time constants for the fusion of single vesicles in various preparations. For tonic and spontaneous exocytosis of synaptic vesicles, it is assumed that vesicles are continuously stimulated with cytosolic Ca$^{2+}$. Activation energies ($\Delta G$) are obtained from the time constant ($\tau$) using: $\tau = \tau_0 \exp(\Delta G/k_B T)$, where $\tau_0 = 10^{-19}$ s. The $k_B T$ unit is the product of the Boltzmann constant and the absolute temperature (353). The pre-stimulus docking requirement bar at the bottom of the figure indicates the time constants that are so short that vesicles must be docked to the plasma membrane before stimulation.
### Table 1. Putative SNARE configurations in various preparations

<table>
<thead>
<tr>
<th>Putative SNARE Configuration</th>
<th>Triggers</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synaptic vesicles (ultrafast)</strong></td>
<td>Ca(^{2+}):Sy t 1, 2, 9</td>
<td>703</td>
</tr>
<tr>
<td><strong>Asynchronous</strong></td>
<td>Ca(^{2+}):Doc2</td>
<td>718</td>
</tr>
<tr>
<td><strong>(Tonic)</strong></td>
<td>Ca(^{2+}):Sy t 1, 2, 9, Syt, 3, 5–57, 10</td>
<td>502, 621, 703</td>
</tr>
<tr>
<td><strong>(Spontaneous)</strong></td>
<td>Ca(^{2+}):Sy t 1, 2, 9</td>
<td>704</td>
</tr>
<tr>
<td><strong>Chromaffin cells (LDCVs)</strong></td>
<td>Ca(^{2+}):Snapin</td>
<td>604, 612</td>
</tr>
<tr>
<td><strong>Islet (\beta) cells (LDCVs:Insulin)</strong></td>
<td>Ca(^{2+}):nd</td>
<td>214, 215, 386, 458, 459, 604, 612</td>
</tr>
<tr>
<td><strong>Exocrine cells</strong></td>
<td>Ca(^{2+}):Sy t 1</td>
<td>43, 162, 182, 275, 680</td>
</tr>
<tr>
<td><strong>(LDCVs)</strong></td>
<td>cAMP: SNAP25</td>
<td></td>
</tr>
<tr>
<td><strong>Lysosomes</strong></td>
<td>Ca(^{2+}):Sy t 7, Doc2</td>
<td>251, 506</td>
</tr>
<tr>
<td><strong>Enlargesomes</strong></td>
<td>Ca(^{2+}):Sy t 4</td>
<td>315, 623</td>
</tr>
<tr>
<td><strong>Neuronal dendrites</strong></td>
<td>Ca(^{2+}):Sy t 4</td>
<td>576, 735</td>
</tr>
<tr>
<td><strong>Astrocytes</strong></td>
<td>Ca(^{2+}):Sy t 2</td>
<td>412, 482, 549, 360, 388, 629</td>
</tr>
<tr>
<td><strong>Basophil, mast cells (LDCVs)</strong></td>
<td>Ca(^{2+}):Sy t 2</td>
<td>412, 482, 549, 360, 388, 629</td>
</tr>
<tr>
<td><strong>T-cells (Immunological synapse)</strong></td>
<td>Ca(^{2+}):Sy t 6</td>
<td>131</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
<td>Ca(^{2+}):Sy t 6</td>
<td>131</td>
</tr>
<tr>
<td><strong>Gastric parietal cells (TV, H(^{+})-ATPase)</strong></td>
<td>Ca(^{2+}):Sy t 6</td>
<td>131</td>
</tr>
<tr>
<td><strong>Kidney duct cells</strong></td>
<td>cAMP: F-actin(?)</td>
<td>71, 276</td>
</tr>
<tr>
<td><strong>Adipocytes, Skeletal muscles (GLUT4)</strong></td>
<td>Insulin:Munc18c</td>
<td>77, 285, 312</td>
</tr>
<tr>
<td><strong>Constitutive exocytosis</strong></td>
<td>None</td>
<td>281, 485, 509</td>
</tr>
<tr>
<td><strong>B cells (IgG), Hela cells</strong></td>
<td>None</td>
<td>281, 485, 509</td>
</tr>
</tbody>
</table>

The four configurations (T, trans-SNARE; B, binary-SNARE; U, unitary-SNARE; C, cis-SNARE) were predicted mainly from the time constants of exocytoses of 0.5–30 ms, 0.03–1 s, and 1–1,000 s, respectively. Qa-, Qbc-, and R-SNARE are three different subtypes of SNARE. Syn, syntaxin; Syt, synaptotagmin.
deal with by the separate fields of neuroscience, endocrinology, immunology, diabetology, and digestive, kidney, and reproductive physiology. Considerable evidence has accumulated that supports the relevance of initial SNARE configurations to the diversity of exocytosis.

We will first discuss the diversity of exocytoses in neurons and other cell types (sects. II and III) and give a comprehensive account of the stages involved in exocytosis (sect. IV). This is followed by a summary of the major proteins that regulate exocytosis (sect. V), and a new general framework for exocytosis based on the distinct initial SNARE configurations that are responsible for its diversity (sects. VI and VII).

II. EXOCYTOSIS OF SYNAPTIC VESICLES

Synaptic vesicles in the presynaptic terminals of neurons undergo exocytosis in four different modes: ultrafast, asynchronous, tonic, and spontaneous. This suggests that the same types of vesicles show markedly different kinetics depending on the mode of exocytosis. We will review the kinetics of each mode of exocytosis in turn.

A. Ultrafast Exocytosis

Neurotransmitters are released from synaptic vesicles at the presynaptic terminal by exocytosis within 1 ms of the arrival of the action potential (22, 442, 479). This ultrafast exocytosis is found only in the presynaptic terminals of neurons (FIGS. 1 AND 2A). This short time scale strongly suggests that the vesicles are already docked to the plasma membrane when the action potential arrives (FIGURE 3A) and that SNAREs are preassembled to some degree. Importantly, there is a structural basis for ultrafast exocytosis, known as the active zone (FIGURE 2A), in which synaptic vesicles are clustered and some vesicles are docked to the plasma membrane (83, 473). The active zone comprises an electron-dense cytoskeletal matrix, referred to as the cytomatrix at the active zone (CAZ), which involves presynaptic dense projections, actin fibers, ribbons, and grids (152, 287, 572). Most of the molecular components that make up the active zone have been identified (sect. V), and some of these are specific to the active zone, for example, RIM, CAST/ELKS, bassoon, piccolo, and liprins (242, 249, 572) (sect. V).

Ultrafast exocytosis of synaptic vesicles harnesses the CAZ proteins in several ways. First, vesicles are localized close to voltage-gated Ca\(^{2+}\) channels such that the vesicles can sense increases in [Ca\(^{2+}\)] near the open pore of the Ca\(^{2+}\) channels before Ca\(^{2+}\) is bound by cytosolic Ca\(^{2+}\) buffers. The shortest distance between the vesicles and the Ca\(^{2+}\) channels is estimated to be between 30 and 60 nm (410). This close localization is often called the Ca\(^{2+}\) domain, or Ca\(^{2+}\) nanodomain (3, 25, 302, 371, 373, 543), and is supported by CAZ proteins (297). Second, synaptic vesicles are effectively replenished during repetitive stimulation, possibly by actin fibers, presynaptic dense projections, and ribbons at the active zone (545). Silencing of the presynaptic terminals is induced when CAZ proteins are inactivated (123, 124, 286). Third, ultrafast exocytosis occurs upon rapid step increases in [Ca\(^{2+}\)], induced by photolysis of a caged-Ca\(^{2+}\) compound (373, 440), suggesting that the exocytotic machinery operates rapidly after binding Ca\(^{2+}\). Ultrafast exocytosis has never been observed in preparations without an active zone, indicating that the active zone is necessary for ultrafast exocytosis.

Vesicles that undergo ultrafast exocytosis upon stimulation by a single action potential are referred to as vesicles in the readily releasable pool (RRP), and vesicles on their way from the site of endocytosis to the active zone are referred to as vesicles in the recycling pool (232, 527). Those vesicles in the presynaptic terminal that are not involved in fast recycling or exocytosis are referred to as vesicles in the reserve or resting pool. The notion of distinct pools of vesicles is useful when describing the status of the vesicles; however, these vesicles are not always pooled or segregated in the cytosol, but rather are scattered and intermingled (142, 232, 527). It should also be noted that the readiness of synaptic vesicles is also dependent on the states of molecules in the plasma membrane and the position of vesicles relative to the membrane. The notion of a vesicle pool is the first approximation for describing the kinetics of exocytosis.

The number of vesicles in the RRP (N) and the release probability of each vesicle (p) can be determined. Katz and del Castillo introduced the binominal statistic to describe the qantal content of vesicles using N and p (24, 140), where N correlates with the number of docked and primed vesicles, and p is dependent on the distance between the vesicles and the Ca\(^{2+}\) channels (410, 678), and on the kinetics of exocytosis after Ca\(^{2+}\) binding with fast Ca\(^{2+}\) sensors, called synaptotagmins. For small presynaptic boutons in the cerebral cortex, the release probability per bouton can be defined. It ranges from 0.1 to 0.9 (60, 410, 431) and correlates with the expression of CAZ proteins (399). The number of vesicles in the RRP is estimated to be ~10 (527), the number in the recycling pool to be ~20, and the number in the reserve pool to be ~200 (232, 268).

B. Asynchronous Exocytosis

Asynchronous exocytosis often follows ultrafast exocytosis and lasts for ~100 ms after the action potential (479). It is enhanced when Ca\(^{2+}\) is replaced by Sr\(^{2+}\) (199). Interestingly, some synapses show asynchronous exocytosis but not ultrafast exocytosis (45). Although there is a debate about whether or not asynchronous exocytosis is mediated by vesicles in the RRP (471, 542), recent studies report that the vesicles responsible for asynchronous exocytosis are the same as those that are responsible for ultrafast exocytosis.
It is therefore likely that the vesicles mediating asynchronous exocytosis are docked to the plasma membrane in a similar manner to the vesicles in the RRP (FIGURE 3A). Thus it is suggested that vesicles need to be docked before stimulation if exocytosis is to be faster than \( \sim 100 \) ms (FIGURE 1). Synaptotagmins confer \( \mathrm{Ca}^{2+} \) dependence on ultrafast exocytosis, whereas Doc2 may confer \( \mathrm{Ca}^{2+} \) dependence on asynchronous exocytosis (718) (sect. VD).

**C. Tonic Exocytosis**

During long-lasting repetitive stimulation, exocytosis becomes tonic and is no longer time-locked to the action potential. Tonic exocytosis is more prominent in the tonic synapses than in the phasic synapses (20, 420) and displays some characteristics that are distinct from the asynchronous exocytosis of vesicles. Tonic exocytosis requires a sustained increase in the bulk \( \mathrm{Ca}^{2+} \) levels (146, 471) rather than the \( \mathrm{Ca}^{2+} \) domains needed for ultrafast exocytosis (502). It is independent of \( \mathrm{Ca}^{2+} \)-dependent activator protein for secretion (CAPS) but dependent on \( \alpha \)-soluble NSF attachment protein (αSNAP) (80), and on VAMP4 instead of VAMP2 (502) (see sect. VA). These characteristics suggest that tonic exocytosis is dependent on the vesicles in the recycling pool (502), but not on the vesicles in the RRP. Exocytosis of nondocked vesicles during tonic exocytosis has been observed in the active zone of bipolar terminals (418, 729). Thus, for tonic exocytosis, vesicles are docked after stimulation (FIGURE 3B) and are continuously resupplied from the recycling pool, with a diffusion constant \( 1.5 \times 10^{-2} \ \mu \text{m}^2/\text{s} \) (257).
The time taken for one vesicle to undergo exocytosis during tonic exocytosis (FIGURE 1) is estimated as follows. The tonic component of exocytosis per second is comparable to the RRP (219, 471), or \( \frac{10}{11} \) vesicles/s/bouton for the small boutons (433). As \( \frac{20}{11} \) vesicles comprise the recycling pool (431), the rate of exocytosis for a vesicle in the pool is \( \frac{0.5}{11} \) s; thus the average time constant of a vesicle is \( \frac{2}{11} \) s during tonic exocytosis (FIGURE 1).

D. Spontaneous Exocytosis

The discovery of miniature endplate potentials led to the idea of exocytosis (24, 140). Miniature endplate potentials are thought to represent the spontaneous exocytosis of the same set of vesicles responsible for ultrafast exocytosis. Despite the simple interpretation that this provides, there are ample new data suggesting that spontaneous exocytosis involves a set of vesicles that are distinct from those involved in ultrafast exocytosis (503). First, spontaneous exocytosis utilizes vesicles in the reserve pool that have VAMP7 (268, 563), VAMP4 (502), and Vps10p-tail-interactor-1a (vti1a) as v-SNAREs (504). This may account for that fact that spontaneous exocytosis is resistant to the deletion of VAMP2 (139, 268) and to treatment with tetanus toxin (TeNT), which specifically cleaves VAMP2 (566). In fact, the two pools of vesicles were differentially labeled in some studies (177, 504, 553), if at all (267, 697). Second, priming proteins affect spontaneous and evoked exocytosis in different ways (TABLE 2). For example, complexin and CAPS (sect. V) play a relatively modest role in spontaneous exocytosis relative to ultrafast exocytosis. Finally, the exact location of exocytosis in the presynaptic terminal may be different for evoked and spontaneous exocytosis (19, 552, 728).

Spontaneous exocytosis is constantly stimulated by resting levels of \([\text{Ca}^{2+}]_i\) (688, 704), and the rate of spontaneous exocytosis can be estimated from the size of the pool from which vesicles undergo spontaneous exocytosis (FIGURE 3, B AND C). With the assumption that spontaneous exocytosis is induced from a reserve pool comprising more than 100 vesicles in small boutons (268), and the rate of spontaneous exocytosis is \(<0.1/s\) per bouton (432), the average time for...
exocytosis is estimated to be >1,000 s (FIGURE 1). The differences between tonic and spontaneous exocytosis are due to differences in \([\text{Ca}^{2+}]\), levels (172, 442).

### III. FUSION OF VESICLES OTHER THAN SYNAPTIC VESICLES

For slow exocytosis, which occurs more than 100 ms after the trigger (FIGURE 1), there is enough time for vesicles to dock with the plasma membrane and for SNARE to be assembled after the trigger. In fact, newly docked vesicles in dock with the plasma membrane and for SNARE to be associated and constitutive exocytosis in a wide variety of preparations.

A. Large Dense-Core Vesicles in Secretory Cells

Substances secreted from nonneuronal preparations are condensed in large dense-core vesicles (LDCVs), which undergo regulated exocytosis. The idea of exocytosis and biogenesis of LDCVs was first proposed for pancreatic acinar cells (476). Unlike synaptic vesicles, LDCVs do not recycle beneath the membrane but are generated in the Golgi apparatus, where they gradually grow larger in preparation for massive secretory events. LDCVs in neuroendocrine cells are relatively small (100–500 nm) but are larger in exocrine and hematopoietic cells (1–2 \(\mu\text{m}\)). LDCVs have an electron-dense matrix that contains secretogranin and chromogranin (38, 245). In chemically fixed preparations, the dense cores often shrink, and apparent halo structures are evident. This shrinkage is very prominent in \(\beta\) cells in the islets of Langerhans, but electron microscopic investigation of quick frozen preparations showed that the halo was absent (153, 494) (FIGURE 2, C AND D); this suggests that the halo is mainly an artifact of chemical fixation. The cytosol of many secretory cells is filled with LDCVs, suggesting that their effective mobilization plays a more important role than the rapid exocytosis of the surface vesicles. Indeed, compound exocytosis is utilized by certain types of cells for efficient mobilization of deep vesicles, as will be described in section III.B.

Stimulus-secretion coupling of secretory cells was first examined in adrenal chromaffin cells (150, 151), in which intracellular \(\text{Ca}^{2+}\) plays a vital role. This discovery led to the \(\text{Ca}^{2+}\) hypothesis of neurotransmitter release (311). In most secretory cells, cytosolic cAMP potentiates \(\text{Ca}^{2+}\)-dependent exocytosis (252, 544, 634). Protein kinase A (PKA) acts as a trigger in some exocrine cells (182, 556) and epithelial cells (175, 414, 630), although kinase systems other than PKA are utilized as triggers in pituitary cells (253), endothelial cells (175, 414, 630), and adipocytes (236, 285). When \(\text{Ca}^{2+}\) is not a major trigger (FIGURE 3D), exocytosis tends to be slow, with time constants between 1 s and 10 min (FIGURE 1).

