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INVITED REVIEW

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Formation, stabilisation and fusion of the readily releasable pool of secretory vesicles

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Abstract Calcium-triggered exocytosis of neurotransmitter or hormone-filled vesicles has developed as the main mechanism for cell-to-cell communication in animals. Consequently, in the course of evolution this form of exocytosis has been optimized for speed. Since many of the maturation processes of vesicles are intrinsically slow, the solution has been to develop a pool of vesicles that are fully matured and can be fused very rapidly upon stimulation. Vesicles in this readily releasable pool are characterized by very low release rate constants at the resting cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and very high release rate constants at stimulated $[Ca^{2+}]_i$. Here I review the kinetic and molecular requirements for the existence of such a pool of vesicles, focusing on chromaffin cells of the adrenal medulla. I discuss how the use of assay methods with different time resolution may lead to fundamentally different conclusions about the role of proteins in exocytosis. Finally, I review recent evidence that the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, formed between proteins residing in the vesicle and the plasma membrane, is involved in formation and stabilization of the readily releasable vesicle pool, whereas synaptotagmin, a Ca^{2+}- and phospholipid-binding vesicular protein, is involved in setting the Ca^{2+} dependence of the fusion process itself. Future studies are likely to focus on the interaction between these two classes of proteins.

Keywords Chromaffin cell · Membrane fusion · Exocytosis · Neurosecretion · Calcium · Capacitance measurement · SNARE · SNAP-25 · Synaptotagmin

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Introduction

The regulated fusion of membrane-bound organelles is a common phenomenon in all living organisms [1]. Most intensely studied is the Ca^{2+}-triggered exocytosis of vesicles containing neurotransmitters or hormones, which forms the basis for cell-to-cell communication in multicellular animals. This form of membrane fusion is characterized by extremely tight control by Ca^{2+} and a very high speed of execution, with fusion following within milliseconds after an increase in the cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) [2, 3]. The optimization of vesicle fusion for speed is an essential and intriguing feature of neurosecretion with important physiological implications. For instance, it is related to the delay in synaptic transmission, which limits the rate at which the brain can process information. At the same time the speed of fusion presents a scientific challenge of matching the time resolution of experimental protocols to the process studied.

Before a transport vesicle fuses with the plasma membrane it must go through several maturation steps, starting with biogenesis of the vesicle and followed by translocation and physical attachment (tethering, docking) to the plasma membrane. Necessarily, some or all of these steps are slow. To allow the cell to respond rapidly to stimulation it needs a reservoir of vesicles that have already passed the slower maturation steps. The existence of vesicles in different maturation steps becomes noticeable upon prolonged stimulation: an initial, rapid "exocytotic burst", which happens on a tens-of-milliseconds scale, is followed by a slower, sustained phase. Most investigators hold that the exocytotic burst represents the release of a pool of vesicles that are "readily releasable" (the readily-releasable pool, RRP), that is, vesicles that can be released in response to a stimulus without further maturation steps. The exocytotic burst subsides as this vesicle pool is depleted. The slower phase of secretion represents vesicles that were in an earlier maturation stage at the onset of stimulation and that are mobilized in a use-dependent manner. This model was first evoked to explain data from the neuromuscular junction [4], but has since

been generalized to other cell types (adrenal chromaffin cells [5, 6, 7], pancreatic β-cells [8] and pituitary melanotrophs [9]). Comparison of the number of vesicles morphologically docked to the plasma membrane, as observed by electron microscopy, and the size of the RPP, as measured electrophysiologically, has led to the conclusion that only a subset of docked vesicles are readily releasable [10, 11]. This indicates the existence of additional maturation steps, usually referred to as "priming", before docked vesicles become release competent. Therefore, at least three vesicle pools can be distinguished: undocked, docked, but unprimed and readily releasable vesicles (Fig. 1).

In recent years much effort has been devoted to the description of the molecular machinery involved in docking, priming and execution of fusion (reviewed in [12, 13, 14, 15]). Here I want to focus on the feature that can be said to distinguish fast Ca^{2+}-triggered secretion from constitutive exocytosis or intracellular fusion reactions: the presence of a sizeable RRP, i.e. a pool of vesicles that have completed the slower maturation reactions and can be released very rapidly in response to an increase in $[Ca^{2+}]_i$. I will discuss the kinetic requirements for fast Ca^{2+}-triggered secretion and the molecular basis for the formation, stabilization and fusion of the RRP as it starts to be unravelled. The focus will be on secretion of large, dense-core vesicles from chromaffin cells of the adrenal medulla (see also [14]).

The readily-releasable vesicle pool: kinetic requirements

If we assume that the processes that lead to the fusion of a vesicle with the plasma membrane consist of a linear sequence of reversible maturation reactions followed by an irreversible fusion reaction (Fig. 1) then the kinetic requirements for fast Ca^{2+}-triggered exocytosis are:

1. A vesicle must undergo a number of maturation steps such that the last (i.e. the fully primed) state before release is stabilized with respect to previous states, i.e. the relative rate constants for entry into (priming, forwards rate) and exit out of (depriming, backwards rate) this state have to be adjusted such that a significant number of vesicles will occupy this state at rest ("1." in Fig. 1).
2. Vesicles in the above-mentioned state/pool (RRP) must be prevented from fusing until the physiological signal (Ca^{2+} trigger) arrives, i.e. the fusion rate constant at basal $[Ca^{2+}]_i$ has to be very low so as not to deplete the RRP ("2." in Fig. 1).
3. When the Ca^{2+} trigger arrives, the fusion rate constant has to be very high to ensure synchronized release ("3." in Fig. 1).

The first point requires that the primed state be relatively stable in order for a significant pool of such vesicles to build up. It also indicates two ways in which release can be regulated: by changing the stability of vesicles in the RRP (through a change of the backwards priming rate) or the forwards rate of priming, the RRP size

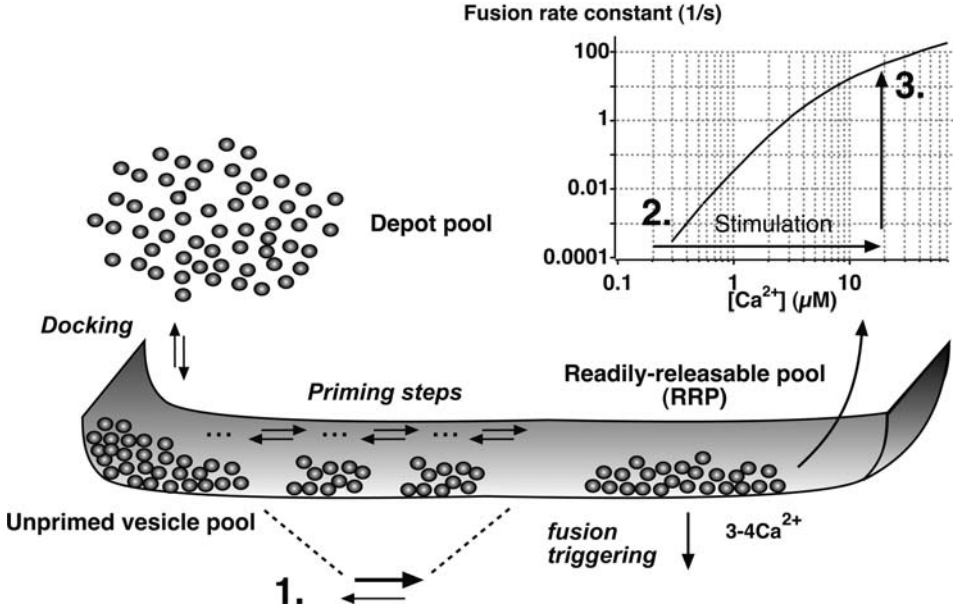

Fig. 1 Kinetic requirements for fast, Ca^{2+}-triggered secretion. Shown is a piece of membrane and the later maturation steps of large, dense-core vesicles. Vesicles from the depot pool dock to the plasma membrane and go through several priming steps until they enter the fully primed or readily releasable vesicle pool (*RRP*). Fusion triggering requires 3–4 Ca^{2+} ions and leads to irreversible exocytosis. The three requirements for fast Ca^{2+}-triggered secretion are (*1.*) that the overall forward and backward priming rates are so adjusted that a RRP of significant size can form in the absence of release, (*2.*) that the fusion rate constant is very low at basal $[Ca^{2+}]_i$, and (*3.*) that it is very high at stimulated $[Ca^{2+}]_i$. Thus the two latter requirements necessitate that the fusion rate constant has a steep dependence on $[Ca^{2+}]_i$ (*inset*, *black curve*)

can be actively regulated by the cell, which can have profound effects on the amount and kinetics of release.