1. Endocrine cells

Endocrine cells secrete hormones into the bloodstream, whereupon the hormones are extensively diluted. The-
fore, the total amount of secretion over a period of minutes is more important than the millisecond time course of exocytosis from the surface layer of the cytosol. In fact, the speed of exocytosis is markedly slower in endocrine cells than the ultrafast exocytosis observed in synaptic vesicles (Figure 1). Moreover, although action potentials trigger exocytosis of LDCVs in many endocrine cells (49, 319, 700), the temporal correlation between each action potential and exocytosis is never as tight as it is in ultrafast exocytosis (112, 745). Endocrine cells secrete hormones into the blood vessels, therefore, and exocytosis was predicted to occur more frequently at plasma membranes facing the blood vessels (465). However, two-photon imaging experiments show that there is no marked spatial regulation of exocytosis, and that exocytosis utilizes the entire plasma membrane (321, 636). In support of this idea, there are no tight junction structures in endocrine glands, and the intercellular space can act as a pathway for hormonal secretions. The next section summarizes the specific features of LDCV exocytosis in different endocrine cell types.

**A) ADRENAL CHROMAFFIN CELLS.** Chromaffin cells develop from postganglionic sympathetic neurons and are often considered as a model system for rapid neuronal secretion (33). Secretion of epinephrine by chromaffin cells plays a major role in the homeostasis of blood circulation when life-threatening events occur. Exocytosis of LDCVs in adrenal chromaffin cells occurs with a time constant of ~1 s when depolarized from a resting level of [Ca$^{2+}$]$_i$ (321, 675). Rapid phases of exocytosis occur when cytosolic Ca$^{2+}$ levels are increased for a few minutes (221, 673). After this “Ca$^{2+}$ priming” process, vesicles undergo more rapid exocytosis, with time constants of 30 ms or less. However, the time course of exocytosis is still 30 times slower than that of ultrafast exocytosis that occurs in synaptic vesicles. This suggests that there are considerable differences in the molecular and cellular bases of the two types of exocytosis (see sect. V). An intensive quantitative investigation of chromaffin from adult bovine adrenal medulla failed to identify preferential docking of LDCVs (Figure 2B), even when the chromaffin cells were primed with Ca$^{2+}$ (494), because the cytosol was already filled with LDCVs. In fact, docking of LDCVs is detectable in embryonic chromaffin cells, in which LDCVs are more sparse (134, 135, 676). Importantly, fast exocytosis in Ca$^{2+}$-primed cells is followed by a slow sustained phase of exocytosis (706, 707), with a time constant similar to that of slow exocytosis in unprimed chromaffin cells; this suggests that nondocked vesicles in chromaffin cells can undergo slow exocytosis (617, 707) (see sect. VI).

**B) PC12 CELLS.** The chromaffin cell line PC12 is often used to investigate the molecular mechanisms underlying exocytosis (26, 32, 36, 99, 120, 185, 205, 305, 323, 346, 391, 499, 591, 622, 625, 656, 657, 744). The kinetics of LDCV exocytosis in PC12 cells are slower (10 s) than in chromaffin cells (1 s) and are not increased by Ca$^{2+}$ priming (309, 322). However, both chromaffin cells and PC12 cells show intensive compound exocytosis, as described in section III (Figure 3E). Despite the fact that exocytosis is extremely slow in PC12 cells, ultrastructural and biochemical studies using cracked cells often show LDCVs docked to the plasma membrane (393). This is an example of the dissociation between the morphological docking and fast kinetics of exocytosis (see sect. VIB).

**C) ISLET $\beta$ CELLS.** The pancreas, or pancreatic islets, contain three types of endocrine cell: $\alpha$ cells (which secrete glucagon), $\beta$ cells (which secrete insulin), and $\delta$ cells (which secrete somatostatin). An increase in [Ca$^{2+}$]$_i$ is the major trigger for secretion in all three cell types (248, 505, 609, 693, 700). $\beta$ cells form the major cell mass in the islets, and insulin secretion is of crucial importance to health and disease as it is the only hormone that facilitates glucose uptake by muscle and adipocytes, leading to a reduction in blood glucose levels. Defective insulin secretion is the major etiology of type 2 diabetes mellitus, and stimulation of insulin secretion is the primary therapeutic target. Insulin secretion is slow and tonic in general and takes place in two phases. The first phase of insulin secretion is induced within 5 min of stimulation and is important for glucose uptake into the liver. The second phase of insulin secretion is tonic and gives rise to glucose uptake into muscles and other tissues. In addition to Ca$^{2+}$, CAMP is an important factor involved in insulin exocytosis (248, 304, 380, 580). The two phases of insulin exocytosis are differentially regulated by PKA (237, 308, 634) and syntaxin (459, 612), and may involve distinct molecular states (458, 633). It is suggested that CAMP-guanine nucleotide exchange factor (GEF), Epac, regulates insulin exocytosis by chronically modulating the availability of insulin granules (590, 733), but not by acutely potentiating insulin exocytosis (238, 597, 603).

Importantly, experiments using caged Ca$^{2+}$ compounds in chromaffin cells (321, 675) and $\beta$ cells (237, 632, 635) revealed that the speed of exocytosis was slow, even when exocytosis was induced by a rapid increase in [Ca$^{2+}$]$_i$, which induces submillisecond exocytosis in synapses (Figure 1). Thus the exocytotic machinery must be inherently slow. Micromolar increases in [Ca$^{2+}$], are often required to trigger exocytosis by secretory cells in synapses, and coupling between Ca$^{2+}$ channels and the fusion machinery may occur, although not as tightly as the coupling involved in synaptic neurotransmitter release (35, 40, 261, 536). PKA increases the number of LDCVs that undergo fast exocytosis in both chromaffin cells (437) and $\beta$ cells (238, 634).

**D) PITUITARY CELLS.** Prominent endocrine cells are present in the anterior and posterior pituitary glands (486). The release of vasopressin and oxytocin from the posterior pituitary is induced via nerves from the hypothalamus, and the release behaviors are similar to those observed for LDCV secretion.
from nerve endings (see sect. IIIC), although LDCVs seldom dock to the membrane [FIGURE 2B]. An interesting characteristic of the posterior pituitary gland is the presence of small synaptic vesicles with unknown functions [FIGURE 2B] (325). The anterior pituitary gland comprises cells that secrete six different hormones: corticotrophs secrete adrenocorticotropic hormone, somatotrophs secrete growth hormone, thyrotrophs secrete thyroid stimulating hormone, lactotrophs secrete prolactin, and gonadotrophs secrete luteinizing hormone and follicle stimulating hormone. Exocytosis of all of these LDCVs is triggered by increases in \([Ca^{2+}]_i\), which are mediated by \(Ca^{2+}\) channels in corticotroph and somatotroph cells, and by \(Ca^{2+}\) release from intracellular \(Ca^{2+}\) stores within thyrotroph, lactotroph, and gonadotroph cells (618), probably in much the same way as in exocrine cells. In addition to \(Ca^{2+}\), PKC is proposed to act as a trigger of exocytosis in gonadotroph cells (253). Compound exocytosis is observed in cells that utilize \(Ca^{2+}\) release from intracellular stores (116).

2. Exocrine glands

Exocrine cells secrete digestive enzymes into the gastrointestinal tract. These cells include the acinar cells in the pancreatic, parotid, mandibular, and lacrimal glands. The secretion of enzymes must be slow and tonic to maintain sufficient concentrations in the extracellular fluids in a regulated manner. Exocrine cells do not express \(Ca^{2+}\) channels; rather, \(Ca^{2+}\) increases mainly occur via \(Ca^{2+}\) release from intracellular stores (619, 726) and \(Ca^{2+}\) influx via the store-operated \(Ca^{2+}\) entry pathways (500).

Secretory granules are localized at the apical region of the cells, where exocytosis is selectively induced. Even though the agonist receptors are localized on the basal side of the cells, \(Ca^{2+}\) release is initiated at the apical pole (303), probably due to diffusion of inositol trisphosphate from the basalolateral membrane towards the apical pole (278, 307, 641). The localization of \(Ca^{2+}\) mobilization from stores coincides well with the exocytosis of zymogen granules, which is apparently advantageous for massive exocytosis from exocrine glands. The \(Ca^{2+}\) increase at the apical pole is as high as 10 \(\mu\)M (279) and is required for the exocytosis of zymogen granules (443), similar to the situation in synapses and endocrine cells.

There is an electron-lucent layer beneath the apical plasma membrane in exocrine glands (273), which excludes the attachment of vesicles to the apical plasma membrane. This electron-lucent layer corresponds with the apical F-actin layer, toward which the acinar cells undergo exocytosis (444). In fact, removal of the F-actin layer by latrunculin facilitates exocytosis (444); thus zymogen granules are not docked to the plasma membrane prior to stimulation.

3. Hematopoietic cells

Most blood cells, including mast cells, eosinophils, basophils, and T-cells, have LDCVs that contain various cytokines and enzymes. Exocytosis in blood cells is believed to depend on cytosolic \(Ca^{2+}\) (412); however, other mechanisms also exist (550) (TABLE 1). The secretion of granules is generally slow in blood cells and secretory granules are nondocked; however, massive exocytosis can be induced by compound exocytosis after stimulation (see sect. IIIC).

Exocytosis from blood cells may occur in a pointed manner toward the target cells. For example, eosinophils are involved in host defense against parasites (84) and are implicated in allergic responses (343). Parasite death is caused by adherence of eosinophils to the parasite surface, followed by the targeted release of cytotoxic granule contents by exocytosis (246). Degranulation in eosinophils occurs via the formation of degranulation sacs, ensuring the release of cytotoxic proteins at a high concentration at focal sites through a single fusion pore (246). It is widely assumed that, in eosinophils and basophils, compound granules are formed by homotypic granule-granule fusion inside the cell, followed by exocytosis and multigranular exocytosis (218). \(Ca^{2+}\) is the trigger for this process in macrophage and dendritic cells (42), whereas kinase signaling is the trigger in basophils and mast cells (629) and at the immunological synapses in cytotoxic T-cells (482). Thrombin triggers exocytosis of LDCVs from platelets, which is essential for thrombosis and is dependent on SNAREs (513).

4. Lysosomal exocytosis

Lysosomes are scattered throughout the cytosol and are rarely docked to the plasma membrane (376). Exocytosis from lysosomes is \(Ca^{2+}\) dependent (352) and occurs in various cells (447, 506). Lysosomal exocytosis is also used for ATP release from glial cells (366, 740) and may play a role in membrane repair (510).

B. Compound Exocytosis

One prominent feature of exocytosis of LDCVs is compound exocytosis, which is defined as exocytosis of more than one vesicle at the same time (492). The advantage of compound exocytosis is the effective mobilization of deep vesicles within the cytosol without the need for transportation to the target plasma membrane, thereby avoiding deformation of cellular structures. This is particularly important for LDCVs, as their large size makes diffusion and transport to the proper release sites in the plasma membrane more difficult. Therefore, compound exocytosis provides the most efficient method of mobilizing LDCVs stored deep within the cytosol. There are two forms of compound exocytosis: sequential and multigranule.
1. Sequential exocytosis

In sequential exocytosis, vesicles fuse with the plasma membrane (preserving their omega-shaped structure to some extent) where they become the target for further exocytosis of deep vesicles within the cytosol (FIGURE 3A). Sequential exocytosis is a highly sophisticated three-dimensional phenomenon, the quantification of which requires a two-photon microscope. The prevalence of sequential exocytosis may, therefore, be underestimated. Sequential exocytosis has been observed in exocrine acinar cells (224, 443, 444), adrenal chromaffin cells (7, 190, 321), PC12 cells (322, 734), β cells (464, 632), pituitary lactotrophs (116), human neuroendocrine Bon cells (652), the nasal gland (469), eosinophils (218), and mast cells (8, 213), and even in bipolar neuroendocrine Bon cells (652), the nasal gland (469), eosinophils (218), and mast cells (8, 213), and even in bipolar synapses (398). Sequential exocytosis is particularly prominent in exocrine acinar cells and chromaffin cells, as described below.

In 1965, structural studies predicted sequential exocytosis in pancreatic acinar cells (273), and the dynamics were identified using two-photon excitation imaging (443) (FIGURE 3A). Interestingly, maintenance of the omega-shaped structure of already-fused granules requires an actin coating (444); otherwise, sequential exocytosis results in vacuoles, akin to those found in acute pancreatitis (444, 507). Thus acinar cells are well prepared for the sequential progression of exocytosis from deep within the cells, maintaining defenses against the harsh environment in the gastrointestinal tract. Interestingly, although actin coating of the granules slows the fusion between the granules, it does not block it (444), suggesting that the granules are not docked to each other or to the plasma membrane, and that the submembrane actin cytoskeleton can dynamically regulate exocytosis.

Two-photon imaging of clusters of chromaffin cells shows that sequential exocytosis results in vacuoles akin to those observed in latrunculin-treated acinar cells, even without latrunculin treatment (321). Such vacuolar structures were seen in earlier studies but were thought to represent endocytotic processes (33). Compound exocytosis can mobilize more than 50% of granules stored in the cytosol. Although compound exocytosis has been observed at the basal surface of cells facing a glass surface using total internal reflection fluorescence (TIRF) imaging (7), it is particularly prominent in tissue preparations in which the extracellular space is faced by an intact extracellular matrix, which blocks diffusion of gels within the granules. After sequential exocytosis, the gel inside the granules swells and forms expanding luminal structures, which bring the vacuolar membrane closer to the cytosolic vesicles and facilitate the progression of exocytosis. Thus mechanical pressure appears to facilitate exocytosis in chromaffin cells.

Studies of exocytosis in acinar and chromaffin cells show that the progression of exocytosis is mostly sequential, and that the speed of fusion of granules in the superficial layer is akin to that of vesicles in the deep cellular layers (321, 444). This suggests that the vesicles in the deep layers have a “fusion readiness” comparable to that of superficial vesicles. Granules are not docked to other granules in the cytosol, supporting the notion that the secretory granules are also not docked to the plasma membrane before stimulation. Moreover, the strictly sequential nature of exocytosis suggests that a key factor must diffuse from vesicle membranes that have already undergone exocytosis to assist exocytosis of vesicles in the deeper cell layers. In fact, diffusion of t-SNAREs into the fused vesicles has been found in chromaffin cells (321), β cells (632), and exocrine cells (493). Thus sequential exocytosis suggests that vesicles are non-docked (see sect. VI).

The fusion pore in primary vesicles plays a pivotal role in sequential exocytosis because all subsequent secretion is mediated through the same initial fusion pore on the original plasma membrane. Indeed, ultrastructural analysis of chromaffin cells shows that the vesicles and plasma membrane are bridged by fine strands, which are preserved even after exocytosis and may represent annexins (438). Similar structures are also found in the anterior pituitary gland, in which sequential exocytosis also occurs (581). In addition, submembraneous F-actin plays a role in stabilizing both the fusion pore and sequential exocytosis in chromaffin cells (321).

2. Multigranular exocytosis

In multigranule exocytosis, vesicles fuse within the cytosol to form the multigranular vesicles that later undergo exocytosis (FIGURE 3B). Typical examples of multigranular exocytosis in hematopoietic cells were given in the previous section. It seems that both multigranular and sequential exocytosis are used by many cells, including mast cells (8), lactotroph cells (116), gastolic parietal cells (175), β cells (262), and synapses (23, 244, 398). Multigranular exocytosis has the advantage of secreting a great number of messengers at the same time; however, unlike sequential exocytosis, the amounts cannot be controlled as precisely. Multigranular exocytosis is a prominent feature of exocytosis from blood cells. Multigranular (multivesicular) exocytosis may also be utilized during synaptic neurotransmitter release; for example, botulinum toxin treatment increases the frequency of giant miniature excitatory postsynaptic potentials (498, 566), suggesting that multivesicular exocytosis may be involved in some pathological states at the synapses.