Recent work has shown that some intracellular membrane fusion events are facilitated by resting Ca^{2+} levels [16] and that even in non-neuronal (epithelial) cells raising $[Ca^{2+}]_i$ can stimulate secretion [17, 18, 19], even though these processes do not reach the speed of "classical" Ca^{2+}-triggered neuroexocytosis. If we want to model these secretory processes according to the same scheme as in Fig. 1, three different reasons for the difference in kinetics between fast (millisecond time scale) Ca^{2+}-triggered neuroexocytosis and these slower, but Ca^{2+}-dependent, membrane fusion processes may be suggested. First, in the slower exocytosis processes the RRP may be destabilized (i.e. the depriming rate is large relative to the priming rate), such that an RRP of significant size can not be maintained or, second, exocytosis may proceed at basal $[Ca^{2+}]_i$, depleting the RRP or, third, the trigger mechanism may be intrinsically slow. A difference in trigger mechanisms is thus only one of several possibilities and, specifically, the importance of backwards priming steps should not be overlooked. If the vesicles in the RRP are unstable (i.e. the depriming rate is high) release is still possible during prolonged stimulation, but the time course will be slow and governed by the priming and depriming rates, regardless of how fast the calcium sensor itself acts.

Ca^{2+}-triggered secretion can be assayed by a number of methods. The most commonly used stimulation methods are influx of Ca^{2+} through Ca^{2+}-permeable channels (by stimulating the cells chemically or electrically), infusion of Ca^{2+}through artificial pores (using permeabilized cells, or a patch pipette) and photorelease of Ca^{2+} from a photolabile calcium cage loaded into the cell. Detection methods either monitor the plasma membrane capacitance, which increases as a result of the addition of vesicular membrane to the plasma membrane area, or they detect the release of vesicular content, either by chemical analysis of the cell medium, by electrochemical means (amperometry), or—in synapses—indirectly by using the postsynaptic cell as a readout.

Releasable vesicle pools in chromaffin cells

When we want to assay the size and fusion kinetics of a releasable pool of vesicles, capacitance measurements have the advantage that they monitor secretion all over the cell membrane, i.e. secretion of the whole population of vesicles and have a high time resolution. To be able to assay the releasable pool size correctly it is necessary to apply a stimulus that is so strong that the entire pool fuses with kinetics that can be distinguished clearly from those of the slower refilling processes. The proper stimulus depends on the cell type and vesicle pool to be assayed. For instance, vesicles co-localized with Ca^{2+}-channels will experience the high local $[Ca^{2+}]_i$ near the mouth of the open channel (Ca^{2+} microdomain, reviewed in [20]) and, consequently, such vesicles will fuse rapidly once the Ca^{2+} channel is open. This pool of vesicles is efficiently assayed by a train of short depolarizations or depolarizations of increasing duration. Such experiments have led to the identification of the so-called "immediately releasable pool" (IRP) [21, 22] of vesicles co-localized with Ca^{2+} channels.

In another stimulation method, flash photolysis of a photolabile Ca^{2+} cage is used to generate a spatially homogeneous increase in $[Ca^{2+}]_i$ [23] (Fig. 2). In such experiments the localization of vesicles relative to Ca^{2+} channels does not matter, making it possible to concentrate on maturation steps. An example of this kind of experiment is shown in Fig. 2A. Following flash photolysis the $[Ca^{2+}]_i$ increases from the hundreds-of-nanomoles/l to the tens-of-µmoles/l range. This is accompanied by an increase in membrane capacitance. If a single vesicle pool is released by the Ca^{2+} jump the expected kinetics are those of a single exponential function:

$$C_m(t) = C_{m,0} + A\left(1 - e^{-t/\tau}\right)$$

where A is the size of the vesicle pool in capacitance units, $C_{m,0}$ the initial capacitance of the cell membrane and τ the time constant of release. Detailed analysis of flash photolysis data from chromaffin cells has shown that a sum of two exponential functions is necessary to fit the increase in membrane capacitance occurring within 1 s after the flash, indicating the presence of two releasable vesicle pools with distinct release kinetics. These vesicle pools have been termed the RRP (release kinetics with a time constant of 20–40 ms at 20 µM Ca^{2+}) and the slowly releasable pool (SRP, release kinetics with a time constant of ~200 ms at 20 µM Ca^{2+}) [22, 24, 25, 26, 27] (Fig. 2A, inset). Upon depletion of the RRP refilling coincides with a decrease in SRP size with a time constant of ~10 s, showing that the two pools are arranged sequentially [22] (see Fig. 3A). Since these pools have been identified by flash photolysis experiments they must represent different maturation properties of the vesicles, and not different localization with respect to Ca^{2+} channels. The existence of two, separate, releasable pools has been confirmed by various molecular manipulations that lead to a selective loss of one of the pools (always the RRP and never the SRP, see below). In chromaffin cells cross-depletion experiments have shown that the IRP forms a subset of the RRP, comprising only around 25% of RRP vesicles [22]. In contrast, in pancreatic α- and β-cells, around 80–90% of RRP granules are co-localized with L-type Ca^{2+} channels [28, 29]. Thus, according to cell type and experimental protocol the forwards kinetic reactions displayed in Fig. 1 can either symbolize vesicular maturation steps or re-localization of the vesicle closer to Ca^{2+}-channels to increase the release probability, or both.

Recently, flash photolysis studies have resolved a small population of vesicles with even faster release kinetics at low $[Ca^{2+}]_i$ than that of the RRP in bovine chromaffin cells: the highly Ca^{2+}-sensitive pool (HCSP) [30]). This pool is interesting because it is the vesicle pool with the

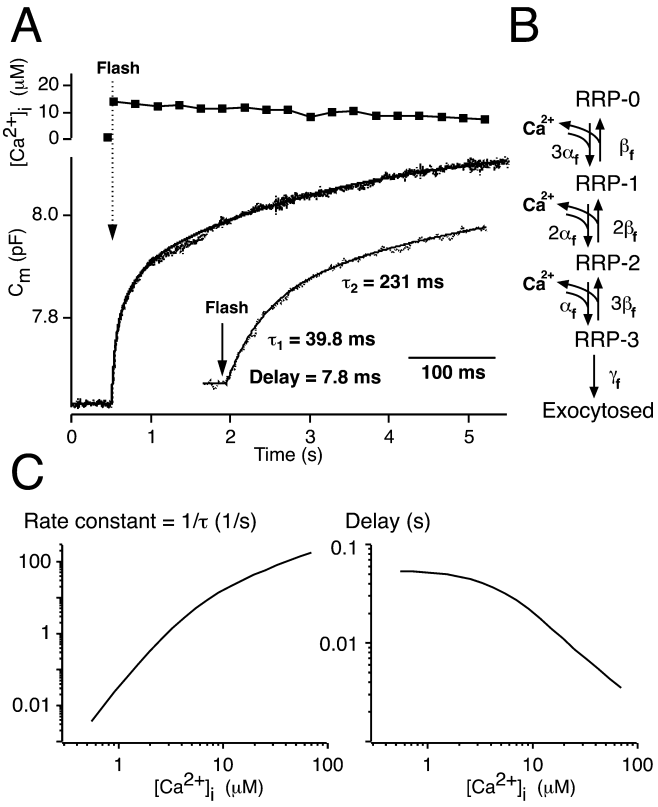

Fig. 2A–C A flash photolysis experiment in a mouse chromaffin cell. **A** Intracellular calcium concentration (*top panel*) and cell membrane capacitance (*lower panel*) during flash photolysis of caged calcium. The flash of UV light (*arrow*) leads to a rapid increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$), and a concomitant increase in cell membrane capacitance. The *line* is a fit of a sum of three exponential components to the capacitance increase. The first two components are shown at higher time resolution in the *inset*. The fastest exponential (time constant τ=39.8 ms) corresponds to fusion of the RRP, the slower (τ=231 ms) to fusion of the slowly releasable pool (SRP). The third component describes the sustained release component. **B** Model of the Ca^{2+} sensor for release from the RRP (α association rate constant for Ca^{2+}, β dissociation rate constant for Ca^{2+}, γ maximal fusion rate). A similar model describes fusion from the SRP [25]. A model with four cooperative Ca^{2+}-binding steps may describe secretion from the RRP more accurately under some conditions, however the kinetic differences between these models are small [110]. **C**Relationship between rate constant for release (*left*) and delay between flash and the start of release (*right*) and $[Ca^{2+}]_i$ as predicted by the model in **B**

highest release probability during small global increases in $[Ca^{2+}]_i$, such as those that accompany activation of IP_3-linked receptors. Furthermore, its size is increased much more than that of the RRP by activation of PKC, indicating a large potential for physiological regulation. The relationships between the RRP, IRP and the HCSP have not yet been elucidated, for instance it is not known whether the HCSP is refilled from the RRP or via a different pathway. For this reason it is not included in Fig. 3A.