C. Large Dense-Core Vesicles in Synapses

Presynaptic terminals contain synaptic vesicles, and LDCVs and electron microscopic analyses show that most of the LDCVs are nondocked to the plasma membrane (FIGURE 2A) (76, 152, 201, 539, 560, 610). Exocytosis of synaptic LDCVs requires repetitive stimulation (583, 692); thus the molecular basis of
LDCV exocytosis in presynaptic terminals is similar to that in endocrine cells. The 20-ms delay in the exocytosis of LDCVs (75) may correspond to the crash fusion events observed in adrenal chromaffin cells (7). Exocytosis of LDCVs is mainly limited by the number of mobile granules and their slow rate of diffusion (81); the mobility of LDCVs is also Ca\(^{2+}\) dependent (583).

**D. Small Vesicles in Nonneuronal Cells**

Ca\(^{2+}\)-dependent exocytosis of small vesicles has been demonstrated in various cells other than synaptic terminals and may be mediated by specific types of vesicles that, like synaptic vesicles, can be labeled with synaptophysin. These vesicles are called “synaptic-like microvesicles” (SLMV; Refs. 290, 439) (FIGURE 2) or “enlargosomes” (117, 118, 305) (TABLE 1). Exocytosis of small vesicles has been studied using capacitance measurements in the cell-attached patch configuration, where single exocytotic events are seen as stepwise increases in the membrane capacitance of neuroblastoma NG108–15 cells (137), pituitary nerve endings (325), pancreatic β cells (379), or PC12 cells (743). The estimated size of the small vesicles ranges from 40 to 100 nm, which is consistent with ultrastructural observations (305, 494) and imaging studies (238, 367). Ca\(^{2+}\)-dependent exocytosis of small vesicles is also found in fibroblasts and muscles, where it may play a role in membrane repair (404, 614). Small vesicle exocytosis in chromaffin cells (706) and PC12 cells (305) is resistant to TeNT but is blocked by TeNT in fibroblasts (587). Enlargosomes may share a common ancestor with synaptic vesicles (537, 587) and appear to be utilized by cells for growth, differentiation, migration, and wound healing (117, 118, 238).

Small vesicle exocytosis is usually characterized using imaging techniques. Small vesicles in PC12 cells selectively contain acetylcholine (ACh), and their exocytosis was visualized by labeling the vesicle with a vesicular ACh transporter labeled with pHluorin (64). Exocytosis of small vesicles from PC12 cells and islet β cells was also studied using two-photon imaging, which revealed that the time constants for exocytosis induced by Ca\(^{2+}\) uncaging were 0.8 and 0.3 s for PC12 cells and islet β cells, respectively (238, 367). These time constants correlate well with the fast component of increases in membrane capacitance (309, 635). The fast component of the capacitance increase in β cells is augmented by cAMP but is resistant to Rp-cAMP (515), and is congruent with the facilitation of small vesicle exocytosis by Epac (238, 544), which regulates small vesicle exocytosis (0.3 s) in β cells more rapidly than PKA regulation of LDCVs (4.5 s) (238). Despite the abundance of Ca\(^{2+}\)-dependent exocytosis of small vesicles in these cells, small vesicles are found nondocked to the plasma membrane before stimulation (305, 494); therefore, they may undergo poststimulus docking (FIGURE 3, B AND C) (see sect. IVA).

Amperometry studies show that the rapid increase in capacitance happens more quickly than LDCV exocytotic events in chromaffin cells (221, 309, 428, 448, 457), PC12 cells (448), β cells (304, 635), CHO cells (447), and mast cells (320) (FIGURE 1). The most likely reason for the dissociation between the capacitance and amperometric signals in these cells is the presence of small vesicle exocytosis. The dissociation is minimized under Ca\(^{2+}\)-priming conditions, in which the basal Ca\(^{2+}\) level is increased to more than 0.3 μM over 3 min (221, 434), although this procedure should not exclude the contribution of small vesicles. It is also reported that the amperometric measurement was affected by a diffusional delay of unknown nature (221), which might reflect sequential exocytosis (321) or the monoamine content of small vesicles. In addition, membrane capacitance measurements record both exocytosis and endocytosis, which are difficult to separate (367). Hence, the kinetic features of LDCV exocytosis from endocrine cells would be better investigated using imaging approaches, which are able to unambiguously distinguish the exocytosis of LDCVs and small vesicles (41, 87, 261, 306, 321, 367, 479).

**E. Regulated Membrane Recycling Systems**

1. **H\(^{+}\)-ATPase trafficking in gastric parietal cells**

Regulated membrane recycling has been proposed as the major mechanism underlying histamine-induced acid secretion from gastric parietal cells, in which H\(^{+}\)-ATPases in endosomal compartments of tubulovesicles (TV) are brought to the apical secretory surface by exocytosis (175). The involvement of SNAREs in this process was recently confirmed using TeNT (300, 349). This recycling is induced by histamine and H\(_2\) receptors, which activate a heterotrimeric G protein Gi, resulting in an increase in cytosolic cAMP levels (175, 253, 368). The signal is then relayed to PKA. The TV membranes are mutually fused, apparently undergoing compound exocytosis, to reorganize the membrane structure. Reorganization of the TVS is dependent on actin fibers, suggesting that cAMP regulates poststimulus docking of TVS to the plasma membrane (FIGURE 3C).

2. **Aquaporin trafficking in kidney-collecting ducts**

Another prominent example of the recycling of transport vesicles can be found in kidney collecting duct cells, where vasopressin raises cytosolic cAMP levels, which results in the exocytosis of vesicles that contain aquaporin-2 into the collecting duct membrane in a PKA-dependent manner. This underlies the major diuretic action of vasopressin (71, 534). This form of exocytosis requires SNAREs, as it is blocked by TeNT (202) and botulinum toxins A, B, and C (501); it is also highly dependent on the subcortical actin layer, and the actions of cAMP may be mediated by depolymerization of the cortical actin layer by inhibition of Rho small GTPases (324, 450). Vesicles containing aquaporin-2...
undergo spontaneous exocytosis, akin to synaptic vesicles. Similar to TV vesicles, zymogen granules in parotid acinar cells express aquaporin and undergo exocytosis induced by cAMP during stimulation with isoproterenol (182, 556).

3. GLUT4 trafficking in adipocytes and skeletal muscles

The actions of insulin promote the uptake of glucose by skeletal muscle and adipocytes via exocytosis of vesicles containing a highly efficient glucose transporter, GLUT4 (174, 236), which is impaired in type 2 diabetes. Exocytosis of GLUT4 is SNARE-dependent (77). The vesicles are stored in the cytoplasm; therefore, insulin stimulation triggers the docking and fusion of GLUT4-containing vesicles to the plasma membrane (30, 285, 708).

4. Trafficking of glutamate receptors in neuronal dendrites

Activity-dependent trafficking of glutamate receptors in postsynaptic dendrites is involved in the synaptic plasticity of glutamatergic synapses (315). Vesicles carrying glutamate receptors are found in the cytosol and undergo Ca$^{2+}$-dependent exocytosis (384, 481). Thus docking and fusion of these vesicles must be induced in a Ca$^{2+}$-dependent manner to enable rapid activity-dependent changes in synaptic functions. Since exocytosis is Ca$^{2+}$-dependent, these vesicles may fall into the same class as enlargosomes (117).

F. Constitutive Exocytosis

Constitutive exocytosis occurs without any particular external stimulation and carries membrane lipids and membrane proteins to the plasma membrane, which is necessary for cell survival (212). TIRF imaging of constitutive exocytosis reveals that it occurs in a manner similar to regulated exocytosis (569). Constitutive exocytosis is mediated by SNAREs (485) (TABLE 1), as is the case for regulated exocytosis, and some secretory cells appear to utilize the same set of SNAREs as are utilized for constitutive exocytosis (TABLE 1, sect. VIC). Thus SNARE-dependent exocytosis can proceed without the need for a specific trigger, and exocytosis can be regulated at any stage during transport, docking, and fusion of vesicles (FIGURE 3, A–C).

G. Fusion of SNARE-Bearing Proteoliposomes

The idea of fusion assay systems that use liposomes bearing SNAREs was first introduced in 1998 (690), when dequenching of membrane fluorescent tracers was used to monitor lipid mixing, which is indicative of membrane hemifusion. Such hemifusion was associated with full fusion content mixing in some studies (445, 665, 682). Liposome fusion can be induced in a Ca$^{2+}$-dependent manner in the presence of a soluble synaptotagmin1 fragment (661) or full-length synaptotagmin1 (347, 685), but the fusion is still slow (1–10 min). A cell-based assay using “flipped” SNAREs reported that SNAREs can promote content exchange (265), but the kinetics are slow (10 min), even when synaptotagmin and complexin are included in the fusion assay. These model systems may explain the slow exocytosis observed in secretory cells, at least with respect to the time course (FIGURE 1).

Recently, a single vesicle fusion system was developed in which lipid mixing and content mixing can be simultaneously studied in vesicles that are linked by SNAREs (341). The fusion is extremely rapid (<250 ms), possibly due to the selection of vesicles that were already docked with each other. The rapid fusion events, coupled with a complete reconstituted system, may allow the study of ultrafast Ca$^{2+}$-dependent fusion in the near future.

IV. KEY CELLULAR STAGES OF EXOCYTOSIS

In this section, we explain some of the key notions of exocytosis that are necessary for understanding the diversity of SNARE-dependent exocytoses. In addition, the concepts of docking, priming, and triggering need some clarification; therefore, we also summarize what is known regarding the intermediate steps involved in membrane fusion and the effects of lipid composition.

A. Docking of Vesicles

1. Definition of docking

The vesicle membrane must make physical contact with the plasma membrane for membrane fusion to occur at any time before or after stimulation (402, 671). For ultrafast exocytosis, such physical or molecular contact is likely formed before stimulation, and the word docking is often used to describe the conceptual state of vesicles before stimulation. Docking, however, can simply mean contact between vesicles and the plasma membrane irrespective of the timing of the stimulation. These two meanings of docking are often used interchangeably, giving the erroneous impression that docking of the vesicles needs to precede the triggering of exocytosis. To avoid this confusion, we use the terms prestimulus and poststimulus docking in the following sections.

2. Prestimulus docking

Prestimulus docking has been studied in neurons and secretory cells using electron microscopy, where vesicles are often

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docked to the site of exocytosis in resting samples (FIGURE 2) (133). Prestimulus docking is considered necessary for fast secretory cells and synapses (699). In fact, the number of docked vesicles in the synapse correlates with the number of vesicles in the RRP (568). SNAREs and Munc18 are the epicenter of exocytosis (see sect. V), and both might be involved in prestimulus docking. In fact, docking of LDCVs in embryonic chromaffin cells was reduced by the deletion of SNAP25 (135), Munc18a (676), or cleavage of syntaxin with BoNTc (134). Consistent with this, docking of synaptic vesicles in the synapses of nematode mutants studied under high-pressure electron microscopy was impaired when syntaxin or Munc18a was deleted (222, 223, 691). However, in mammalian (69, 270, 571, 670, 686) and fly (67, 577) synapses, SNAREs and Munc18a are dispensable for docking, indicating that prestimulus docking is more dependent on CAZ proteins, such as RIM (297, 330) and Munc13 (595). Therefore, prestimulus docking does not specify the state of SNAREs.

Prestimulus docking is also found in cells that show slow exocytosis, such as PC12 cells, in which docked and nondocked granules undergo exocytosis over the same time course (317). Moreover, prestimulus docking does not hasten insulin exocytosis in β cells (310, 655, 719). If prestimulus docking does not hasten exocytosis in slow secretory cells, then why are vesicles found tightly docked to the plasma membrane in PC12, chromaffin, and pituitary cells? One idea is that prestimulus docking assists sequential exocytosis (see sect. III.B) by stabilizing the fusion pore of the primary exocytotic vesicles (FIGURE 3E) (321, 322, 632). Such docking is mediated by annexins (306, 438, 581), which can tightly link vesicles with the plasma membrane.

Another mechanism for docking or tethering vesicles to the plasma membranes involves Rab, a small GTpase, and other tethering proteins (68, 72, 272, 725). Such loose molecular contacts may play an important role in directing vesicles to their proper targets, because SNARE interactions are too promiscuous (32, 558, 714) to explain the specificity of vesicle trafficking.

3. Poststimulus docking

Most vesicles stored in the cytosol are nondocked to the plasma membrane in synapses and secretory cells. Indeed, most synaptic vesicles are nondocked in the presynaptic terminals (like LDCVs in synapses) in endocrine and exocrine cells. The same is true for small vesicles in nonsynaptic preparations, such as transport vesicles in gastric parietal cells and kidney duct cells, as described in section III. Live imaging has directly demonstrated that Ca\(^{2+}\) can induce poststimulus docking of vesicles for exocytosis, referred to as a “crash fusion event,” in adrenal chromaffin cells (7, 671), islet β cells (310, 459, 460, 590), PC12 cells (317, 555), and mast cells (31). Such crash fusion events are found even in synaptic vesicles, where they are involved in the tonic exocytosis of ribbon synapses (257, 418, 729). Because TIRF imaging reaches at least 40 nm from the plasma membrane, and SNARE complexes are only 12 nm in length, it is unlikely that ternary-SNARE complexes form before stimulation during crash fusion events (353). Crash fusion events are dominant during insulin exocytosis from β cells (310, 590), where the rates of granule arrival at, and departure from, the submembrane space are two orders of magnitude higher than the release rates. Such dynamics may determine the rate of exocytosis (239).

4. Submembranous actin layers and exocytosis

We can infer whether pre- or poststimulus docking is involved in exocytosis from the dependence of exocytosis on submembranous actin fibers. If vesicles are already docked before stimulation, removal of the actin layer should not affect exocytosis. Indeed, latrunculic A treatment, which disrupts actin fibers (122), does not affect ultrafast exocytosis from the active zone (426, 545), but it augments spontaneous exocytosis (268, 426). These results are consistent with the idea that ultrafast exocytosis is mediated by docked vesicles and spontaneous exocytosis occurs from nondocked vesicles in the reserve pool (268, 503). Similarly, disruption of subcortical actin is reported to augment exocytosis from adrenal chromaffin cells (653), β cells (647), exocrine acinar cells (444), gastric parietal cells (175), and kidney duct cells (71, 450). Deletion of Munc18a reduces morphological docking of LDCVs in embryonic adrenal chromaffin cells (676) because Munc18a removes the subcortical actin layers (133, 651). Treatment with latrunculin A restores morphological docking, but not exocytosis, suggesting that Munc18a removes the subcortical actin layer for docking in addition to priming SNARE assembly (69, 135, 270, 571, 686).

Prestimulus docking is a morphological concept and is absolutely necessary only for ultrafast exocytosis (FIGURE 1); however, it neither necessarily accompanies tight SNARE assembly nor hastens exocytosis. Thus we should better specify the initial states of the SNARE assembly, rather than the “docking” of vesicles, wherever possible.

B. Priming of Exocytosis

Priming of exocytosis generally pertains to the molecular and cellular processes that facilitate stimulus-induced fusion reactions. Priming in permeabilized chromaffin cells (259) and PC12 cells (393) requires Mg-ATP. Priming is often assumed to follow vesicle docking in these preparations and in the active zone (446, 451); however, priming can precede docking. For instance, ATP-dependent disassembly of SNAREs by N-ethylmaleimide-sensitive fusion protein (NSF) is necessary for exocytosis (336, 392, 705), and such priming reactions precede the docking of vesicles. In addition, there is a specific priming process called Ca\(^{2+}\)
priming (673, 677), which is induced by moderate increases in \([\text{Ca}^{2+}]\), and markedly hastens exocytosis, presumably by inducing prestimulus docking and assembly of SNARE complexes in synapses and chromaffin cells (442). Priming can even be positional, as the vesicles need to be brought close to the \(\text{Ca}^{2+}\) channels to trigger ultrafast exocytosis (442, 678).

Many priming molecules that regulate SNAREs have been identified (80, 392), including Munc18, Munc13, CAPS, complexin, and snapin (sect. V). Thus priming can be defined as a molecular process that facilitates exocytosis by regulating SNAREs. It should be noted that, since SNARE assembly may occur after stimulation in slow secretory cells (sect. VI), the same molecule used for priming of ultrafast exocytosis may be utilized after stimulation for slower exocytosis (see FIGURE 8). For instance, although CAPS is normally assumed to be involved in prestimulus SNARE assembly in PC12 cells, it also facilitates \(\text{Ca}^{2+}\)-dependent SNARE assembly after stimulation (317). Many other examples are presented in section VI. Thus priming proteins regulate exocytosis and SNARE assembly both before and after the triggering of exocytosis.

### C. Triggering of Exocytosis

#### 1. Physiological triggering

SNARE-dependent exocytosis can occur constitutively without stimulation, meaning that exocytosis can be regulated at any stage from transport, SNARE assembly, and fusion. Once such regulation, or “clamp,” is removed, exocytotic reactions may proceed to fusion. Exocytosis can be triggered by an increase in \([\text{Ca}^{2+}]\), (479) or cAMP (630), activation of kinases (253, 285, 629), or even changes in membrane voltage (731, 732). Such stimulation can be very rapid and transient, or repetitive and tonic, lasting for minutes or hours, for example, tonic exocytosis of synaptic vesicles and insulin exocytosis. Exocytosis can be regulated at multiple stages (597, 634).

#### 2. \(\text{Ca}^{2+}\)-dependent triggering steps

Synaptotagmins and Doc2 are \(\text{Ca}^{2+}\) sensors for \(\text{Ca}^{2+}\)-dependent exocytosis (see sect. VD). Synaptotagmins sense increases in \([\text{Ca}^{2+}]\), caused by influxes of \(\text{Ca}^{2+}\) through \(\text{Ca}^{2+}\) channels located close to the site of exocytosis (\(\text{Ca}^{2+}\) domain) for ultrafast exocytosis (see FIGURE 8). Exocytosis evoked by a single action potential, however, is rather exceptional, and \(\text{Ca}^{2+}\)-dependent exocytosis is usually induced by an increase in \([\text{Ca}^{2+}]\), which occurs diffusely over space and time. This applies even to synaptic neurotransmitter release, particularly for tonic and spontaneous exocytosis. In endocrine and exocrine cells, increases in \([\text{Ca}^{2+}]\) can be slowly achieved by \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) stores. In these cases, \(\text{Ca}^{2+}\) induces poststimulus docking and, therefore, acts upstream of SNARE assembly (633), as is the case for other triggers such as cAMP. Thus triggering is achieved by resumption of SNARE assembly, which is halted at various stages depending on the cell type (see sect. VI). Thus exocytotic vesicles experience the following stages (FIGURE 8): 1) SNARe disassembly and partial SNARE assembly, 2) triggering, 3) poststimulus SNARE assembly, and 4) the fusion reaction that occurs in parallel with the final SNARE assembly.

### D. Membrane Dynamics

The free energy required for the fusion of a vesicle to the membrane has been theoretically estimated as \(40–120 \, k_B T\), where \(1 \, k_B T = 4 \times 10^{21} \, \text{J}\), the product of the Boltzmann constant and the absolute temperature (119, 338). This is the same order of magnitude as the energy supplied by the hydrolysis of ATP (38 \(k_B T\)) and the reduction in the free energy of SNAREs after zipper (35 \(k_B T\)) (353). Thus the fusion reaction and SNARE assembly have comparable energy barriers, consistent with the SNARE hypothesis of exocytosis.

Membrane fusion proceeds via the following three steps: 1) initial contact, 2) formation of hemifusion intermediates, and 3) opening of the fusion pore (FIGURE 4) (119, 332). Molecular dynamic simulations suggest that the process of hemifusion and fusion pore opening are very rapid after initiation (181, 452). In contrast, forming the initial contact may take a long time, and the narrow fusion pore can be stable over seconds. It is likely that fusion is arrested before initial contact, at least for slow exocytosis, and possibly for all forms of exocytosis.

#### 1. The initial step of membrane fusion

The two membranes must make physical contact for membrane fusion to occur. In biological fusion reactions, such contact is actively guided by tethering molecules in the active zone (725), including small G proteins (Rabs) (281), myosin V, microtubules, and F-actin (594). This is to ensure the selectivity of the fusion events. Conversely, the contact zone is crowded with membrane-associated proteins, such as the subcortical actin meshwork (337), membrane integral proteins, and adaptor proteins, which cover 50% of the surface of the plasma membrane (102). In principle, the intimate contact involved in fusion requires the opening of protein-depleted patches in the opposing membranes. These proteinaceous obstacles are dynamic, allowing access to vesicles for only a short period of time (138), and these dynamics can regulate the kinetics of exocytosis. In the active zone, CAZs may promote the initial contact.

Examples of initial contact formation are found in many biological tissues, including exocrine acinar cells, in which exocytosis is induced toward the cortical F-actin layer.
within a few seconds of stimulation (444). The rate of exocytosis in secretory cells may be regulated by the frequency of vesicles approaching the plasma membrane (138, 239). Interestingly, vesicle movements are increased before fusion (6, 138), suggesting that lipidic contact may be made immediately before exocytosis. The initial contact between vesicles with the plasma membrane may be induced 40–100 ms before exocytosis (7, 239, 310, 317, 555, 590). Thus the transport of vesicles and the submembraneous cytomatrix are major factors determining the dynamics of exocytosis.

The shortest distance between the two membranes would be 2–3 nm for a lipid bilayer at full hydration (598), and the initial step of membrane fusion may be point-like local contact between the two membranes (FIGURE 4A) (159, 598) from which the hemifusion stalk intermediate (FIGURE 4B) is formed. The early metastable and transition states are mostly inferred from molecular dynamics simulations, as the transitional state is too fast to directly observe. The prestalk fusion intermediate may be initiated from only one lipid molecule, whose two hydrocarbon chains are inserted into the opposing membranes (598). Such reactions must be facilitated by the buckling of the plasma membrane and end cap formation (FIGURE 4A) (402) by proteins such as SNAREs, synaptotagmin (269, 387, 582), or Doc2 (208) (see sect. V). The end cap, or membrane dimple, was reported in ultrastructural studies of secretory cells (425) and TIRF microscopy (10).

In ultrafast exocytosis of docked synaptic vesicles, the osmotic pressure generated by a hypertonic sucrose solution (HTSS) can induce exocytosis (67, 532). Positive pressure also enhances exocytosis from chromaffin cells (321). The positive pressure forces the vesicles closer to the plasma membrane, which might facilitate initial contact formation of the two lipid bilayers (179).

Another factor that may affect contact formation is the curvature of the vesicle membranes. Small vesicles with a greater curvature can more readily form a contact with the plasma membrane (FIGURE 4, D AND E). This may explain why the rate of exocytosis of larger vesicles is slower than

![FIGURE 4. Schematics depicting membrane dynamics during exocytosis. A: end-cap formation. Fusion is initiated by contact between the vesicle and the plasma membrane. B: a stalklike hemifusion intermediate. C: the fusion pore. Fusion pore opening is facilitated by an inverse cone-shaped lipid in the outer leaflet of the membrane (red arrowhead) or a cone-shaped lipid in the inner leaflet (blue arrowhead). D and E: preferential contact by smaller vesicles due to reduced steric hindrance and a larger membrane curvature. F: membrane tension assisting the opening of the fusion pore. Smaller vesicles may generate a larger tension to facilitate the opening of the fusion pore.](http://physrev.physiology.org/issue/92/10/1929)
that of the small vesicles (FIGURE 1). Although this discrepancy in the rate of exocytosis may be due to the different types of SNAREs expressed, exocytosis of small vesicles in cells lacking neuronal SNAREs is as fast as the exocytosis of LDCVs that have neuronal SNAREs (<1 s), suggesting that the size of the vesicles contributes to the kinetics of exocytosis.

2. Hemifusion intermediates

After the initial physical contact between the two membranes, a local lipidic connection between the proximal (contacting) monolayers of the fusing membranes is formed from a pointlike contact (FIGURE 4A) (331). This hemifusion intermediate is called the stalk intermediate and is currently thought to adequately describe the transitional stage of membrane fusion (103, 280). Molecular dynamics simulations have predicted similar or lower energy barriers (377, 452, 598). The stalk intermediate can rapidly lead to either opening of the fusion pore or to stable hemifusion diaphragms (402).

The hemifusion diaphragms can be fixed and their ultrastructures confirmed (375, 727). Such structures are detected in protein-free bilayers (100, 106, 381, 716), SNARE-incorporated lipid bilayers (2), and biological membranes (350, 413, 602, 701). Vesicles in the active zone might already be hemifused (727). The fusion pore can rapidly open from the stalk intermediate; however, the hemifusion diaphragm may be a dead-end structure if no tension is applied. In fact, a recent single vesicle imaging study directly demonstrated that hemifusion of some vesicles does not proceed to full fusion (341, 701).

E. Fusion Pores

Opening of the fusion pore that connects the vesicle cavity with the extracellular space is a critical step in exocytosis, and it is induced from the stalk intermediates (FIGURE 4B). The early stage of fusion pore opening was identified in ultrastructural studies (463) and later revealed to be semi-stable using membrane capacitance measurements, which enable rapid and quantitative measurement of fusion pore sizes between 0.2 and 2 nm (357, 425, 441). The fusion pore normally rapidly dilates to induce irreversible full fusion for complete secretion and insertion of membrane lipids and proteins, whereas it shuts again and becomes semi-stable before undergoing full fusion.

1. The lipidic nature of the fusion pore

The semi-stable nature of the fusion pore was initially interpreted as suggesting that the fusion pore was made of gap junction-like proteins spanning the two membranes (65, 160, 280). The V0 membrane sector of the V-ATPase is one such candidate in yeast vacuolar fusion (489, 663) and in Drosophila synapses (250). It has, however, since been revealed that the role of V0 ATPase may be indirect (405, 695), possibly by helping SNAREs to open the fusion pore (160). Another candidate was the oligomerized syntaxin transmembrane domain (225, 227). It needs to be explained, however, how a tightly oligomerized transmembrane domain (TMD) can reversibly disassemble again to dilate the fusion pore. Also, the protein spanning the other bilayer has not been identified for the V0-ATPase or syntaxin TMD.

In contrast, many lines of evidence suggest that the fusion pore is mostly, if not entirely, lipidic. First, the semi-stable nature of the fusion pore has been demonstrated using pure lipid bilayers (94). Second, there is considerable transfer of lipid bilayers during flickering of the fusion pore (424), before the opening of a 1.4-nm fusion pore (636), and before diffusion of vesicle proteins (640). Finally, exocytosis depends critically on the composition of membrane lipids, in the same manner as predicted by lipid structure (see sect. IVF). This is difficult to reconcile with a purely proteinaceous model.

2. How does the fusion pore open?

It is theoretically predicted that fusion pore opening and growth consume energies of each ~40 kBT (119, 338), which is larger than the formation of hemifusion intermediates (104). In addition, fusion pore opening and growth depends on membrane tension (FIGURE 4F). Such tension may be supplied by the elastic stress of the curved plasma membrane (FIGURE 4D) (402) and/or by the elastic stress on the membranes of curved vesicles (FIGURE 4F) (424). In small vesicles, the membrane tension can be large, which facilitates the opening of the fusion pore. This provides another possible explanation as to how the size of vesicles may affect the kinetics of exocytosis (FIGURE 1). All three steps (FIGURE 4, A–C) of membrane fusion are facilitated by increasing membrane curvature and tension.

If the energy supplied by the proteins exceeds that of membrane fusion, the fusion pore will rapidly dilate and reach full fusion (636). If the two energies are similar, the fusion pore can be closed after the initial opening, or can flicker open and closed. These behaviors have been observed using membrane capacitance measurements (171, 247, 291, 441), amperometry (5, 746), and fluorescence imaging (321, 636). Fusion pore flickering has also been reported in proteoliposomes (722). Such transient opening may be sufficient for neurotransmitter release, and a short recycling pathway has been postulated (736). Short and narrow fusion pore opening, however, is not sufficient to release large molecule hormones (37, 636) or supply membrane proteins. Thus transient opening of a fusion pore is considered a fusion failure for hormonal secretion. The major population of vesicles in adrenal chromaffin cells (322) and pancreatic β cells (380, 638) undergo full fusion, particularly in intact preparations, whereas kiss-and-run exocytosis is
more abundant in dissociated culture preparations in which exocytosis was measured at the membrane facing the coverslip (659). Since full collapse of vesicles into the plasma membrane occurs rapidly (1 s) (321), their exocytosis may result in water secretion (658).

The stability of the fusion pore depends on a variety of factors other than membrane tension. The lifetime of the fusion pore is affected by its cargo (416, 417). Full fusion is favored by stronger stimulation in some preparations (517, 561, 736), while kiss-and-run events are favored by stronger stimulation in the other preparations (4). Exocytosis of LDCVs containing a brain-derived neurotrophic factor (BDNF) is full fusion in dendrites and kiss-and-run fusion in axons (397). SNAREs, and many SNARE-interacting proteins, including Munc18a (173, 292), syntaxin-1A (579, 742), Doc2 (178), SNAP25 (163, 436), VAMP2 (66, 316), syntaxin (227), SNARE complex (226), and complexin (9, 17), affect fusion pore properties (see sect. V). This supports the idea that SNARE-related proteins impinge on the opening of the fusion pore. It is not always easy for fusion proteins to supply a tension that is sufficient to readily overcome the energy required for fusion pore opening and dilation (589), and the lifetime of the fusion pore may contribute to the time courses of secretion (415).

F. Lipid Composition

The kinetics of exocytosis are profoundly affected by lipid composition, which exert either direct physical effects on membrane dynamics or indirect effects on the membrane proteins.

1. Direct effects on membrane structure

The plasma membrane is composed of various phospholipids and cholesterol, the concentrations of which reach molar levels and physically affect exocytosis. The composition of membrane lipids varies in different cells, different subcellular domains, and in each leaflet of the bilayer. Synaptic phospholipids are enriched in unsaturated fatty acids, such as arachidonic acid, and comprise 10% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), 60% phosphatidylserine (PS), 60% phosphatidylinositol (PI), and 15% sphingomyelin (SM) (130). Each lipid can be characterized quantitatively by the curvature of the monolayer that it spontaneously forms and can be classified into one of three groups according to the shape of that monolayer. Some membrane lipids are flat, such as PE and SM. Most are cone-shaped, such as PC and cholesterol, and unsaturated free fatty acids, and generate a negative curvature of the membrane monolayer (FIGURE 4C) (188). Finally, lyso-phosphatidylcholine (LPC), which is generated by phospholipase A2 (PLA2), and phosphatidylcholine 4,5-bisphosphate [PI(4,5)P2], which is generated by phosphorylation of PI, are inverse-cone-shaped and impart a positive curvature to the membrane (FIGURE 4C). The curvature of lipids affects the two membrane leaflets in an opposing manner (FIGURE 4C). For example, abundant PEs in the inner leaflet facilitate negative curvature and fusion (101, 329), whereas those in the outer leaflet prevent negative curvature and fusion. Conversely, inverse-cone-shaped lipids in the inner leaflet hinder negative curvature and fusion (411) and exocytosis (105), whereas those in the external leaflet promote exocytosis.

One valuable example is the effect of snake presynaptic phospholipase A2 neurotoxins (SPANs), which cause exhaustive exocytosis and paralyze neuromuscular junctions (525). This action appears to operate downstream of SNARE complex formation (526). The toxin hydrolyzes PC into LPC and fatty acids (408, 526). Inverse-cone-shaped LPCs remain confined to the external leaflet of the presynaptic membrane, whereas fatty acids have a high rate of transbilayer movement and will partition between the two membrane leaflets. The cone-shaped fatty acids in the inner leaflet of the plasma membrane facilitate the formation of hemifusion intermediates, whereas the inverted cone-shaped LPCs in the outer leaflet of the plasma membrane promote opening of the fusion pore (FIGURE 4C). These effects of SPANs can be fully mimicked by the extracellular application of LPC and free fatty acids (FFA) (525). This illustrates that a change in lipid composition can have serious consequences in terms of synaptic exocytosis.

2. Lipid effects on membrane proteins

Many effects of membrane lipids on SNAREs and synaptotagmins have been reported (130, 695). Some of these are listed below.

Phosphoinositides such as PIP2 profoundly modulate membrane dynamics, including signal transduction, the membrane cytoskeleton, dynamin, and ion channels (143). Thus PIP2 may affect exocytosis indirectly via the cytoskeleton (50). PIP2 increases the speed of synaptotagmin binding to the plasma membrane (28, 565), and binds to CAPS (374), Mint (462), and rabphilin3 (114), which might result in stabilization of the fusion pore (200). PIP2 also influences both the spatial and functional properties of syntaxin at the plasma membrane (430, 666).