Distinguishing between pool sizes and fusion kinetics in chromaffin cells is complicated by the fact that the sizes of both the SRP and the RRP are regulated by $[Ca^{2+}]_i$, due to Ca^{2+} dependence of the priming reaction (references in Table 1). This presents the challenge of distinguishing between two sequential Ca^{2+}-dependent processes (priming and exocytosis triggering). Since the $[Ca^{2+}]_i$ relevant for the regulation of RRP size is lower (0.1–2 μM) than for triggering (several μmolar) this problem was solved by applying a two-step increase in $[Ca^{2+}]_i$: slow photolysis of caged calcium induced by a fluorescence light source for increasing $[Ca^{2+}]_i$ to the 0.1–2 μM range and subsequent stimulation by flash photolysis leading to a rapid jump to >4 μM, depleting the RRP and SRP and allowing the determination of RRP and SRP size as a function of basal $[Ca^{2+}]_i$ [25]. These studies showed that the RRP size increases as a function of basal $[Ca^{2+}]_i$ up to 1–2 μM, at which the fusion rate (which depends on the third power of $[Ca^{2+}]_i$) approaches and finally surpasses the refilling rate (which depends on the first power of $[Ca^{2+}]_i$), leading to a fall in RRP size at higher $[Ca^{2+}]_i$[25]. Thus both the RRP and the SRP dependence on Ca^{2+} follow a bell-shaped curve, with a maximal size of ~140 vesicles in each pool at 500–700 nM Ca^{2+} (mouse chromaffin cells, [25]). The SRP and RRP have separate Ca^{2+} sensors that can be modelled by the sequential binding of 3 Ca^{2+} ions to the sensor, followed by an irreversible triggering reaction ([25]; Figs. 2B and 3A, see also below). Thereby there are at least three Ca^{2+}-dependent reactions leading to fusion in the chromaffin cells, two thereof in parallel (SRP and RRP Ca^{2+} sensors) and one (priming) in series with both triggering reactions (Fig. 3A).

The vesicle pool interpretation of secretion data has its limitations. Newly formed secretory vesicles may "jump the queue" and be released in preference to older vesicles [31], a finding that can not be accounted for in a simple, linear pool model. However, this process happens on a much longer time scale (hours) than most electrophysiological experiments.

In the above I have assumed that the vesicles fusing during different phases of secretion are equivalent, catecholamine-containing vesicles arranged in a linear maturation scheme. Chromaffin cells, however, also have other populations of vesicles. Following depletion of catecholamine-containing vesicles at $[Ca^{2+}]_i$<50 μM, calcium jumps to >100 μM release another ("intermediate") vesicle population that does not contain catecholamines as assayed by amperometry [26]. Earlier studies of chromaffin cells in other laboratories have shown that the capacitance signal precedes the amperometric signal by up to 0.5 s during the first, but not during later stimulations [32] and that the amperometric signal corresponding to the fast phase of secretion, while present, is smaller than expected from the capacitance increase [33]. It was therefore concluded that after flash photolysis of caged Ca^{2+} secretion of vesicles not containing catecholamines would confound capacitance measurements. A detailed comparison of amperometric and capacitance signals following flash photolysis in Neher's laboratory yielded the opposite conclusion, however, that the early rising phase (caused by fusion from the RRP) also could be explained by fusion of catecholamine-containing vesicles [34]. More recently we have studied

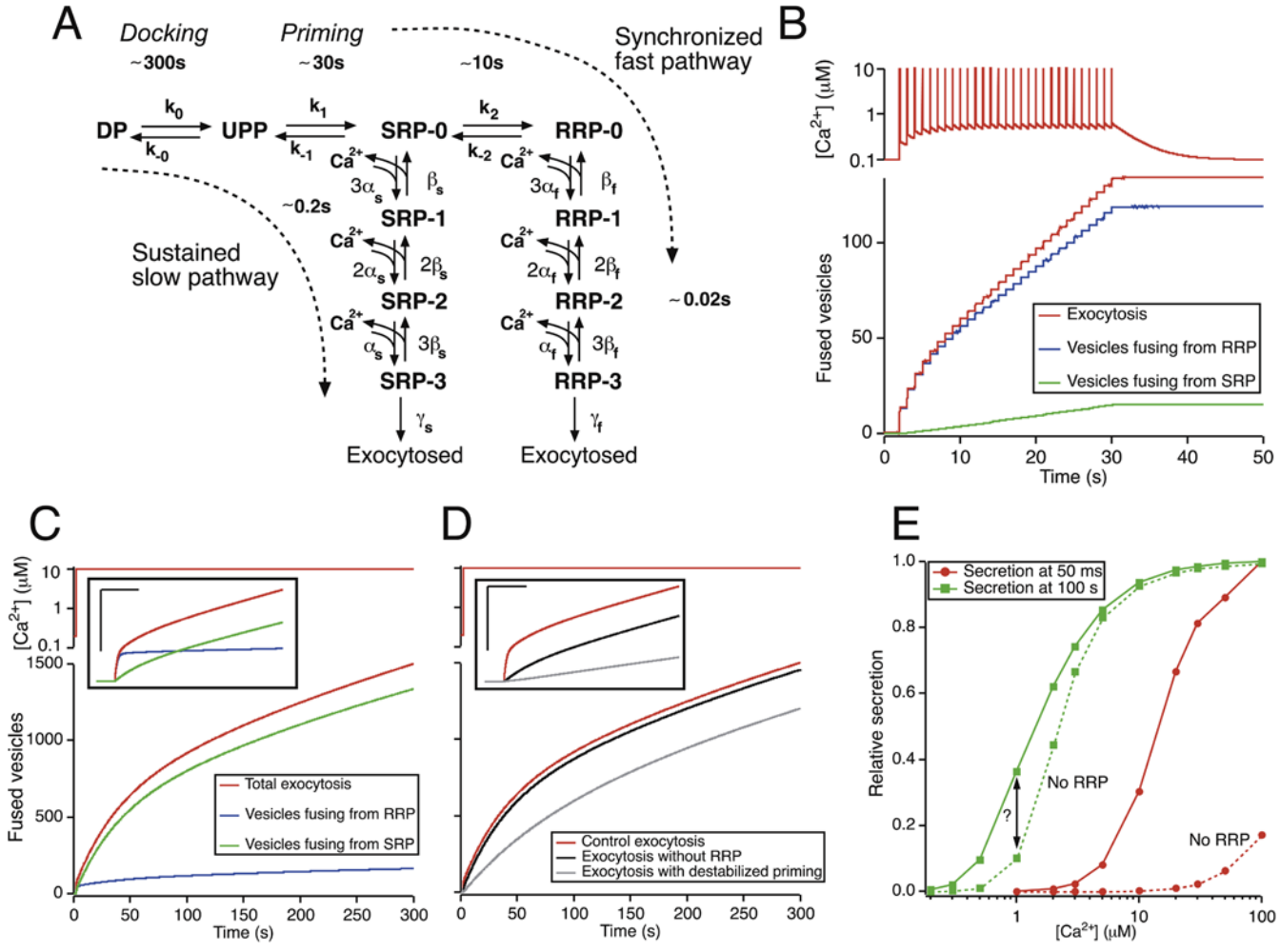

Fig. 3A–E A synchronized, fast and a sustained, slow pathway for secretion in chromaffin cells. **A** Kinetic model for secretion of large dense-core vesicles from chromaffin cells, constructed mostly with information from capacitance measurement studies (*DP* depot pool, *UPP* unprimed pool, *SRP-i* slowly releasable pool with *i* Ca^{2+} ions bound, *RRP-i* readily releasable pool with *i* Ca^{2+} ions bound). *Horizontal steps* symbolize maturation reactions, *vertical steps* triggering reactions. The priming rate is Ca^{2+} dependent, modelled as $k_1=[Ca^{2+}]\cdot r_{max}/([Ca^{2+}]+K_d)$, where r_{max} is the maximal rate of vesicle priming (at saturating $[Ca^{2+}]$) and K_d the dissociation constant for Ca^{2+}. The approximate characteristic times for each process are given; for triggering the values at 20 μM are shown. **B** Simulation of the model in **A** by a train of 10 μM calcium jumps lasting 40 ms, delivered at 1 Hz, simulating a series of depolarizations. After each "depolarization" $[Ca^{2+}]_i$ subsides immediately, simulating the collapse of the local Ca^{2+} microdomains, and leaves an additive increase in basal $[Ca^{2+}]_i$ of 150 nM that is then cleared from the cell with a time constant of 3 s. This stimulation pattern gives rise to jump-like increases in the number of fused vesicles. Most vesicles fused through the RRP pathway. Simulations were made using a 4th-order Runge-Kutta scheme with automatic step size control. **C** Simulation of the model in **A** under conditions of a step-like increase in $[Ca^{2+}]_i$. To decrease simulation times a simplified model was used (also in **D**), in which the triggering steps were not modelled directly but replaced by the corresponding mean rate at the appropriate $[Ca^{2+}]$. The *inset*shows the result during the first 5 s of stimulation (*vertical scale* 100 vesicles; *horizontal scale* 1 s). Parameters for simulation: k_0=0.002 s^{-1}, k_{-0}=0.0041 s^{-1}, r_{max}=0.03 s^{-1}, K_d=2.3 μM, k_{-1}=0.05 s^{-1}, k_2=0.12 s^{-1}, k_{-2}=0.1 s^{-1}, α_f=4.4 μM^{-1} s^{-1}, β_f=56 s^{-1}, γ_f=1450 s^{-1}, α_s=0.5 μM^{-1} s^{-1}, β_s=4 s^{-1}, γ_s=20 s^{-1} [22, 25, 82]. The initial size of the vesicle pools were DP: 2000, UPP: 787, SRP: 38, RRP: 45 (vesicles). **D** Simulations under conditions in which the RRP was destabilized (k_{-2} increased from 0.1 s^{-1} to 1000 s^{-1}, *black trace*), or where priming was destabilized (k_{-1} increased from 0.05 s^{-1} to 2 s^{-1}, *grey trace*). All other parameters as in **C**, the initial pool sizes were changed according to the new rate parameters. **E** Secretion evaluated 50 ms (*red* traces) or 100 s (*green traces*) after a step-like increase in $[Ca^{2+}]$ from 0.2 μM to different values. The secretion was normalized to the value obtained at 100 μM Ca^{2+} for each condition. Note that the apparent Ca^{2+} sensitivity is higher when evaluated at 100 s than at 50 ms. The *dotted lines* show the results after eliminating the RRP

chromaffin cells from synaptotagmin 1 knock-out mice (in which the RRP is missing [35]) using simultaneous capacitance and amperometry measurements. These studies have shown that in the absence of an RRP the fastest part of the amperometric signal is reduced proportionally. Both capacitance and amperometric signals are restored upon synaptotagmin 1 over-expression (G. Nagy, unpublished data). These data show definitely that the RRP, as studied in our laboratory, consists of catecholamine-containing vesicles. The likely reasons for the discrepancies in the literature are, first, that if cells are stimulated from a low basal $[Ca^{2+}]_i$ the RRP (and the SRP) are essentially empty, and thus catecholamine secretion follows after a considerable delay because of vesicle

Table 1 Manipulations that change the size of the readily releasable vesicle pool(s) (*RRP*) in chromaffin cells (*NSF N*-ethylmaleimide-sensitive factor, *PIP_2* phosphatidyl-inositol-4,5-bisphosphate, *BIS* bisindolylmaleimide, *SNAP-25, -23* synaptosomal-associated protein-25 kDa, -23 kDa, *SNARE* soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)

Molecule/manipulation	Direction of change	Method	Proposed mechanism	References
Ca^{2+}	↑	Infusion/photo-release/hormone treatment	Increased priming rate, PKC-dependent and -independent	[5, 6, 7, 25, 48, 120, 121]
ATP	↑	Infusion of non-hydrolysable analogues	Maintaining NSF activity [64]; synthesis of PIP_2 in membrane [93, 95, 96]	[5, 26, 43, 44, 45] but see [10]
PKC	↑	Phorbol ester stimulation/BIS inhibition/Ca^{2+} increase	Actin depolymerization/increased priming rate	[30, 46, 47, 48, 49, 122]
PKC P-site mutants	↓	Over-expression SNAP-25-S187C	Decreased refilling after release	[49]
PKA	↑	Pharmacology	Increasing pool stability	Conflicting evidence [50, 51, 52, 53]
PKA P-site mutants	↑	Over-expression Snapin-S50D		[123]
	↓	Over-expression Snapin-S50A		
	↓	Over-expression SNAP-25-T138A	Decreased pool stability	[53]
Temperature (37 °C)	↑	Permeabilized cells, patch-clamp	Increased priming rate, overfilling	[124, 125]
Munc13-1	↑	Over-expression	Increased priming rate	[82]
Munc18-1	↓	Knock-out	Munc18 promotes docking, the effect on RRP is indirect.	[68]
	↑	Over-expression		[68]
Synaptotagmin 1	↓	Knock-out mouse	Required for fusion of RRP, but not SRP	[35]
SNARE complex	↓	Infusion of clostridial neurotoxins	Cleaving SNAREs	[26]
	↓	Infusion of antibody	Block of SNARE assembly	[27]
	↑	Infusion of α-SNAP	Increased SNARE availability	[45, 121]
SNAP-25	↓	Knock-out mice and over-expression	Pool stability decreased	[67]
	→	Over-expression a isoform	Pool stability normal	
	↑	Over-expression b isoform	Pool stability increased	
	↓	Δ9 expression	Block of RRP	[60]
SNAP-23	↓	Over-expression	Pool stability decreased	[67]
Synaptic vesicle protein 2	↓	Knock-out mice	Interaction with synaptotagmin or SNAREs	[126]

priming and second, that if photorelease of caged Ca^{2+} results in $[Ca^{2+}]_i$>50–100 μM, the capacitance trace will be confounded by rapid endocytosis (>50 μM in bovine cells) and the intermediate component of vesicles mentioned above (>100 μM). Thus, to study catecholamine-releasing vesicles in isolation the $[Ca^{2+}]_i$ both before and after stimulation must be kept within certain limits.

Effect of stimulation regime: fast and slow pathways for fusion

The three Ca^{2+}-dependent reactions connected with exocytosis lead to marked differences in secretory behaviour depending on the stimulation regime. Thus, assaying exocytosis by fast techniques (capacitance measurements) can lead to fundamentally different conclusions than when exocytosis is assayed by intrinsically slower techniques, such as calcium infusion into cracked-open cells followed by analysis of the cell medium. Figure 3B–E illustrates this point by simulating the vesicle pool model in Fig. 3A assuming different stimulation regimes. The model in Fig. 3A was constructed on the basis of the identified vesicle pools in chromaffin cells (above), combined with models for the two Ca^{2+} sensors leading to fusion from the SRP and the RRP. In Fig. 3B the model was stimulated by a series of phasic action-potential-like stimuli (10 μM calcium jumps lasting 40 ms, delivered at 1 Hz). Model simulations show that this stimulation regime leads to step-like increases in the number of fused vesicles (Fig. 2B, lower panel). The steps have decreasing amplitude, indicating secretory depression as a result of depletion of the RRP. Most vesicles fused come from the RRP, due to the very fast RRP calcium sensor (Fig. 3B, blue trace). The SRP calcium sensor has a higher calcium affinity than the RRP sensor [25], but it is too slow to lead to significant fusion under conditions of rapidly fluctuating $[Ca^{2+}]_i$; the calcium signal will decay and calcium dissociate from the SRP sensor before fusion can succeed.

When calcium is increased stepwise (for instance by infusion of calcium into cracked-open cells, or by flash photolysis of caged Ca^{2+}) the result is very different. Consider first the situation in which $[Ca^{2+}]_i$ is jumped instantaneously to 10 μM (Fig. 3C, D). The insets in Fig. 3C and D show the response during the first 5 s after stimulation, which would correspond to the result observed when stimulating cells with flash photolysis of caged Ca^{2+} and assaying secretion with capacitance measurements (compare with Fig. 2A) [14]. The exocytotic burst originating from fusion of the RRP is complete within 0.1 s, followed by fusion of the SRP. After both pools have fused secretion continues with slower time course, however during this sustained phase, more than 90% of the vesicles now fuse by the SRP pathway! Consequently, when secretion is observed over the course of minutes, secretion from the SRP dominates (Fig. 3C). At this time scale we note a phase with a time constant of ~30 s, which corresponds to the priming reaction, followed by a near-linear phase, corresponding to vesicle docking. Thus, if secretion is assayed by slow techniques (such that the events shown in the inset stay unobserved) one will effectively be observing vesicles fusing through the SRP pathway with a time course characteristic for priming and docking reactions. Since docking of new vesicles is not likely to happen during infusion experiments in cracked-open cells [36], in this case only one or more priming reactions will be observed.