Synaptic vesicles are enriched with cholesterol (30–40% of membrane lipids), which is known to modify the TMD of VAMP to form a parallel dimer rather than a scissor-like conformation and, consequently, to facilitate lipid mixing (649). Moreover, cholesterol serves as a strong stimulus for hemifusion but acts as a mild stimulus for pore opening and expansion during SNARE-mediated fusion (93). Cholesterol enhances spontaneous exocytosis, but suppresses evoked exocytosis in synapses (687).
t-SNAREs are involved in triggering exocytosis. For example, PIP2
Membrane lipids have a direct effect on ion channels, which
the vesicle membrane and the increased size of the RRP.
due to the interaction between VAMP and sphingosine in
vates synaptic vesicle exocytosis (129). This effect may be
Sphingosine facilitates SNARE complex assembly and acti-
vents its dilation (741).
Membrane lipids have a direct effect on ion channels, which
are involved in triggering exocytosis. For example, PIP2
modulates K+ channels and arachidonic acids modulate Ca2+
channels (180).

**V. PROTEINS REGULATING ULTRAFAST EXOCYTOSIS**

In this section, we describe the molecular bases of ultrafast exocytosis. Exocytosis is completely abolished by deletion of SNAREs, NSF, Munc18, and Munc13, and partially impaired by deletion of complexin, CAPS, and snapin. The same molecules are utilized for slower exocytosis, but they may be used at different stages of the process, as described in section VI. Specific CAZ proteins are required for the organization of exocytosis in the active zone, but not for exocytosis itself.

**A. SNAREs**

SNAREs are the minimal machinery required for membrane fusion (356, 533, 620, 690) and are the targets of the clostridial neurotoxins that cause tetanus and botulism (51, 52, 358, 564). These neurotoxins specifically cleave SNAREs and completely block ultrafast exocytosis of synaptic vesicles (89, 270, 282, 546). Loss of SNARE proteins results in a complete loss of Ca2+-evoked ultrafast exocytosis in *Drosophila* (67, 139, 577), *C. elegans* (540), and mice (571, 686). Injection of the syntaxin SNARE motif causes strong inhibition of ultrafast exocytosis in the squid giant synapse (456). SNAREs are associated with the plasma membrane voltage-gated Ca2+ channels responsible for the Ca2+ influx that triggers the exocytosis of synaptic vesicles (44, 541, 723).

SNAREs are characterized by a conserved heptad repeat of ~60 residues called a SNARE motif. VAMP2 contains one SNARE motif adjacent to the COOH-terminal TMD (FIGURE 5A). Syntaxin contains a large NH2-terminal region that consists of three autonomously folded α-helical bundles (H_{abc} domain), an unfolded linker, a SNARE motif (also called a H3 domain), and a COOH-terminal TMD (FIGURE 5, A AND D). The H_{abc} domain binds to the SNARE motif intramolecularly to form a closed conformation, which is stabilized by Munc18 (155) (FIGURE 8). SNAP25 possesses two SNARE motifs separated by a short linker containing palmitoylated cysteines, which are responsible for membrane anchoring (538) (FIGURE 5A).

The individual SNARE motifs are largely unstructured in solution, but when four SNARE motifs are mixed, they assemble into a stable α-helical ternary complex. The crystal structure of the SNARE complex reveals a parallel four-helix bundle with all COOH termini exposed on one side (628) (FIGURE 5B). Four charged residues from individual SNARE proteins form ionic interactions at the center of the complex (called zero layer); one arginine (R) from VAMP2, one glutamine (Qa) from syntaxin, and two glutamines (Qb and Qc) from SNAP25. Therefore, SNAREs are classified into four groups: R-, Qa-, Qb-, and Qc-SNAREs (165).

Assembly of the SNARE complex proceeds from the NH2-terminal to the COOH-terminal transmembrane region in a zipper-like fashion and induces membrane fusion. It is not known how far the zippering is completed before ultrafast exocytosis is triggered, but the resulting SNARE complex in *trans* (trans-SNARE complex) is expected to bring vesicles and the plasma membranes into close apposition for ultrafast exocytosis (283) (FIGURE 8). The SNARE complex is remarkably stable; therefore, the energy derived from its formation can be used to overcome energy barriers for membrane fusion (229). This energy can be passed to the TMDs to locally disrupt lipid membranes (613).

**B. Munc18**

Sec1/Munc18 (SM) proteins are essential for intracellular membrane trafficking (233, 264, 455). Deletion of Munc18a, a major neuronal SM protein in mammals, completely blocks evoked and spontaneous synaptic transmission (670). The binding partner of SM proteins is syntaxin1 (FIGURE 5, C AND D) (192, 193, 234, 491). There are three distinct modes of Munc18a binding with syntaxin1 (82). First, Munc18a binds to closed syntaxin1. The horsehoe-shaped cavity of Munc18–1 binds to closed syntaxin1 (FIGURES 5D AND 8). Munc18a may clamp syntaxin1 to block nonspecific SNARE complex assembly during intracellular trafficking by shielding syntaxin1 from intracellular SNARE partners (407). Munc18 may act as a chaperone for syntaxin (193, 235, 490) because when Munc18a is deleted, syntaxin expression is reduced by 70% (78, 650, 691). Second, Munc18a binds with the NH2-terminal sequence of syntaxin (59, 156, 484, 508). An open
mutant of syntaxin (syntaxin LE mutant; L165A, E166A), which disrupts the closed conformation, can interact with Munc18a, whereas syntaxin lacking the NH2-terminal sequence cannot and fails to assemble a SNARE complex (523). Thus the binding of Munc18a to the NH2-terminal region of syntaxin is critical during SNARE assembly as it allows the transition of syntaxin1A from the inactive to the active conformation (FIGURE 8). Finally, Munc18a directly interacts with the ternary-SNARE complex (154, 318, 586) (FIGURE 8) to facilitate the regulation of SNARE functions.

C. NSF and SNAP

A tight SNARE complex (cis-SNARE complex) remains in the plasma membrane after exocytosis and needs to be disassembled and recycled to enable another round of fusion events. Disassembly of the SNARE complex is mediated by the NSF (Sec18p in yeast), and the interaction between the SNARE complex and NSF requires SNAPs (Sec17p in yeast). NSF is a soluble homo-hexameric ATPase, which contains an NH2-terminal domain and two ATP-binding
domains. Although overall sequence similarity between SNARE subtypes is limited, all SNARE complexes are disassembled by αSNAP and NSF (12, 385, 475, 601). The extreme stability of the SNARE complex means that NSF has to use energy derived from ATP hydrolysis to disassemble it (FIGURE 8). Three SNAPs are recruited from the cytoplasm to every one SNARE complex in the membrane, and the resulting SNAP/SNARE complex recruits a NSF heteromer, leading to the disassembly of the SNARE complex (189, 230, 231, 256, 601).

In mammals, SNAPs form a small protein family with three isoforms, termed α, β, and γSNAP, in which αSNAP is the most abundant and ubiquitous isoform (115). Neither deletion of βSNAP nor a reduction in αSNAP levels affects nerve cell viability, differentiation, or function (80); however, constitutive deletion of αSNAP in mice leads to embryonic lethality (91, 259). αSNAP/NSF acts at the priming stage during synaptic transmission (313, 363, 578). Mutations in NSF block synaptic transmission due to accumulation of syntaxin and SNARE complexes in synaptic vesicles (362, 363). Microinjection of NSF peptides that inhibit the ATPase activity of NSF also inhibited synaptic transmission, and did so in a few seconds before ultrafast exocytosis (336).

D. Synaptotagmins and Other Ca2+ Sensors

Synaptotagmin1 is enriched in synaptic vesicles and is the major Ca2+ sensor for ultrafast exocytosis (22, 95, 144, 328, 364, 453, 621, 724), but not for asynchronous exocytosis (194). Presynaptic microinjection of synaptotagmin peptides or anti-synaptotagmin antibodies inhibits synaptic transmission in squid (53, 187, 419).

Synaptotagmin1 contains two tandem C2 domains (C2A and C2B), which are homologous to the regulatory C2 region of protein kinase C. The crystal structure of the two C2 domains shows that they have a very similar β-sandwich structure (FIGURE 6B) (169, 191, 626, 627). Both domains bind Ca2+ via loops located at the top of the β-sandwich. Five acidic amino acid residues on the two loops form three Ca2+-binding sites on the C2A domain and two Ca2+-binding sites on the C2B domain (169, 584). Both C2 domains bind phospholipids in a Ca2+-dependent manner (488, 621). Synaptotagmin1 interacts with other synaptotagmins (70, 186, 487, 702), including syntaxin1 (29, 396), SNAP25 (29, 567, 739), VAMP2 (184), a syntaxin1/SNAP25 binary complex (29, 520, 660), and the SNARE complex (29, 57, 125, 739). It has been shown that bacterially expressed synaptotagmin1 shows nonspecific binding in vitro (662).

Ca2+ binding to the C2B domain is more important for transmitter release than Ca2+ binding to the C2A domain. Disrupting Ca2+-binding sites on the C2B domain has a dramatic effect on ultrafast exocytosis (382, 449), whereas disruption of Ca2+-binding sites on the C2A domain has a small effect (167, 615). Ca2+ bound to the C2B domain of synaptotagmin1 promotes binding between two membranes due to its highly positive electrostatic potential. The positively charged sites on the C2B domain directly interact with the negatively charged phospholipid head groups, and may result in local membrane bending for initiation or acceleration of membrane fusion (14, 387).

Asynchronous (194, 365, 449) and spontaneous (364, 704) exocytoses are augmented in neurons lacking synaptotagmin1. This suggests that asynchronous and spontaneous exocytoses are mediated by Ca2+ sensors other than synaptotagmin1. Attractive candidates for a slow Ca2+ sensor are the double C2 domain (Doc2) proteins (467, 468). Doc2 proteins are soluble proteins, which are enriched in synapses. Doc2 has three isoforms, Doc2α, Doc2β, and Doc2γ (186, 547). Like synaptotagmin1, these proteins contain C2 domains but have relatively high affinity for Ca2+ (478). Knocking out Doc2 in cultured hippocampal neurons results in normal evoked responses, but reduces spontaneous release frequency by 50% (208). Doc2 may be a Ca2+ sensor for asynchronous exocytosis (718) and may also be involved in spontaneous exocytosis (209, 478). Synaptotagmin1 acts as a Ca2+ sensor for spontaneous exocytosis, although its deletion increases spontaneous exocytosis as it also clamps a more sensitive Ca2+ sensor (704).

E. Complexin

Complexins/synaphtins are soluble proteins with a molecular mass of 19–20 kDa, which are expressed in the brain (277, 403, 637). Deletion of complexin results in a 70% reduction of ultrafast exocytosis (254, 271, 295, 389, 400, 511, 710–712, 717) and facilitates spontaneous exocytosis (295, 400, 717). This is akin to the effects of synaptotagmin1 (194) (TABLE 2), except that it can be overcome by an increase in external Ca2+ concentration. Complexin does not bind Ca2+ but directly associates with synaptotagmin1 (643). Complexin binds with the assembled SNARE complex at the central helix with nanomolar affinity (644), and this binding is necessary for complexin function (400, 644, 711). The crystal structure of the complexin/SNARE complex shows that the central helix of complexin binds to the surfaces of VAMP2 and syntaxin in an anti-parallel fashion (58, 97) (FIGURE 6C). The NH2-terminal complexin region is more important for ultrafast exocytosis than the COOH-terminal region (295, 711). The NH2 terminus of complexin also binds to the COOH terminus of the SNARE complex to facilitate ultrafast exocytosis (400, 711) and priming of synaptic vesicles (717).

In addition to these mechanisms, complexin inhibits liposome or cell-cell fusion by clamping full zippering of the trans-SNARE complex (195, 562, 639). In fact, the accessory helix (residues 26 to 47) of complexin (FIGURE 6D) can interact with the membrane-proximal region of the SNAREs
to prevent their zipperng (196) and may cross-link SNAREs into a zig-zag array (294, 334, 354). The complexin clamp may be competitively relieved by Ca$^{2+}$/H$^{+}$-bound synaptotagmin1, thereby inducing fast Ca$^{2+}$/H$^{+}$-evoked membrane fusion (400, 639, 717).

**F. Munc13**

Munc13 proteins are large soluble proteins that are necessary for ultrafast exocytosis (16, 21, 518, 669). Munc13 proteins contain multiple domains including C$_2$ domains.

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**FIGURE 6.** A: the primary structures of mouse synaptotagmin1 and Doc2. The number of residues is indicated above each diagram. TMR, transmembrane region; MID, Munc13–1 interacting domain. B: the crystal structure of the C2 domains [Protein data bank (PDB) ID 1BYN, ID 1K5W] (169, 584). The connection between the C2A domain and the C2B domain was drawn by hand. The spheres represent Ca$^{2+}$/H$^{+}$. C: the primary structure of mouse complexin I. NT, NH$_2$-terminal region; CT, COOH-terminal region. D: the crystal structure of a SNARE complex and complexin (PDB ID 1KIL) (97).
(C2A, C2B, and C2C), a RIM-binding domain (RBD), a calmodulin-binding domain (CBD), a C1 domain (C1), and Munc homology domains (MHD1 and MHD2) (FIGURE 7). The RBD may take part in the Munc13/RIM interaction to recruit Munc13 to the active zone (11), and the CBD and C2 domains may be involved in Ca\(^{2+}\)-dependent short-term plasticity (293). The C1 domain of Munc13 proteins binds diacylglycerol and mediates the action of phorbol esters during exocytosis (46, 390, 516).

The MUN domain (residues 859–1531) partially rescues release in Munc13 knockout mice (39), which is further augmented by a domain encompassing the C2C domain (383). Ultrafast exocytosis in a C. elegans UNC13 null mutant was partially rescued by overexpression of the open syntaxin1 LE mutant (518), indicating that UNC13 facilitates the open configuration of syntaxin. Such action may be mediated by the MUN domain, which can bind the NH\(_2\)-terminus of syntaxin (47), and to heterodimeric and heterotrimeric SNARE complexes (211, 378, 694). The MUN domain also interacts with the NH\(_2\)-terminal domain of Doc2 (466) and mediates Ca\(^{2+}\)-dependent recruitment of vesicles to the target membrane synergistically with diacylglycerol (207, 251).

The Munc13 family comprises brain-specific Munc13–1, Munc13–2, and Munc13–3 and nonneuronal Munc13–4. Munc13–1 is utilized in phasic synapses in which the RRP is full, and Munc13–2 is utilized in tonic synapses, in which the RRP is gradually refilled after repetitive stimulation (531). A splice variant of Munc13–2, ubMunc13–2, and Munc13–4 are ubiquitously expressed (327) and may be necessary for exocytosis of vesicles in nonneuronal cells (251, 513).

G. CAPS

CAPS was originally isolated from brain cytosol as a factor required for Ca\(^{2+}\)-triggered LDCV exocytosis (679). Deletion of CAPS reduces ultrafast exocytosis by 70% by reducing the RRP (288, 514), suggesting that CAPS is also in-

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**FIGURE 7.** The primary structures of priming and CAZ proteins. The number of residues is indicated above each diagram. From top to bottom: mouse Munc13–1, CAPS1, snapin, tomosyn, RIM1, CAST1, bassoon, and piccolo. C2A, C2A domain; CBD, a calmodulin-binding domain; C1, C1 domain; MHD, Munc homology domain; C2C, C2C domain; C2, C2 domain; PH, PH domain; CC, a predicted coiled-coil domain; SNARE, VAMP-like SNARE motif; ZF, zinc-finger domain; PDZ, PDZ domain.
involved in ultrafast exocytosis. CAPS is an evolutionarily conserved multidomain protein, for example, Caenorhabditis elegans has a single CAPS isoform (UNC-31), and vertebrates have two closely related isoforms (CAPS1 and CAPS2). The pleckstrin homology (PH) domain interacts with PIP2 (206). The CAPS protein exhibits sequence similarity to the MUN domain of Munc13–1 (47, 327) (FIGURE 7), which is suggested to be a tethering factor (355). CAPS binds independently to each of the three SNARE proteins (126) and markedly accelerates SNARE-dependent liposome fusion in vitro by promoting transSNARE complex assembly.

H. Snapin

Snapin was identified as a SNAP25-binding protein and is expressed in both neuronal and nonneuronal cells (85, 274) (FIGURE 7). Microinjection of a SNAP25-binding site peptide derived from snapin inhibits synaptic transmission of cultured superior cervical ganglion neurons (274). The SNAP25-binding site peptide of snapin also blocks the interaction between synaptotagmin1 and the SNARE complex. Deletion of snapin reduces excitatory postsynaptic currents (EPSC) by 70% and slows EPSC kinetics (477). Deletion of snapin also leads to a marked reduction in the amount of synaptotagmin1/SNARE complex in the mouse brain (477). These results suggest that snapin stabilizes the coupling between synaptotagmin1 and the SNARE complex during Ca2+/H11001-triggered ultrafast exocytosis at central nerve terminals. The synaptotagmin1/SNARE complex, which also contains snapin, does not include complexin (477), suggesting that snapin and complexin exclusively regulate the synaptotagmin1/SNARE complex.

I. Tomosyn

Tomosyn is a 130-kDa cytosolic protein identified as a syntaxin-binding protein in rat brain cytosol (183). Tomosyn is a negative regulator of exocytosis because its overexpression causes a significant reduction in exocytosis (27) and its deletion causes enhanced neurotransmitter release (158, 548) through increased synaptic vesicle priming (198, 203, 204, 401). Tomosyn comprises two domains: a large conserved NH2-terminal domain and a COOH-terminal SNARE motif, which is homologous to the VAMP2 SNARE motif (395) (FIGURE 7). The COOH-terminal SNARE motif can form a ternary-SNARE complex with syntaxin and SNAP25 in the same manner as the neuronal SNARE complex (240, 495). Thus tomosyn can compete with VAMP2 for assembly with binary-SNAREs. The crystal structure of Sro7p, a yeast homolog of tomosyn, reveals that the NH2-terminal 14 WD40 repeats fold into two seven-bladed β-propeller domains (241) (FIGURE 7), which directly bind to synaptotagmin1 in a Ca2+/H11001-dependent manner and inhibit Ca2+/H11001-dependent exocytosis (713).

J. Other CAZ Proteins

1. RIM

RIM (Rab3-interacting molecule) is a binding partner for Rab3 (683), a highly abundant protein in synaptic vesicles. Deletion of RIM strongly reduces presynaptic Ca2+/H11001 channel density and the size of the RRP (228, 297). RIM is a large soluble protein that contains various domains including a zinc-finger, PDZ, and C2 (FIGURE 7). RIM1 also binds to several other proteins, including cAMP-GEFII, a cAMP sensor (474), RIM-binding proteins (RIM-BPs) (684), Munc13–1 (48), synaptotagmin, SNAP25, N-type Ca2+/H11001 channels (121, 297), and alpha-liprins (370) to form a protein scaffold in the presynaptic nerve terminal. This suggests that RIM tethers N- and P/Q-type Ca2+/H11001 channels to presynaptic active zones via a direct PDZ domain-mediated interaction and acts during vesicle priming by interacting with Munc13. RIM may also facilitate SNARE assembly by reversing autoinhibitory homodimerization of Munc13 (141).

2. CAST

CAZ-associated structural protein (CAST) directly or indirectly binds CAZ proteins to form a large molecular complex at the active zone (249, 461) (FIGURE 7). CAST also binds to RIM1 and, indirectly, to Munc13–1 to form a ternary complex. The last three amino acids (I, W, and A) are critical for binding to the PDZ domain of RIM1. Bassoon, another CAZ protein, is also associated with this ternary complex. Thus there exists a network of protein-protein interactions between CAZ proteins (461). Deletion of CAST selectively affects the exocytosis of inhibitory synapses by reducing the size of the RRP (296).

3. Bassoon

Bassoon is a large protein containing two NH2-terminal zinc-finger domains, several coiled-coil domains, and a stretch of polyglutamines at its COOH terminus (646) (FIGURE 7). The zinc-finger domains bind to the prenylated Rab acceptor protein-1 (PRA1) (79, 394), a molecule that interacts with rab3A and VAMP/synaptobrevin II in vitro (166). Bassoon is localized at the CAZ of excitatory and inhibitory synapses (519, 646) as well as at the base of retinal ribbon synapses (61). In the retinas of bassoon knockout mice, the presynaptic ribbons are not anchored to the plasma membrane and are free-floating (145). The interaction between bassoon and RIBEYE, a ribbon-specific protein, is required for the formation of photoreceptor ribbon synapses (645).
4. Piccolo

Piccolo is a 500-kDa protein containing zinc-finger domains. It is structurally related to bassoon, but it also contains PDZ and C2 domains (90, 166) (FIGURE 7). The piccolo zinc-finger domains directly interact with PRA1 (79, 166, 394). Deletion of piccolo and bassoon impairs vesicle clustering without directly affecting neurotransmitter release (429).

Thus 10 different proteins are required for ultrafast exocytosis. They may appear redundant if they were only utilized for ultrafast exocytosis; in reality, however, they support different forms of exocytosis, even in the active zone, and

![Diagram of SNARE complex](http://physrev.physiology.org/)
pleiotropic exocytoses in a variety of other preparations (see sect. II), as described in the next section.

VI. MOLECULAR MODELS OF EXOCYTOSIS

A. Different trans-SNARE Configurations

The neuronal SNAREs (syntaxin-1, SNAP25, and VAMP2) assemble to form a ternary complex that bridges the vesicle and the plasma membrane at some point during exocytosis. To produce sufficient force for membrane fusion, the trans-SNARE assembly cannot be as tight as the cis-SNARE assembly. Indeed, the trans-SNARE complex can be reversibly disassembled (432, 707). The trans-SNARE state may be the initial configuration required for ultrafast exocytosis in the active zone and fast exocytosis in neuroendocrine cells.

1. Energetics of SNARE-mediated ultrafast exocytosis of synaptic vesicles

The entire process of ultrafast exocytosis is depicted by the energy profile of a SNARE complex in Figure 8. Hydrolysis of ATP generates 38 \( k_B T \) of energy and induces disassembly of the SNARE complex, whose zipperping produces at least \( \sim 35 \) \( k_B T \) of energy needed for hemifusion of pure lipid bilayers (333); the formation and expansion of the fusion pore requires even more energy (119). Thus hydrolysis of multiple ATPs and SNAREs likely contributes to the exocytosis of single vesicles.

The two forms of the ternary-SNARE complex, cis- and trans-, are depicted in Figure 8. Disassembly of cis-SNARE by NSF generates a “unitary-SNARE” state, in which each SNARE is not bound to other SNAREs. Subsequent assembly of t-SNAREs (syntaxin and SNAP25) generates a “binary-SNARE” complex, and then a v-SNARE (VAMP2) assembles with the binary complex to form a ternary trans-SNARE complex connecting the vesicle with the plasma membrane. Priming processes represent a progression from the cis-SNARE state to the trans-SNARE state, which favors the membrane fusion reaction. From the length of time taken to form the RRP (10–20 s), we estimate that the activation energy of the unitary and binary states is \( \sim 20 \) \( k_B T \) (80, 532) (Figure 1). Likewise, the activation energy of exocytosis is predicted to be 20 \( k_B T \) for vesicles in the RRP at resting levels of \([Ca^{2+}]_i\), which undergo exocytosis with a time constant of \( \sim 10 \) s (373). These resting transitions in SNARE states are depicted as gray lines in Figure 8.

If the trans-SNARE complex represents the initial state that triggers exocytosis, it needs to be protected from further SNARE assembly and fusion. Although synaptotagmin1 and complexin may have a clamping role (108, 195, 197, 341, 562, 639, 661, 717), the accumulation of vesicles at the active zone is unaffected in mice lacking synaptotagmin1 or complexin, suggesting that these molecules alone may not explain the arrest of fusion. It is also possible that SNAREs (341, 685) and CAZ proteins may act structurally as the fusion clamp. Also, the heptagonal CAZ grid encasing the vesicles at the active zone (152) may sterically hinder membrane fusion. Such barriers can readily be overcome by alteration of the lipid composition (525, 654) (see sect. IVF) or osmolarity (see below).

Exocytosis from the trans-SNARE state can be triggered by \( Ca^{2+} \) sensors, such as synaptotagmin1 and Doc2. Accounting for a fusion rate of 1 ms, the last energy barrier for the trans-SNARE configuration for ultrafast exocytosis in the presence of \( Ca^{2+} \) activation is 14 \( k_B T \) (Figure 8, trans-SNARE configuration). Importantly, exocytosis in the active zone can be induced by a HTSS (80, 532), even in mice lacking synaptotagmin1 (704) or complexin (511). This suggests that the trans-SNARE complex takes a particular organization for ultrafast exocytosis (217) such that HTSS mimics the action of synaptotagmin1 and complexin. HTSS may shrink the cytosol (532) and force the plasma membrane closer to the vesicles, facilitating the initial contact between the two membranes. This may be the role of \( Ca^{2+} \)-dependent binding of synaptotagmin1 and Doc2 on the membrane phospholipids (208, 269). The mechanical pressure may be utilized for synaptic functions, as is the case for chromaffin cells (321). Importantly, Munc18a, Munc13, and CAPS are necessary for HTSS-induced exocytosis (Table 2).

FIGURE 8. The energy profile of SNARE proteins in the cytosol, where they exist in three distinct states: trans-SNARE, binary-SNARE, and unitary-SNARE. Top panel shows a schematic illustration of SNARE and Munc18 during exocytosis of a vesicle. Blue circles represent vesicles. Munc18 catalyzes the transition from the unitary-SNARE to the cis-SNARE. The free energy profiles of a set of SNARE proteins and associated molecules are predicted from the time constants of transitions between the states as described in the text and are depicted in the bottom three panels. The gray trace in each panel depicts the default profile for that configuration. The black and red traces show the energy profiles in resting and stimulated cells, respectively. In the trans-SNARE configuration in the active zone, Munc13 assists in the formation of the binary complex, and RIM, CAPS, and PIP2 promote formation of the trans-SNARE complex. Fusion is prevented by CAZ, SNAREs, synaptotagmins, and complexin, whereas it is triggered by \( Ca^{2+} \) binding with synaptotagmins and Doc2. In the binary-SNARE configuration, exocytosis is triggered from the binary-SNARE state. Cytomatrix and tomosyn prohibit the formation of the trans-SNARE complex, whereas CAPS and PIP2 promote it after stimulation. \( Ca^{2+} \) reduces the energy barrier for the trans-SNARE and fusion steps. In the unitary-SNARE configuration, exocytosis is initiated from unassembled SNAREs. The motility of the vesicles in the cytomatrix and assembly of unitary-SNAREs are regulated. Triggers promote vesicle motility and SNARE assembly, which is assisted by priming proteins, such as Munc13, CAPS, and snapin. Distinct initial SNARE configurations can naturally account for the enormous kinetic diversity (1 ms to 100 s) of exocytosis.
indicating that they are involved in the formation of tight SNARE assemblies for ultrafast exocytosis. Ultrafast exocytosis shows only one kinetic component when induced by the uncaging of caged Ca\textsuperscript{2+} in the calyx of Held (678), whereas it shows two kinetic components when induced by depolarization, reflecting the heterogeneous distribution of synaptic vesicles relative to Ca\textsuperscript{2+} channels. Such heterogeneous distribution of vesicles, however, may not exist in small boutons or inhibitory synapses (543).

2. Different trans-SNARE states

Compared with ultrafast exocytosis in the active zone, exocytosis in adrenal chromaffin cells is slower. Membrane capacitance measurements indicate that the fastest time constant in Ca\textsuperscript{2+}-primed adrenal chromaffin cells is 30 ms (673, 675). Nevertheless, it was suggested that SNAREs in adrenal chromaffin cells are in the trans-state because rapid capacitance increases were resistant to botulinum neurotoxins (BoNTs) (706) and an antibody against free SNARE (707), neither of which act on assembled SNAREs. The slow kinetics of LDCV exocytosis from Ca\textsuperscript{2+}-primed chromaffin cells may be partly due to the large size of the vesicles; 500 nm compared with 40 nm in the synapse (FIGURE 3, D AND E). Moreover, CAZ is absent in chromaffin cells, and exocytotic vesicles contact with an ordinary submembraneous cytomatrix. The distance between chromaffin granules and the plasma membrane in the active zone may be functionally larger than for synaptic vesicles. As a result, the trans-SNARE complex is looser than that formed for ultrafast exocytosis. Ca\textsuperscript{2+} priming may help to remove part of the cytomatrix to facilitate trans-SNARE complex formation and speed up exocytosis; otherwise, exocytosis is slow in chromaffin cells, with a time constant of ~1 s.

By conforming to slow kinetics, exocytosis in chromaffin cells does not require synaptotagmin1 (674) and Munc13–1 (TABLE 2). The time constant for the capacitance increases was limited to 30 ms and did not decrease upon overexpression of CAPS and Munc13–1 (369). To date, HTSS has not been reported to induce exocytosis in endocrine cells (54, 427). Replacement of synaptotagmin1 with synaptotagmin2 slows exocytosis (434), whereas it hastens the ultrafast exocytosis in synapses (703). Thus the trans-SNARE states in Ca\textsuperscript{2+}-primed chromaffin cells have molecular bases distinct from those in ultrafast exocytosis.

Two distinct trans-SNARE states have been distinguished in Ca\textsuperscript{2+}-primed chromaffin cells (706), in which the increases in the membrane capacitance induced by large increases in [Ca\textsuperscript{2+}], show two kinetic components, with time constants of 30 and 300 ms. Rigorous kinetic analyses indicated that the two components could be ascribed to two distinct trans-SNARE complexes: tight and loose. These states were interconvertible in a Ca\textsuperscript{2+}-independent manner, with a time constant of 4 s (675). The fast trans-SNARE state was selectively eliminated by BoNTA (706), COOH-terminal mutation of SNAP25 (608), and deletion of synaptotagmin1 (674) and was slowed by a R233Q mutation in synaptotagmin1 (605), replacement of SNAP25a with SNAP25b (607), and replacement of the SNAP25b COOH-terminal linker with SNAP23 (436). The amplitude of the fast component, but not the slow component, of the capacitance increases was regulated by PKA-mediated phosphorylation of SNAP25 (437). These results suggest the existence of two distinct trans-SNARE states in Ca\textsuperscript{2+}-primed chromaffin cells.

The amplitude of both components of the capacitance increase was reduced by TeNT, BoNTC, -D, and -E (706); depletion of PIP\textsubscript{2} (421); complexin (86); CAPS (370); synaptenin (642); NH\textsubscript{2}-terminal mutation of SNAP25 (608); and expression of tomosyn (720, 721) and was augmented by PKC-dependent phosphorylation of SNAP25 (435). These delineate the common priming steps for the two trans-SNARE states, which bear some similarity with those of ultrafast exocytosis.

Importantly, the kinetics of exocytosis varies according to the subtype of Ca\textsuperscript{2+} sensor in the synapses and secretory cells. In synapses, synaptic currents are fastest with synaptotagmin2, and slower with synaptotagmin1 and synaptotagmin9 (703). Importantly, although synaptotagmin3, -5, -6, -, and -10 do not rescue ultrafast exocytosis, they can evoke slow or tonic exocytosis upon repetitive stimulation (703). In agreement with the results in synapses, deletion of synaptotagmin1 and -7 impair fast and slow capacitance increases, respectively, in Ca\textsuperscript{2+}-primed chromaffin cells (573). The difference between synaptotagmin1 and synaptotagmin7 arises, in part, from subtle sequence changes (709). Cochlear hair cells utilize otofelin together with specific SNAREs as a fast Ca\textsuperscript{2+} sensor (289, 454, 535). Asynchronous exocytosis may be mediated by Doc2 and is suppressed by synaptotagmin1 and complexin (TABLE 1) (717).

Interestingly, the kinetics of exocytosis are markedly altered by the linkers between the SNARE motif and the TMDs of VAMP (66, 136, 217, 316, 406) and syntaxin (668), and by the linker between the two SNARE motives in SNAP25 (69, 436). Thus the linker regions outside the SNARE motif play an important role in the kinetics of membrane fusion mediated by SNAREs, in line with the idea that SNARE assembly can be induced up to the transmembrane regions (613).