The reason for the difference between the result in Fig. 3B and C is that the scheme in Fig. 3A offers two alternative routes for fusion. Under conditions of sustained calcium increases (above a certain level), the SRP sensor will be faster than the (presumably Ca^{2+}-independent [25]) conversion from the SRP to the RRP. Primed vesicles will fuse through the SRP pathway without maturing into the RRP. Therefore, if rapid techniques are not used to monitor secretion within the first 0.1 s of stimulation, properties of the RRP or its calcium sensor cannot be assayed. Consider, for instance, the situation in which the formation of the RRP is blocked by deletion of synaptotagmin 1 [35], the presumed RRP calcium sensor. With a high time resolution the fast part of secretion is gone (black trace, inset in Fig. 3D), however at later times no difference in secretion will be noticeable (Fig. 3D, compare red and black traces). Actually the difference between control secretion and secretion after deletion of the RRP is even smaller than expected from Fig. 3C (compare red and green curves), because some of the vesicles that fused through the RRP in Fig. 3C are fusing through the SRP in Fig. 3D. Accordingly, synaptotagmin 1 is essential for rapid secretion from chromaffin cells when secretion is assayed by capacitance measurements [35], whereas experiments performed with slower techniques would suggest that synaptotagmin 1 is not necessary for exocytosis in neuroendocrine cells [37, 38, 39].

Figure 3E shows the effect of time resolution of the investigative protocol on the identified Ca^{2+} dependence of secretion. The model in Fig. 3A was simulated during a Ca^{2+} step and secretion evaluated 50 ms or 100 s after the step. The normalized secretion curves show that at the *lower* time resolution measurements (100 s) a *higher* Ca^{2+} sensitivity of secretion is identified. In this case secretion is dominated by the K_d for the (relatively slow) priming reaction, assumed to be 2.3 μM [25]. The K_d for the fast Ca^{2+} trigger is higher (12 μM), however priming, and not triggering, is rate-limiting for the cumulative secretion within a 100-s window after stimulation. Consequently the size of the RRP has little influence on secretion measured with this protocol. When measuring 50 ms after the Ca^{2+} step the apparent Ca^{2+} sensitivity is lower, reflecting the fast Ca^{2+} sensor (Fig. 3E, red curve). If we now assume that the RRP is deleted, we get a very dramatic effect with the high-resolution measurement (dotted red curve). At lower $[Ca^{2+}]$, the effect of deleting the RRP is also noticeable with the slow protocol, because at these concentrations the SRP sensor is now so slow that the vesicles will mature into the RRP and fuse using the faster RRP Ca^{2+} sensor. However, it is not clear whether the model in Fig. 3A is correct in this case (black double

arrow with question mark in Fig. 3E). The model for the SRP and RRP calcium sensors in Fig. 3A was constructed on the basis of flash photolysis experiments with $[Ca^{2+}]_i \geq 4$ μM, because at lower concentrations secretory rates become so low that fusion of the SRP and RRP could not be distinguished with certainty [25]. The secretory rates from the SRP and RRP at lower $[Ca^{2+}]_i$ are thus based on extrapolation. One possibility is that the SRP calcium sensor actually has a shallower dependence on Ca^{2+} at lower concentrations than the RRP sensor. If this is the case (as suggested by the left-hand points in Fig. 5B in [25]), then the SRP sensor may actually be as fast or faster than the RRP sensor at low calcium concentrations, such that the SRP pathway will dominate also at lower concentrations.

A further complication in distinguishing fast and slow pathways for fusion is the very slow fusion kinetics of the frequently used PC12 cells compared with chromaffin cells [40]. In an attempt to increase temporal resolution in a cracked PC12-cell assay, fast, rotating-disc-electrode voltammetry was used for fast on-line detection of liberated catecholamines [41]. However, in spite of increased temporal resolution secretion still occurred on a 10-s time scale, even at 100 μM Ca^{2+} [41], indicating that an equivalent of a chromaffin cell RRP (or even an SRP) is not found in PC12 cells. One possibility (see above) is that in these cells primed vesicles are unstable (i.e. the rate of depriming is high) such that a standing pool of primed vesicles is absent. The grey trace in Fig. 3D simulates this possibility. Consequently, the kinetics of release from this cell type may reflect the Ca^{2+}-dependent priming reaction exclusively [42].

The interpretation of infusion experiments given here differs from the classical interpretation [5, 43, 44], according to which the first phase of release (with a time constant of tens of seconds to a few minutes) is due to the triggering of Ca^{2+}-dependent exocytosis, whereas the second phase represents ATP-dependent priming. What I have illustrated here by simulating the model in Fig. 3A (constructed on the basis of capacitance measurements) is that the meaning of the terms "triggering" and "priming" depends on the stimulation regime. What we call "RRP triggering" in flash photolysis experiments is not likely to show up at all in infusion experiments using slow techniques, and the first detectable phase in infusion experiments may correspond to what is known as priming in studies using faster techniques (and which is also Ca^{2+} dependent, see above). Part of the problem is semantic: the term priming is used by many authors to denote that step which precedes the most rapid one detected by their assay. In some cases this represents an ATP-dependent step preceding the Ca^{2+}-dependent reaction under study. In contrast, when using capacitance measurements, the term priming has been used to denote the process of refilling the releasable vesicle pools. In spite of the different methods, though, many findings about earlier maturation steps agree between the two methods, for instance the ATP-dependence of priming [26, 43] and the stimulating effect on priming of small amounts of Ca^{2+} [5, 25], but no agreement should be expected when assaying properties that are relevant only to the fusion of the RRP.

The readily-releasable vesicle pool: molecular requirements

Table 1 summarizes the molecular manipulations that have been shown to change the size of the RRP in chromaffin cells, with a focus on capacitance measurements. In the following I will comment on a few aspects of special interest.

Results from both patch-clamp and infusion experiments show that ATP is required for the priming reaction and in its absence the RRP is depleted [5, 26, 43, 44, 45] (but see [10]). As mentioned above a prolonged increase in basal $[Ca^{2+}]_i$ leads to increased priming and, consequently, to a larger RRP (Table 1). Part of this effect is caused by Ca^{2+}-dependent activation of PKC [46, 47, 48, 49], but even in the presence of PKC inhibitors Ca^{2+} still increases RRP pool size through an unknown mechanism [48]. The dependence of priming on basal $[Ca^{2+}]_i$ is an elegant mechanism by which the same signal that causes release also accelerates refilling of the releasable vesicle pools to counteract depletion. Furthermore, hormone-induced release of calcium from intracellular stores is efficient in augmenting RRP size, even though the $[Ca^{2+}]_i$ may not reach the secretion threshold [7]. This phenomenon may serve to integrate longer-lasting signals encoding the overall physiological state with acute stimulation of the adrenal gland. The function of cAMP in chromaffin cells is more controversial, since cAMP has been found to stimulate secretion in most (e.g. [50, 51]), but not all (e.g. [52]), studies on chromaffin cells. In recent flash photolysis experiments PKA inhibitors decreased the size of the RRP, whereas cAMP infusion was without effect [53]. The findings in different laboratories can be resolved if we assume that PKA is constitutively active in some, but not in other, investigations, but this hypothesis remains to be tested. The effects of Ca^{2+}, PKA, PKC and ATP on exocytosis account for the physiological regulation of exocytotic strength. Recent investigations have attempted to identify the proteins/lipids making up the exocytotic machinery itself, which also constitute the most likely downstream targets of the regulators.

The neuronal soluble *N*-ethylmaleimide (NEM)-sensitive factor attachment protein receptor (SNARE) complex is formed between the vesicle-SNARE synaptobrevin (anchored in the secretory granule) and the target-SNAREs synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin (anchored in the plasma membrane) in preparation for exocytosis. It consists of a twisted coiled-coil bundle made up of four α-helices (the so-called SNARE domains), one each from synaptobrevin and syntaxin and two from SNAP-25 [12, 54, 55]. The α-helices of synaptobrevin and syntaxin are oriented in parallel, such that formation of the complex brings the membranes into close contact. The complex is extremely stable in solution [56] and its formation may liberate

enough energy to drive membrane fusion. Indeed, SNARE proteins reconstituted into lipid vesicles suffice to cause fusion in vitro, albeit with very slow kinetics [57]. The SNARE complex is necessary for exocytosis as demonstrated by infusion of clostridial neurotoxins that cleave one or more SNARE proteins and block secretion in chromaffin cells [26, 58, 59]. Infusion of an antibody that prevents full SNARE complex formation causes the loss of the RRP, indicating that full SNARE complex formation is necessary to form the RRP, but not the SRP [27] and implying, therefore, that the SRP may only require a "loose" SNARE complex. Alternatively, binding of the antibody to the (partially) assembled SNARE complex may prevent the binding of other components, like synaptotagmin. The light chain of botulinum toxin A (BoNT/A) cleaves the last 9 amino acids from the C-terminal end of SNAP-25 and infusion with this toxin slows the exocytotic burst, consistent with the loss of the RRP [26]. Similar results are obtained when the corresponding deletion mutant of SNAP-25 (SNAP-25 Δ9) is over-expressed in bovine chromaffin cells [60]. These findings may possibly be explained by the observations that the last 9 amino acids in SNAP-25 are necessary for binding to synaptotagmin 1 [61], which is necessary for formation of the RRP [35].

Non-productive *cis*-SNARE complexes, which are formed during fusion when the anchors of the three SNAREs become located in the same membrane, are dissociated by the concerted action of α-SNAP and the ATPase NEM-sensitive factor (NSF) [62]. Infusion of α-SNAP increases secretion from chromaffin cells [63]. In flash photolysis experiments α-SNAP introduced via a patch pipette increases the size of the RRP, whereas infusion of NEM, which inhibits NSF, blocks the refilling of the RRP after one round of release [45]. Work with cracked-open PC12 cells has also indicated that α-SNAP/NSF activity precedes fusion and thus can account for part of the ATP-dependence of the priming reaction [64]. These data seem to indicate that the availability of free SNAREs regulates secretion at an early recruitment/priming step. However, recent work on isolated membrane sheets formed by ultrasound disruption of PC12 cells has shown that syntaxin and SNAP-25 are free to form SNARE complexes with exogenously added synaptobrevin immediately after disruption, but then become unavailable over the course of minutes due to the formation of *cis*-SNARE complexes in the plasma membrane [65]. This means that in vivo the disruption of spontaneously forming *cis*-SNARE complexes may not be rate-limiting for the priming reaction because of a high basal NSF/α-SNAP activity. In cracked-open cells—or to a smaller extent after dialysis against a patch pipette for several minutes—NSF and α-SNAP diffuses out, such that subsequently availability of SNAREs may become rate limiting for the priming reaction. The situation is exacerbated by stimulation, since this causes the accumulation of *cis*-complexes in the plasma membrane and increases the requirements for NSF and α-SNAP. A role for NSF/α-SNAP in dissociating *cis*-SNARE complexes after but not before fusion has been indicated by studies in *Drosophila*[66].

Recently, we have shown that fast, Ca^{2+}-triggered secretion is abolished in the SNAP-25 knock-out mouse, and that it can be restored (rescued) by short-term viral over-expression of SNAP-25 [67]. Most knock-out mice for exocytotic proteins have a lethal phenotype and have so far been studied using acute adrenal slices from embryos or newborn animals [35, 68]. In our study we developed a preparation of isolated chromaffin cells from single mouse embryos and showed that secretion is restored by over-expression of SNAP-25 using Semliki Forest Virus [67, 69]. This general approach allows the study of a number of different mutations and isoforms of exocytotic proteins without interference from endogenous proteins, provided that the knockout mouse is available.

SNAP-25 has two splice variants which differ at nine amino acid positions; the SNAP-25a variant is present in the embryonic brain and in neurosecretory cells (including chromaffin cells), while the SNAP-25b variant dominates in the adult brain [70]. Over-expression of the SNAP-25b variant increases the size of the two releasable vesicle pools, SRP and RRP, threefold compared with wild-type chromaffin cells, whereas the fusion kinetics of the pools are unchanged [67]. The sustained secretion component, which represents the priming rate at high $[Ca^{2+}]_i$, is also unchanged. We therefore suggested that it is not the (forwards) priming rate that is changed by the isoform, but rather the (backwards) depriming rate (Fig. 3A), indicating that the two SNARE complexes, containing SNAP-25a or SNAP-25b, confer different stability to the primed vesicles. Over-expression of the closely related, but almost ubiquitously expressed SNAP-23 isoform [71, 72] leads to a slight increase of sustained secretion in SNAP-25 knock-out cells, but an exocytotic burst is still absent. When over-expressed in control (SNAP-25-expressing) cells SNAP-23 decreases secretion by abolishing the exocytotic burst. The combination of over-expression in knock-out and wild-type cells therefore shows that SNAP-23 can compete with SNAP-25 for participation in secretion.

By analogy with the difference between SNAP-25 isoforms we have suggested that SNAP-23 and SNAP-25 may differ in their ability to stabilize primed vesicles; such vesicles being unstable when SNAP-23 participates in secretion, leading to a very small standing pool of primed vesicles (see Fig. 1 and grey line in Fig. 3D). So, by exchanging SNAP-25, which is characteristic of cells with fast Ca^{2+}-triggered exocytosis, with SNAP-23, which is present in most cells, we can convert the cell from a fast, Ca^{2+}-triggerable state to a state in which it displays exocytosis on a much slower timescale, reminiscent of constitutive exocytosis. The stabilization of primed vesicles is one of the three principal kinetic possibilities for the difference between fast Ca^{2+}-triggered and constitutive exocytosis (Fig. 1) and this property may be encoded in the SNARE complex used for fusion. This function of the neuronal SNARE complex in stabilising primed vesicles is so far a hypothesis, since it is inferred from the comparison of the sustained rate of release and the

steady-state pool sizes. Direct measurement of primed vesicle stability has not been accomplished so far. Other possibilities are that in the SNAP-23 over-expressing cells the Ca^{2+} dependence of priming is changed to higher values, or that the maximal triggering rate of the Ca^{2+} sensor is substantially decreased. It is still unknown whether the differences in secretory behaviour between SNAP-25a, SNAP-25b and SNAP-23 are caused by endogenous properties of the three alternative SNARE complexes, or by different binding of auxiliary factors.

Other recent findings indicate that SNAP-25 may be a regulator of exocytosis. Mutation of the only known PKC site in the SNARE complex (Ser^{187} in SNAP-25) change vesicle pool recovery [49]. Over-expression of a phosphomimetic mutant (S187E) accelerates the sustained component of secretion, whereas a mutation mimicking the non-phosphorylated state (S187A) slows refilling of the releasable vesicle pool. However, the over-expression of the phosphomimetic mutant does not interfere with the ability of PKC inhibitors to reduce secretion, indicating the involvement of other PKC targets in the regulation of release. Mutation of a PKA site (Thr^{138} in SNAP-25) to alanine decreases the SRP and RRP size, as does block of PKA [53]. Notably, mutating the PKA site to aspartate to mimic the phosphorylated state, diminishes the effect of PKA inhibitors on secretion, indicating that phosphorylation of Thr^{138} is indeed involved in the effect of PKA in chromaffin cells.

All the above findings indicate that SNAREs are involved in setting pool sizes or sustained rates of secretion, i.e. they have a role in the priming step (NEM and α-SNAP infusion [45], phosphorylation mutations [49, 53]) and possibly the maturation step between SRP and RRP (BoNT/A treatment [26], SNAP-25 Δ9 over-expression [60]). These findings supports a model in which the SNARE complex "zipping" from the N-terminal end to the C-terminal membrane anchors of syntaxin and synaptobrevin stabilizes vesicles in the primed state. Possibly part of the released energy upon SNARE complex formation is used to keep the vesicle primed, such that exocytosis is rapid upon arrival of the Ca^{2+} signal. A puzzling observation is the fact that neither the SNAP-25 isoforms nor phosphorylation mutants studied so far in our laboratory, nor infusion of α-SNAP or NEM that increases or decreases SNARE availability, respectively, changes the rate of fusion from the RRP or from the SRP [45, 49, 53, 67], as might have been expected were the SNARE complex assembly to lead directly to fusion. This could lead to the proposition that the SNARE complex is involved in priming, but not execution of fusion, as suggested from work on vacuolar fusion [73]; however another possibility is that none of these manipulations changed SNARE complex assembly itself ('zipping').