3. How many SNARE complexes are required for exocytosis?

Proteoliposome studies show that a single trans-SNARE complex is capable of inducing exocytosis (56, 665), whereas other studies report that 3 (148) or between 5 and 11 (299) are required. Smaller numbers might reduce the fusion rate (299). In living cells, at least two SNARE complexes are involved in synaptic transmission (596), and three to five SNARE complexes are involved in exocytosis in
PC12 cells (227, 266) and Ca\textsuperscript{2+}-primed chromaffin cells (423). Oligomers of SNAREs purified from the brain comprise three to five SNARE complexes (110, 521). The number of SNAREs is not a simple determinant of the kinetics of exocytosis, given that two or three SNAREs might be enough for fast exocytosis. Consistently, the amplitude, but not kinetics, of exocytosis are affected by BoNTC and TeNT (546). The number of SNAREs may affect the stability of the fusion pore (589). It is also possible that release probability is regulated by the number of VAMP2 molecules, given the fact that each synaptic vesicle has 70 of them (638).

4. SNARE oligomerization

Oligomerization of SNARE complexes is found in many preparations. Star-shaped oligomerized SNAREs have been purified from the brain (521). They have also been formed in an aqueous solution (164). Ring-shaped SNARE complex oligomers have been detected by atomic force microscopy (AFM) and electron microscopy when full-length SNARE was expressed in liposomes (109). Such oligomers could be disassembled by NSF (284). These SNARE complexes were all cis-SNARE. Also, electron paramagnetic resonance (EPR) analysis demonstrated the formation of SNARE oligomers preceding hemifusion (375).

There are several potential mechanisms for the oligomerization of SNAREs. Oligomerization of SNAREs may be induced by TMD (227, 255, 335, 342), SNARE motifs (409, 593), or complexin (644). Recent liposome experiments demonstrate that SNAREs form zig zag-shaped oligomers with the help of complexin (294, 334, 354). It remains to be seen whether such oligomers and large changes in supramolecular structures can be utilized for ultrafast exocytosis. Alternatively, the WD40 domains of tomosyn induce oligomerization of SNAREs and inhibit synaptic transmission (548). Deletion of tomosyn reduces SNARE oligomerization by 30%, and oligomerization of SNAREs may play an inhibitory role. Another possibility is that Ca\textsuperscript{2+}-induced oligomerization of synaptotagmin may induce synaptic vesicle fusion via assembly and oligomerization of SNARE complexes (96, 361), either by direct binding (111, 702) or by indirect binding mediated by complexin (643).

Unlike SNAP25 and SNAP23 in animal cells, Qb-SNARE and Qc-SNARE are separate molecules in plants (359). It is possible that the two Q-SNAREs are linked in animal cells to promote the efficiency of membrane fusion events for more rapid activity of animal cells relative to plant cells. The linker region may just bring the two SNARE motives of SNAP25 closer to allow assembly, but it may also have another role. The oligomerization of SNAREs can be achieved by cross-linking (or domain swapping) of SNARE complexes through two distinct SNARE domains (Qb and Qc) in two SNAP25 molecules. The existence of such a biochemical state was postulated in early studies (164, 340, 497, 644) but has met with several criticisms. First, SNARE coiled-coil structures can be formed without a SNAP25 linker region (627). Second, a single molecular Förster resonance energy transfer (FRET) study showed that the occurrence of domain swapping is infrequent at very low (<100 pM) molecular concentrations (694). Third, in chromaffin cells, SNAP25 with two mutations in distinct SNARE motifs was effective on the capacitance increases only when both mutations were in the same SNAP25 molecule (423). Finally, isolated SNARE domains of SNAP25 can rescue exocytosis of PC12 cells (98, 558) and proteoliposomes (480). These arguments indicate that domain swapping is not necessary to form the SNARE complex, may need higher concentrations of SNAP25 (340), and may be specifically utilized for ultrafast exocytosis. If the SNAP25 domains are swapped, the SNAREs are placed in close proximity to the fusion pore, which may boost efficient formation of hemifusion and fusion pore opening. Domain swapping may be assisted by the fact that the Qb-SNARE motif of SNAP25 first binds with syntaxin, leaving the Qc-SNARE motif free (524, 694). The domain swapping model must, therefore, be directly examined in the active zone in the future.

Taken together, many studies show that there are many diverse trans-SNARE configurations, even for neuronal SNAREs (syntaxin1, SNAP25, and VAMP2), in terms of 1) the tightness of trans-SNARE complex, 2) the kinetics of poststimulus SNARE assembly, 3) the number of trans-SNARE complexes for each exocytosis event, 4) oligomerization, and 5) domain swapping, all of which depend on priming proteins and sensors. Also, the initial conformation of lipid bilayers (FIGURE 4) may be different depending on the trans-SNARE configurations.

B. Non-trans-SNARE Configurations

In the previous section we described the molecular mechanisms of exocytosis induced within 100 ms after stimulation, where prior formation of a trans-SNARE complex seems a reasonable assumption. If exocytosis is slower, however, such assumptions are no longer valid, and there are many lines of evidence for other initial SNARE configurations. The trans-SNARE configuration, however, has been adopted as the sole model of regulated exocytosis in most studies, and no other possibilities have been systematically explored. Therefore, we will first summarize the reasons for considering non-trans-SNARE configurations and then provide some examples and mechanisms in detail.

1. Rationale for non-trans-SNARE configurations

1) There is no direct proof or quantitative demonstration of prestimulus trans-SNARE complex formation for exocytosis, even in the active zone. The key structural prob-
lem is related to how tight SNAREs are assembled so that the remaining SNARE assembly can induce ultrafast fusion.

2) There is huge kinetic diversity in exocytosis (1 ms to 1,000 s), even though the same set of SNARE proteins is used by neurons and neuroendocrine preparations (FIGURE 1, TABLE 1). Such diversity can be naturally explained if initial configurations other than trans-SNARE states are considered (FIGURE 8). Otherwise, vesicles for slow exocytosis must be bound to the membrane by trans-SNARE complexes for an unnecessarily long time before exocytosis, which renders mobilization of vesicles stored in the cytosol extremely inefficient. Hence, trans-SNARE configurations may be physiologically relevant only for ultrafast or fast exocytosis with time constants shorter than 100 ms.

3) TIRF and confocal experiments have shown that vesicles are nondocked to the plasma membrane before stimulation in synaptic vesicles in ribbon-type synapses in bipolar terminals (257, 418, 729) and LDCVs in secretory cells (7, 310, 317, 555, 590, 671) (see sect. IVA), indicating that SNAREs are not assembled. In secretory cells, small vesicles are nondocked but undergo fast Ca$^{2+}$-dependent exocytosis (see sect.IID).

4) Some vesicles in secretory cells, such as PC12 and β cells, show tight morphological docking, but the docking does not affect (126, 655, 719), or even slow, exocytosis (310) (sect. IIIA). This suggests that SNAREs are not preassembled in the docked vesicles.

5) Sequential exocytosis is found in a variety of secretory cells and neurons (sect. IIIB) and is best explained by the assembly of v-SNARE with t-SNAREs, which are supplied from the plasma membrane into the fused vesicle membrane (see sect. VID).

6) Stimulus-induced assembly of SNAREs during exocytosis has been demonstrated both biochemically (43, 603, 698) and using FRET imaging (633) (see sect. VID).

7) The tonic (80) and spontaneous exocytosis (268) of synaptic vesicles likely involves nonprimed and nondocked vesicles (see sect. VIB2).

8) SNAREs for cAMP-dependent slow exocytosis have been identified in many cells (630) (TABLE 1; sect. IIIE), in which vesicles are apparently nondocked to the membrane prior to stimulation.

9) Constitutive exocytosis also utilizes SNAREs (485) (TABLE 1), indicating that nondocked vesicles can undergo fusion without any stimulation, and that any step in between maturation, transport, tethering, docking, and fusion can be used to control exocytosis (see sect. IIIF).

10) Proteoliposome experiments show that SNAREs are sufficient for slow exocytosis (689) and that the rate-limiting step for fusion can be a Ca$^{2+}$-dependent docking step (599) (see sect. VIC). Fast exocytosis can be induced only when docked vesicles are selected as the initial state (341).

2. Tonic exocytosis of synaptic vesicles

Tonic exocytosis is induced by repetitive action potentials in the active zone and is not time-locked to each action potential as is the case for slow exocytosis in endocrine cells. The rate of tonic release is determined by the availability of unitary-SNAREs through αSNAP and βSNAP (80). Thus, during tonic exocytosis, vesicles reside within the recycling pool (557). This involves the disassembly of cis-SNAREs, transport of vesicles to the active zone, and assembly of trans-SNARE complexes. In fact, nondocked vesicles undergo exocytosis during tonic stimulation underneath the ribbon in bipolar terminals (418, 730).

3. Spontaneous exocytosis of synaptic vesicles

Spontaneous exocytosis involves vesicles in the recycling and reserve pools, which are characterized by VAMP7 (268) and vti1a as a Q-SNARE (504). Interestingly, spontaneous exocytosis shows markedly different molecular characteristics to ultrafast exocytosis. Spontaneous exocytosis is resistant to knockout of VAMP2, SNAP25, RIM, and CAPS and is enhanced by knockout of synaptotagmin1 (TABLE 2). It is also inhibited by deletion of Doc2 (208, 478). Mutation of the linker region in VAMP2 by insertion of 12 or 24 residues between the SNARE motif and transmembrane region affected ultrafast exocytosis, but not spontaneous exocytosis (136). Cholesterol has opposite effects on ultrafast and spontaneous exocytosis (687). These results indicate that molecular events downstream of Ca$^{2+}$ stimulation of vesicles in the reserve pool are very different from those involved in Ca$^{2+}$ stimulation of vesicles in the RRP. The finding that the actin depolymerizing agent latrunculin A increased spontaneous exocytosis but did not affect ultrafast exocytosis (268, 426) suggests that the vesicles responsible for spontaneous exocytosis are nondocked to the plasma membrane (see sect. IVA4). Thus spontaneous exocytosis does not seem to selectively utilize trans-SNARE configurations, unlike ultrafast exocytosis.

4. Slow exocytosis in secretory cells

Although relatively fast exocytosis can be induced in Ca$^{2+}$-primed chromaffin cells, sustained exocytoses follow a fast exocytotic burst (673, 706). This is thought to be mediated by vesicles with unassembled SNAREs. Ca$^{2+}$-induced SNARE assembly before exocytosis has been directly demonstrated in β cells (633).

Ca$^{2+}$-dependent exocytosis is slower in secretory cells with nonneuronal SNAREs and synaptotagmins than in those
with neuronal SNAREs (283). Table 1 lists the SNAREs and synaptotagmins that have been identified in immunohistochemical or knockout experiments. Of particular note, islet β cells utilize neuronal SNAREs, but not synaptotagmins 1, 2, 7, or 9 (214, 215). Insulin vesicles are nondocked before exocytosis (310, 590) and therefore utilize non-trans-SNARE configurations (633). Nondocked vesicle exocytosis is generally found in other cells that utilize nonneuronal SNAREs and synaptotagmins. For example, postsynaptic dendritic exocytosis utilizes syntaxin4, SNAP23, and synaptotagmin4 (315, 623). Synaptotagmin7 and -10 are utilized for insulin-like growth factor and glucagon secretion in the brain (88) and α cells in the islets (216), respectively. Exocrine acinar cells utilize syntaxin2, SNAP23, and VAMP2 for primary exocytosis of zymogen granule exocytosis (43). Glial exocytosis may be mediated by lysosomes (352, 740) utilizing syntaxin1, SNAP23, and VAMP3 (cel-lubrevin) (576) and is regulated by synatotgamin4 (735). Thus nonneuronal SNAREs and synaptotagmins may be optimized for Ca^{2+}-dependent non-trans-SNARE configurations.

In epithelial and hematopoietic cells, exocytosis is not triggered by Ca^{2+}, and vesicles are rarely attached to the plasma membrane. Exocytosis from these cells is mainly mediated by nonneuronal SNAREs (Table 1). For instance, exocytotic insertion of aquaporin in kidney epithelial cells is triggered by χAMP and mediated by syntaxin4, SNAP23, and VAMP2 or -7 (TI-VAMP) (276). Gastric parietal cells utilize χAMP to trigger H^+ -ATPase insertion, which is mediated by SNAP25, syntaxin3, and VAMP3 (cel-lubrevin) (551), and synaptotagmin2 (412), and phosphorylation of SNAP23 plays an important role in triggering exocytosis induced by IgE (629). The immunological synapses of T-cells utilize syntaxin7 and VAMP7 (260, 482). Exocytosis of vesicles containing GLUT4 in adipocytes and skeletal muscles is mediated by syntaxin4 and SNAP23, and triggered by tyrosine phosphorylation of Munc18c by insulin receptors (15, 285), and the docking step is regulated by PI3-kinase (30). A syntaxin3/SNAP23/VAMP8/Munc18b complex is utilized for regulated exocytosis in exocrine cells and platelets and for constitutive exocytosis. These exocy-toses are not mediated by the trans-SNARE configuration: exocytosis is slow, vesicles are not docked before stimulation, and exocytosis is often potentiated by removal of subcortical actin layers (see sect. IVA).

4. Different non-trans-SNARE configurations

If exocytosis is initiated by non-trans-SNARE configurations, there are at least three possible initial states for exocytosis: binary-SNARE, unitary-SNARE, and cis-SNARE (Figure 8). It is not easy to identify which of these states is used for the initial configuration, and more than one configuration may be utilized for a particular type of exocytos-sis. To support their existence, however, we will scrutinize possible mechanisms involving each of the three non-trans-SNARE configurations in the next section. Slow exocytosis provides us with valuable examples to aid our understanding of the processes of SNARE assembly because it proceeds gradually after it is triggered, unlike the ultra-fast exocytosis.

C. Binary-SNARE Configurations

The binary t-SNARE acceptor complex of the two plasma membrane SNAREs, syntaxin and SNAP25, has been proposed as an intermediate stage of SNARE assembly (520, 522, 524). Further assembly of VAMP2 to form a trans-SNARE complex is the next stage of the fusion reaction (Figure 8). We will describe existing lines of evidence that the binary-SNARE state can act as the initial configuration for exocytosis in many secretory phenomena. Such binary-SNARE configurations are particularly suited for mobilizing a large pool of vesicles, which are stored in the relatively superficial layers of the cytosol.

1. Biochemistry of the binary configuration

Binary-SNARE states are formed when the Munc18a/syn-taxon1 complex binds to SNAP25, a process catalyzed by Munc13 (378) (Figure 8). A binary-SNARE complex can efficiently induce trans-SNARE complex formation upon increases in [Ca^{2+}], because synaptotagmins bind with the binary-SNARE complex (29, 520, 660), as well as with membrane phospholipids (488, 621), in a Ca^{2+}-dependent manner. Such binding has a micromolar requirement for [Ca^{2+}], in accordance with the [Ca^{2+}], requirement of exocytosis in synapses and endocrine (635, 674) and exocrine cells (443). The binding of synaptotagmins with the plasma membrane supplies VAMPs within the vesicles to the binary complex in the plasma membrane. VAMP2 can rapidly assemble with the binary complex (496).

Proteoliposome experiments indicate that Ca^{2+} can stimulate docking of vesicles (722) by synaptotagmin (113, 347, 599) and can induce full fusion in 30 ms (682) (Figure 3C). A binary-SNARE configuration can act as the initial configuration for Ca^{2+}-dependent fusion events in proteoliposome, because t-SNAREs frequently form the binary complex in proteoliposomes (496), and because binary-SNARE states are stabilized by many priming proteins, including complexin, synaptotagmin, Munc13, and Munc18 (694). A new liposome assay system using a microfluidic device and low ionic strength solutions revealed that synaptotagmin can act as a distance regulator to control the assembly of the binary t-SNARE complex and VAMP in a Ca^{2+}-dependent manner (667). This suggests that binary configurations account for exocytosis with time constants between 30 ms and 1 s (Figure 1, Table 1).
2. Possible examples of the binary configuration in synapses and endocrine cells

TIRF images of endocrine cells show that clusters of syntaxins and SNAP25 often overlap to form binary complexes (157), but not ternary complexes (524). In β cells, a FRET probe of SNAP25 identified a high FRET region, in which binary-SNAREs were formed, and exocytosis was faster than in a low FRET region. Crash fusion events in β cells occur within 40 ms after docking of vesicles to the plasma membrane (7, 310, 590, 651), suggesting a binary configuration based on rapid time courses (FIGURE 8). The rapid (10–100 ms) exocytosis of nondocked LDCVs in synapses (74) also fits with the idea of a binary configuration. Graded ribbon-type synapses in bipolar terminals also display exocytosis of nondocked vesicles during tonic stimulation (418, 730). Such sustained vesicular release at high rates is also observed in mossy fiber terminals in the cerebellar granule layer (557), and may be generally applicable to tonic exocytosis in synapses (80). Binary-SNAREs might act as release sites, the formation of which is facilitated by endocytosis (263). In β cells and bipolar terminals, it has been proposed that exocytosis is limited by the diffusion of vesicles (239, 257). Thus the binary-SNARE configuration appears to be utilized for various forms of Ca\(^{2+}\)-dependent exocytosis (TABLE 1).