A direct function of the SNARE complex in triggering release has been suggested on the basis of infusion experiments in PC12 cells [74, 75]. Infusion of botulinum toxin E (BoNT/E) was used to cleave endogenous SNAP-25, thus blocking secretion. Infusion of a 65-aa fragment encompassing the C-terminal of SNAP-25 (S25-C) rescues secretion [74]. In a stage-specific assay for ATP-dependent priming and Ca^{2+}-dependent triggering reactions the C-terminal fragment rescued secretion only when present together with Ca^{2+} during the triggering step. This was taken to indicate that zipping-up of the SNARE complex occurs during triggering after Ca^{2+}-entry [74]. Infusion of the syntaxin SNARE motif inhibits secretion from cracked-open PC12 cells only when the N-terminal part of the motif is present [76]; the C-terminal part alone does not inhibit secretion. These experiments imply that the zipping-up of the SNARE complex (proceeding from the N- towards the C-terminal end) occurs after Ca^{2+} triggering, and that no partially assembled intermediate is present before Ca^{2+} infusion [76]. However, as we have seen (Fig. 2), in this assay system it is hard to distinguish between a Ca^{2+}-dependent priming step and the triggering step itself (as we understand these concepts from capacitance measurements). Furthermore, as pointed out above no kinetic correlate of the RRP has been found in PC12 cells, possibly because vesicles can not be stabilized in the primed state. It may thus be impossible to distinguish priming and triggering steps kinetically in this cell type. It should also be noted that some of the SNAP-25 mutations studied in our laboratory show effects on several phases of secretion; for instance SNAP-25 Δ9 not only eliminates the RRP but also slows the sustained component of release and largely blocks the response to a second stimulation [60] (see [77] for another example). The cracked-cell assay is more likely to assay the two latter effects, rather than the former. Thus from the viewpoint of measurements on chromaffin cells it is an unresolved question whether zipping of the SNARE complex plays a direct role in triggering fusion, or whether the fully zipped SNARE complex is a "priming machine" that may act as an intermembrane nucleation centre for triggering factors such as synaptotagmins (see also below).

Munc13 is a presynaptic protein with a diacylglycerol-binding C1 domain (reviewed in [78]). It was identified as a priming factor by the observation that in Munc13-1 knock-out mice synaptic vesicles cannot fuse, even though they appear to be docked properly to the plasma membrane [79]. All docked vesicles are thus, apparently, in the unprimed pool. The mechanism of Munc13 action is thought to be through interaction with syntaxin. Syntaxin contains an autonomously folded N-terminal domain (H_{abc}) in addition to the C-terminal SNARE motif, and adopts a "closed" conformation in solution, which is incompatible with SNARE complex formation [80]. Munc13 binding to the N-terminal domain may open syntaxin and make the SNARE motif available for SNARE complex formation [78]. This has been demonstrated elegantly by the rescue of an Unc13 mutant by an open form of syntaxin in *Caenorhabditis elegans* [81]. Munc13 over-expression in chromaffin cells increases the size of the RRP and SRP by a factor of four and also accelerates the sustained component of release [82]. These effects are consistent with a function of Munc13-1 in stimulating the (forwards) priming rate. It has been shown

recently that the enhancement of synaptic transmission in autaptic hippocampal neurons by β-phorbolesters is mediated by their binding to the C1 domain of Munc13 and not by PKC, indicating that Munc13 plays an important role in synaptic plasticity [83]. Whereas the role of Munc13 is well established in neurons, whether Munc13 is an endogenous priming factor in chromaffin cells is still unknown, since expression levels appear to be low [82]. Furthermore, the effect of β-phorbolester in chromaffin cells is completely blocked by the PKC inhibitor bisindolylmaleimide I [46], which is without effect in hippocampal neurons [83].

Apart from a SNARE complex, all membrane fusion reactions require a so-called Sec1/munc18-like (SM) protein, which in Ca^{2+}-triggered exocytosis is Munc18-1/nSec1 [84]. Munc18-1 is an arc-shaped protein that binds with nanomolar affinity to syntaxin in the closed conformation, but not to the assembled SNARE complex, implying that it may be a negative regulator of SNARE complex formation and exocytosis; indeed, over-expression in *Drosophila* decreases secretion at the neuromuscular junction [85]. In contrast, infusion or over-expression of Munc18 in PC12 and chromaffin cells fails to inhibit secretion [86]. In chromaffin cells from adrenal slices of the Munc18-1 knock-out mouse secretion is inhibited dramatically due to a defect in vesicle docking [68]. In that study over-expression of Munc18 increases secretion by increasing both the size of the releasable vesicle pools and the rate of priming. If Munc18 functions exclusively in docking, the effect on the size of the SRP and RRP may be indirect and due to an increased pool of docked, but unprimed vesicles, which, in a reversible reaction scheme (such as the one in Fig. 3A), in turn will increase the size of the SRP and RRP. Thus, there is no evidence from these experiments that Munc18 acts directly during priming. A role for Munc18-1 in vesicle docking has been supported by a recent, thorough study in *C. elegans* [87]. Since formation of the SNARE complex does not seem to be necessary for docking (morphological vesicle docking in chromaffin cells is normal in the absence of SNAP-25 [67]), this means that Munc18-1 must have another function than solely regulation of SNARE complex formation [68].

The peripheral vesicular membrane protein CAPS (calcium-dependent activator protein for secretion) may be a native priming factor in neurosecretory cells [42]. An antibody against CAPS blocks secretion from rat melanotrophs [88] and bovine chromaffin cells [89]. An interesting feature of CAPS is that it binds to large, dense-core vesicles and phosphatidylinositol 4,5-bisphosphate (PIP_2)-containing lipid membranes via different domains [90, 91]. Since PIP_2 is found mainly in the plasma membrane [92] this suggests a possible mechanism by which CAPS could link vesicles to the membrane and promote vesicle priming (but see [91]). A role for PIP_2 in priming was first indicated by studies of permeabilized cells [93, 94]. In digitonin-permeabilized chromaffin cells infusion of PLC decreases the levels of inositol phospholipids and inhibits secretion [93]. After PC12 cells are permeabilized secretion runs down due to the escape of two cytosolic proteins necessary to produce PIP_2, phosphatidylinositol-4-phosphate-5-kinase and phosphatidylinositol transfer protein [94, 95]. The maintenance of inositol phospholipids may account for part of the dependence of vesicle priming on ATP [93, 95, 96]. In pancreatic β-cells infusion of PIP_2 through a patch pipette increases RRP size [97]. The increase (but also the basal size) is blocked by CAPS antibodies, which means that CAPS acts at or after the PIP_2-dependent step [97]. Another possible binding partner for PIP_2 is synaptotagmin [98, 99], the double C2-domain containing integral vesicular protein which is usually assumed to be the calcium sensor for exocytosis (see below). Delineating the role of specific protein-lipid interactions in the cell will be one of the important future challenges in exocytosis research.

Fusion of the releasable vesicle pools

Stimulation of neurons and neurosecretory cells by flash photolysis of caged Ca^{2+} has made it possible to study the kinetics of RRP fusion during step-like increases in $[Ca^{2+}]_i$ [2, 3, 9, 24, 100, 101, 102]. Secretion from the RRP starts after photorelease of Ca^{2+} with a measurable delay and then proceeds with a near-exponential time course until depletion of the pool (Fig. 2A, inset). By employing flashes of different intensities the $[Ca^{2+}]_i$ can be stepped to different values to assay the calcium-dependence of the release kinetics. These studies have shown that increasing the post-flash $[Ca^{2+}]_i$ increases the exponential rate constant for release (i.e. $1/\tau$, where τ is the time constant for the exponential time course) (Fig. 2C, left) and decreases the delay (Fig. 2C, right). These two relationships can be reproduced if the Ca^{2+}-triggering step is modelled as a sequence of Ca^{2+}-binding events to the Ca^{2+} sensor, followed by an irreversible (and Ca^{2+}-independent) fusion step [24, 25, 101, 102] (Fig. 2B). The values of the association-rate (on-rate, α), the dissociation-rate (off-rate, β) as well as the rate constant for the final fusion step (γ) can be obtained by fitting the model directly to the observed relationships [2, 25, 101, 102]. Similar methods could be used for description of the "alternative" Ca^{2+} sensor that causes fusion from the SRP [25].

This biophysical characterization of the Ca^{2+} sensor(s) for exocytosis sets the stage for the search for molecular manipulations that can change properties of the Ca^{2+} sensor or, in other words, we can now start to identify the molecular counterparts for the two last kinetic requirements for fast, Ca^{2+}-triggered exocytosis ("2." and "3." in Fig. 1). However, whereas many manipulations change the size of the releasable vesicle pools (Table 1), there are very few studies using high time-resolution techniques that have demonstrated conclusively an effect on the kinetics or Ca^{2+} sensitivity of the triggering step of release from the RRP or SRP.

Synaptotagmin 1 is a vesicular protein containing two C2-domains, C2A and C2B, that are involved in Ca^{2+}-

dependent phospholipid binding, as well as binding to two components of the SNARE complex, syntaxin and SNAP-25. In addition, the C2B domain is involved in Ca^{2+}-dependent multimerization, phosphoinositide binding, binding to endocytic proteins and Ca^{2+}channels (reviewed by [103, 104, 105, 106]). For a long time the function of synaptotagmin 1 as a Ca^{2+} sensor for exocytosis has been assumed: deletion of synaptotagmin 1 abolishes fast secretion in *Drosophila* [107], *C. elegans* [108] and mouse [109]. In mouse hippocampal neurons the slow part of secretion persists in the absence of synaptotagmin 1, implying that synaptotagmin 1 may be the Ca^{2+} sensor for fast release [109]. In chromaffin cells from synaptotagmin 1 knock-out mice the RRP is missing, whereas the SRP persists [35]. Inspecting the model for secretion from chromaffin cells (Fig. 3A), these observation would be consistent with the function of synaptotagmin as a Ca^{2+} sensor for the RRP, since in its absence the RRP should not be able to fuse. However, the alternative explanation, that synaptotagmin 1 is required for the conversion from the SRP to the RRP without acting as a Ca^{2+}sensor itself, cannot be ruled out. Data from neuronal preparations also have not been able to distinguish between these possibilities since a similar heterogeneity of release-ready vesicles exists in neurons and, importantly, the influence of synaptotagmin 1 on this heterogeneity has not been determined.

Taking advantage of the biophysical characterization for the calcium sensor(s) we recently tested the role of synaptotagmin by performing Ca^{2+} uncaging experiments in chromaffin cells from a knock-in mouse carrying a mutant synaptotagmin 1 [110]. The charge-neutralizing mutation in the C2A domain (R233Q) decreases the apparent Ca^{2+}affinity for phospholipid binding by a factor of two (i.e. a doubling of K_d), while Ca^{2+}-dependent binding to syntaxin is unchanged [111]. This mutation thus provides a test of the two alternative hypotheses put forward above: that synaptotagmin either regulates the interconversion of SRP vesicles into RRP vesicles, or that synaptotagmin 1 is itself the calcium sensor for RRP fusion. In the first case the mutation should have an effect on the relative sizes of RRP and SRP pools, whereas in the latter case properties of the RRP calcium sensor should be changed. Following flash photolysis of caged Ca^{2+} secretory delays were longer and secretory rates lower in the R233Q knock-in, indicating a clear effect on the Ca^{2+} sensor. The relative sizes of the SRP and RRP pools are unchanged (in fact the size of both pools are larger in the knock-in, possibly due to overfilling caused by reduced release at basal Ca^{2+}). Calcium ramps created by slow photolysis of the Ca^{2+} cage have been used to probe secretion at low $[Ca^{2+}]_i$. These data show that the knock-in starts to secrete at a higher $[Ca^{2+}]_i$ than wild-type animals. Using the mathematical model for the fast calcium sensor we could show that all of these effects could be accounted for by a twofold increase in the K_d for Ca^{2+} binding to the fast Ca^{2+} sensor. These data support a role for synaptotagmin 1 as the RRP calcium sensor and also support the idea that it is Ca^{2+}-dependent phospholipid binding that triggers secretion, since this was the feature changed by the mutation. Recently, however, it has been shown that this mutation also changes binding to SNAP-25, but a primary role in membrane interaction during exocytosis is still presumed [112]. The mutation does not, however, address the question whether the C2A or C2B domain is more important for triggering, since when present in the double C2A-C2B domain it changes the overall Ca^{2+}-dependent phospholipid binding [111, 112]. Several recent studies have addressed the effect of mutating the C2 domains of synaptotagmin 1 in neuronal systems [111, 113, 114, 115, 116, 117]. These studies have shown conclusively that Ca^{2+} binding to the C2 domains is critical for the function of synaptotagmin 1, and the picture appearing is that Ca^{2+} binds simultaneously to the double C2A-C2B domain. However, the role of the different binding partners of synaptotagmin (phospholipids, SNAREs, multimerization) in exocytosis and endocytosis is controversial.

Despite the above findings, many questions about synaptotagmin function still persist. For instance, as shown in Fig. 3 it is hardly possible using calcium infusion and slow methods to discern the properties of the RRP calcium sensor. The question therefore is, why do manipulations of synaptotagmin, or its of its binding to SNAP-25, lead to marked changes in secretion when investigated using such methods [39, 99, 118]? A possible explanation is an upstream function of synaptotagmin 1, either in a priming step or at the level of the SRP Ca^{2+} sensor. Indeed, the identity of the SRP Ca^{2+} sensor is another open question. In the R233Q knock-in we noted changes in the kinetics of SRP fusion also, even though we consider these data to be less reliable than those relating to RRP fusion [110]. On the other hand, deletion of synaptotagmin 1 does not eliminate SRP fusion [35], so that another protein or protein complex must be involved.

As discussed above ("2." in Fig. 1), an important prerequisite for fast, Ca^{2+}-triggered secretion is that vesicles be prevented from fusing at the resting $[Ca^{2+}]_i$. This is the one feature for which the least information is currently available. The knock-out of synaptotagmin 1 increases the rate of fusion of small synaptic vesicles under basal conditions ("mini rate") in *Drosophila* [107, 113]), but not in the mouse [109]. Furthermore, certain mutations of synaptotagmin 1 lead to an even more severe phenotype than complete removal of synaptotagmin [113, 116], implying that synaptotagmin 1 may be a fusion clamp at lower $[Ca^{2+}]_i$, while at the same time triggering secretion at higher $[Ca^{2+}]_i$ [113]. However, the situation is complicated by the presence of more than a dozen synaptotagmin isoforms, as well as other Ca^{2+}-binding proteins. Another interpretation of these data is thus that, in the absence of synaptotagmin 1, another Ca^{2+} sensor substitutes and has a higher Ca^{2+} affinity (so that release at basal $[Ca^{2+}]_i$ increases), but a lower maximal rate of release (γ) (so that release at stimulated $[Ca^{2+}]_i$ decreases) than synaptotagmin 1. In this case certain synaptotagmin mutations may be even more severe than deletion of the gene altogether, since the presence of a non-functional,

mutant protein may prevent substitution by another protein/isoform.

Yet another open question is whether synaptotagmin triggers secretion alone, or in a functional interplay with other proteins, such as the SNARE complex. As discussed above most manipulations of SNARE proteins in chromaffin cells change pool sizes or rates of replenishment, but not the rate constant of fusion of the vesicles. There is, however, one exception; in a previous study we mutated three charged amino acids in SNAP-25, which were located on the outside of the SNARE complex and, in the crystal structure, bind to a strontium ion. This mutation causes slower secretion [77], implying that this site on the SNARE complex either participates directly in secretion triggering, or that it couples to a calcium trigger, such as synaptotagmin. Since with the mutation we can only distinguish one releasable vesicle pool with properties quite similar to those of the wild-type SRP, whether the mutation causes slower fusion through a modified RRP pathway, or whether the faster fusing vesicle pool (the RRP) is lost altogether and the remaining secretion mediated by the SRP pathway can not be determined. Thus it is not clear whether the mutation has an effect on pool size (deleting the RRP), on release kinetics (slowing secretion) or, possibly, both.

If the zipping up of the SNARE complex participates directly in fusion triggering, as suggested several years ago [119], a model must be found in which Ca^{2+} binds to synaptotagmin 1, and yet most of the energy required for fusion is delivered by the SNARE complex. This suggests a complex interplay, in which synaptotagmin 1 binding to the plasma membrane will stimulate SNARE complex zippering [106], however direct evidence for this model is missing.

Conclusion

The kinetic prerequisites for fast, Ca^{2+}-triggered exocytosis are (1) the formation and stabilization of a readily-releasable pool of vesicles, (2) the prevention of fusion of the RRP vesicles at basal [Ca^{2+}] and (3) very fast fusion triggering of RRP vesicles at elevated [Ca^{2+}]. Recent evidence shows that SNAREs and, by inference the SNARE complex, is involved in forming and stabilising the RRP. This process can be regulated, as shown by the different sizes of the releasable vesicle pools induced by SNAP-25 splice variants and phosphorylation mutants. Auxiliary factors such as Munc13, Snapin, and synaptic vesicle protein 2 (SV2) also appear to regulate the RRP size, possibly through interaction with the SNARE complex, whereas Munc18 has an upstream role in vesicle docking. The triggering of fusion at stimulated [Ca^{2+}] seems to be caused by binding of Ca^{2+}-loaded synaptotagmin 1 to the plasma membrane, whereas the role of other synaptotagmin interactions remains controversial. The prevention of fusion at resting [Ca^{2+}] is so far not understood: synaptotagmin 1 may serve this function via specific domains, but the existence of multiple isoforms makes for alternative explanations. The most interesting open question is how—and whether—the energy released by SNARE complex formation can drive membrane fusion, and yet the triggering function be subserved by another protein (synaptotagmin).

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