3. How is the binary-SNARE configuration clamped?

Several potential mechanisms are considered, which may prevent the binary complex from formation of trans-SNARE complexes in the resting state (FIGURE 8).

1) Vesicles cannot form trans-SNARE complexes if the plasma membrane region for exocytosis is already occupied with vesicles in the RRP. This is the case for nondocked vesicles in the active zone and in Ca\(^{2+}\)-primed chromaffin cells (673, 706). In many other preparations, however, vesicles do not seem to form trans-SNARE complexes, even though the membrane region is available (see sects. III and IV).

2) A major barrier to the binary complex forming the trans-SNARE complex in the resting state may simply be due to the low incidence of the collision of vesicles with the binary complex in the plasma membrane. SNARE-mediated fusion events occur slowly, even in proteoliposomes in which diffusion of liposomes is unlimited (496, 667, 689), unless the vesicles are already sited close to each other (341, 682). In the cytosol of living cells, vesicle movement is retarded by the cytoskeleton and organelles, and the approach of vesicles to the plasma membrane is restricted by the submembrane cytomatrix. Even though the vesicles reach the plasma membrane, the v-SNARE may not collide with the binary-SNARE complexes (138, 239) as their residence time is short. In tonic synapses, Munc13–2 allows the formation of a trans-SNARE complex only after repetitive stimulation (531).

3) The binary state is specifically stabilized by soluble VAMP-like proteins, such as tomosyn (183) and amisyn (559), which can bind to binary complexes and stabilize them. Their binding can be competitively removed by VAMP, which leads to exocytosis. Thus these proteins control the speed of exocytosis in the binary mode to avoid exhaustion of vesicles. In fact, overexpression of tomosyn causes a significant reduction in exocytosis in PC12 cells (120, 240), chromaffin cells (721), adipocytes (696), β cells (107, 738), and neurons (27). Likewise, VAMPs with the longin domain (LD), such as VAMP7, will be less reactive with the binary acceptor complex (672).

D. Unitary-SNARE Configurations

Unitary-, or free-SNAREs, are a highly reactive form of SNARE complex (FIGURE 8) and are an obvious candidate for the initial SNARE state. We call them unitary-SNAREs to avoid the possible misunderstanding that such SNAREs are completely free and not bound to other molecules, such as Munc18. Such a configuration is considered to be the default in plant biology (359). The unitary-SNARE configuration has a great advantage in inducing slow and compound exocytosis for intensive mobilization of vesicles from deep within the cytosol.

1. Biochemistry of unitary configurations

Unassembled SNAREs can be generated from ternary- and binary-SNARE complexes (243), oligomerized SNAREs (256, 456, 472, 483), and syntaxin/SNAP25/tomosin heterotetramers (240, 548). Thus unitary-SNAREs are dynamically maintained and may be generated shortly before exocytosis is triggered (336). Unassembled syntaxin can be chaperoned by Munc18a, which bundle SNARE motifs and Habc for stabilization (715).

The vesicle membrane protein, cysteine-string protein (CSP), chaperones SNAP25, helps proper folding of SNAP25 for SNARE formation, and prevents ubiquitylation and proteosomal degradation of SNAP25 (585). In fact, knockout of CSPα in neurons reduces SNAP25 levels and induces synaptic degeneration (168). In β cells, CSP is expressed in insulin granules, and overexpression suppresses insulin exocytosis (73). Thus unitary-SNAREs are bound to their respective chaperones and are protected from SNARE assembly.

In spite of the clamping role of Munc18a, proteoliposome experiments demonstrate that Munc18a facilitates the formation of the binary-SNARE and trans-SNARE complexes (378, 528, 586). Munc13 induces dissociation of Munc18 from closed syntaxin and the formation of binary- and ternary-SNARE complexes (378, 518). Unsaturated fatty acids act on syntaxin in its closed conformation and facilitate exocytosis (see sect. IVF2). If vesicles contact with the plasma
2. Possible examples of unitary configurations in endocrine cells and synapses

t-SNAREs are found unassembled (unitary), but clustered and reactive, in the plasma membranes of PC12 cells (36, 220, 344, 345, 524), where syntaxin1 is likely associated with Munc18a (600). There are abundant t-SNAREs surrounding the docked vesicles in PC12 cells (36), and they are dynamically diffusing (326). Thus SNAREs are unassembled, even though vesicles are docked in PC12 cells, which may account for the long latency of exocytosis (10 s) in these cells (FIGURE 1). Poststimulus assembly of SNAREs has been reported in β cells of the islets (603).

Ca\(^{2+}\)-dependent assembly of t-SNAREs was demonstrated in β cells in the islets using a FRET probe. There are two regions in the plasma membrane of β cells in which SNAREs are unitary or binary (633). Exocytosis from the unitary-SNARE region is slow and is associated with an increase in FRET levels, indicating that SNAP25 is assembled with syntaxin, and that the unitary-SNARE configuration is utilized for the slow phase of insulin exocytosis. TIRF imaging of endocrine cells often shows that vesicles are docked for a short time after stimulation (<3 s), but before fusion events in β cells (310, 590) and PC12 cells (555), consistent with FRET results indicating that fusion is induced 3 s after SNARE assembly in the unitary-SNARE configuration (633).

Sequential exocytosis is most simply explained by the unitary-SNARE configuration. The spread of sequential exocytosis into the cytosol may be best explained by the diffusion of SNAP23 or SNAP25, as syntaxins are integral membrane proteins and may have more difficulty passing through the narrow fusion pores. In fact, redistribution of SNAP25 and SNAP23 has been demonstrated in endocrine cells (322, 632) and mast cells (213). Syntaxin may also be a diffusive factor, particularly when the fusion pore is widely open, as has been reported in exocrine cells (493). Exocrine acinar cells express syntaxin3 and VAMP8 for granule-granule fusion during sequential exocytosis (43, 224), and the unitary-SNARE configuration may be utilized during multigranular exocytosis. In neutrophils, SNAP23 is expressed in secretory vesicles (592), perhaps providing a reason for the abundance of multigranular exocytosis (218), in which vesicle-vesicle fusion precedes exocytosis.

It is proposed that the sustained phase of exocytosis observed after an increase in \([\text{Ca}^{2+}]\), is mediated by unitary-SNAREs in chromaffin cells (706) and in β cells (633). Sustained exocytosis is considered to represent assembly of SNAREs from the unitary states, as it is blocked by BoNTs and antibodies (705–707). The sustained phase is augmented by Munc13–1 (18, 747) and Munc13–2 in chromaffin cells (747) and in β cells (298), suggesting that the binary-SNARE assembly is facilitated by Munc13 in a Ca\(^{2+}\)-dependent manner (FIGURE 8). The sustained phase is also facilitated by CAPS (369, 611) and PI\(_3\) (421), suggesting that CAPS also facilitates SNARE assembly after the Ca\(^{2+}\) trigger (FIGURE 8). The sustained phase is greater with SNAP23 than with SNAP25 (436, 607), consistent with the idea that SNAP23 is involved in the unitary-SNARE configuration.

cAMP-dependent exocytosis is likely mediated by unitary-SNARE configurations (TABLE 1). This is because exocytosis must spontaneously proceed from unitary-SNAREs, with constitutive exocytosis mediated by syntaxin4/SNAP23/VAMP8/Munc18b (485) and slow exocytosis induced by Ca\(^{2+}\), cAMP, and kinases. The long time constants of cAMP-dependent exocytosis (between 1 and 1,000 s; FIGURE 1 AND TABLE 1) can be explained by the time required for the unitary-SNAREs to be assembled and induce exocytosis (FIGURE 8). SNAP23 can induce only slow exocytosis (>1 s) (607); therefore, SNAP23 may not allow a stable binary-SNARE state (176). The slow time course of spontaneous exocytosis in the synapse (1,000 s) suggests that it is at least partly mediated by the unitary configuration (FIGURE 8) or even the cis-SNARE configuration.

3. How is the unitary-SNARE configuration clamped?

There are several possible mechanisms for preventing the unitary-SNARE configuration from undergoing further SNARE assembly in the resting state (FIGURE 8).

1) The availability of unitary-SNAREs is regulated. For example, tyrosine phosphorylation of Munc18c, which insulates syntaxin (235, 378, 737), is the major trigger of exocytosis of vesicles containing GLUT4 (15, 285). Likewise, phosphorylation of SNAP23 is a key factor regulating exocytosis in mast cells (629). Exocytosis in salivary glands is triggered by cytosolic cAMP and phosphorylation of VAMP2 by PKA (182).

2) SNARE assembly itself may be regulated. Glucose-induced insulin exocytosis is potently regulated by PKA in a manner that is dependent on snapin (603). Snapin may allow SNARE assembly only when it is phosphorylated by PKA in β cells. Snapin can associate with SNAP23 and is able to form a ternary complex with SNAP23 and syntaxin4 (85), which may be involved in various forms of slow exocytosis.

3) Access of the vesicles to the plasma membrane may be a limiting factor for binary-SNARE complex formation. Vesicle proteins such as synaptotagmins, Doc2, and VAMP2 bind to t-SNAREs (184, 314, 567) and may facilitate formation of the binary- and trans-SNARE states (326). Such
access is limited until cAMP-dependent removal of the submembranous cytomatrix has occurred, including the subcortical actin layer (324, 450).

E. Cis-SNARE Configurations

1. Sperm acrosome exocytosis

The acrosome of sperm cells undergoes exocytosis within a few minutes after contact with the zona pellucida of an oocyte, which is a crucial step for fertilization. The initial trigger for acrosomal exocytosis is Ca$^{2+}$ entry into the cytosol (131). Then, Rab3 is activated and triggers NSF/αSNAP-dependent disassembly of cis-SNARE (372) (which is composed of syntaxin1A, SNAP25, and VAMP2) in both the acrosome and the plasma membrane (648). The resulting disassembled SNAREs reassemble to form trans-SNARE complexes between the acrosome and the plasma membranes. Ca$^{2+}$ entry is followed by Ca$^{2+}$ release from the acrosome, which in turn induces fusion of acrosome with the plasma membrane in a synaptotagmin6-dependent manner (131). αSNAP also sequesters syntaxin and blocks trans-SNARE conformation (529). cis-SNAREs are also involved (see sect. VI). The unitary- and binary-SNARE modes may be widely utilized for slower exocytosis in synapses and secretory cells. The initial SNARE configurations appear to be the major factor governing the kinetics of exocytosis and are determined by the factors B–G below. These factors may also affect the kinetics of exocytosis by regulating poststimulus SNARE assembly.

2. Other possible cases

There are abundant cis-SNAREs in synapses (521) and yeast vacuoles (664), and they may be used as a reservoir of SNAREs that can be used on demand. Indeed, NSF and αSNAP act 1–5 s before fast exocytosis in synapses (336), and SNAREs are recycled during tonic release (80). Tonic release is dependent on αSNAP, suggesting that cis-SNAREs are also utilized during tonic release (80). The action of NSF may be regulated by rab3 (131), which is required for recycling (348) and used for priming and biogenesis of LDCVs in chromaffin cells (34, 574, 705). Cytosolic Ca$^{2+}$ promotes short-term plasticity (422) and recovery from depletion of RRP (147, 616, 681) in various synapses, partly by activating cis-SNAREs.

VII. KINETIC DETERMINANTS

We have described the major factors affecting the kinetic diversity of exocytosis in various secretory vesicles in neurons and secretory cells. We can now summarize the major determinants of the time constants of exocytosis after stimulation based on all the data described in this review.

A. The Initial SNARE Configurations

There is tremendous diversity in the initial assembly states of neuronal SNAREs. This critically affects the time constants of exocytosis. The more tightly formed the SNARE complex is, the sooner exocytosis occurs after stimulation. Neuronal SNAREs are able to form the trans-SNARE state before stimulation only in the active zone, but this is not their sole mode of action, as nondocked vesicles may also be involved (see sect. VI). The unitary- and binary-SNARE modes may be widely utilized for slower exocytosis in synapses and secretory cells. The initial SNARE configurations appear to be the major factor governing the kinetics of exocytosis and are determined by the factors B–G below. These factors may also affect the kinetics of exocytosis by regulating poststimulus SNARE assembly.

B. Subtypes of SNAREs and Priming Proteins

Neuronal SNAREs mediate rapid exocytosis, whereas nonneuronal SNAREs, such as VAMP4, syntaxin2, and SNAP23, mediate slower exocytosis and are associated with ubiquitous priming proteins such as Munc18b and Munc13–4. In preparations with nonneuronal SNAREs, vesicles are nondocked and the trans-SNARE complex is not formed before stimulation. SNARE linker regions may also affect the fusion processes directly (sect. VI). The number of SNARE complexes may regulate the probability of exocytosis.

C. Ca$^{2+}$-Sensor Subtypes

Even if cells utilize neuronal SNAREs, the speed of exocytosis is dependent on the type of Ca$^{2+}$ sensor used: from fastest to slowest, the speed of the synaptotagmins is 2 >1 >9 >7 >6 (703). Doc2 causes slower exocytosis than synaptotagmin1. It is thus likely that the triggering proteins also regulate the initial configurations of SNAREs (sect. VI).

D. Vesicle Distance From the Ca$^{2+}$ Channels

The localization of vesicles relative to the Ca$^{2+}$ channels profoundly affects the kinetics of exocytosis (sect. II). This localization is regulated by SNAREs, and by priming and triggering proteins.

E. Submembraneous Cytomatrix

The cytomatrix at the active zone (CAZ) allows trans-SNARE configurations. In non-trans-SNARE configurations, the diffusion properties of vesicles and the distance between the vesicles and the plasma membrane also play a major role in the kinetics of exocytosis. The submembra-
nous cytomatrix directly regulates exocytosis after triggering (see sect. VI).

F. Vesicle Size

There is a general tendency that smaller vesicles undergo faster exocytosis (FIGURE 1). This may be attributable to the following three factors: smaller vesicles may accumulate larger energy for membrane bending, which may be released during fusion. The membrane curvature is larger in smaller vesicles, and contact between the two membranes can be more easily achieved in small vesicles (see sect. IV). Finally, more rapid diffusion of small vesicles in the cytosol allows faster exocytosis in non-trans-SNARE configurations (see sect. III).

G. Lipid Composition

The lipid composition of both the vesicles and plasma membranes profoundly affects the fusion readiness of vesicles by affecting both membrane fusion and the formation of SNARE complexes (524) (sect. IV).

VIII. CONCLUSIONS

We have described the molecular and cellular mechanisms of SNARE-dependent exocytosis that underlie the diverse experimental results shown for synapses, endocrine, exocrine, epithelial, hematopoietic, muscle cells, and model systems. This diversity can be mostly ascribed to distinct initial SNARE configurations, although one must admit that the molecular mechanisms underlying the clamping and triggering of SNARE assembly have not been fully elucidated. The diversity of initial configurations will provide us with valuable opportunities to scrutinize the individual steps of the common SNARE assembly processes for exocytosis and membrane trafficking. As such, experimental determination of initial SNARE states holds the key to understanding exocytosis, even though it is technically demanding. Direct FRET measurement of SNARE organization in the active zone and in secretory cells would go a long way to achieving this goal. If various molecules are incorporated in the assay, single molecular FRET analyses will be informative in identifying the precise molecular interactions between molecules. Single vesicle liposome assays may provide an opportunity to study ultrafast exocytosis in vitro, as the molecular composition can be controlled. These in vitro methodologies have a tendency to give weighted views of the actual in vivo phenomena. Super-resolution imaging, such as PALM, STED, and RESOLFT (210), will help to characterize high-order molecular structures and vesicle dynamics. Two-photon FRET/fluorescence lifetime imaging will yield key information from intact tissues. Armed with these new tools and ideas, we believe that a better understanding of exocytosis will be achieved in the next decade.

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