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DYNAMICS OF SECRETION

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INTRODUCTION

Exocytosis of specific molecules – the neurotransmitters – is the primary mean of chemical communication between neurons and their effectors. Neurotransmitters are stored in two vesicular organelles localized at the presynapse (i) the synaptic vesicles (SVs) and (ii) the dense-core vesicles (DCVs). SVs are most abundant in neurons and, ultrastructurally, they appear as small clear vesicles of uniform diameter (around 50 nm), which store fast acting neurotransmitters (glutamate, GABA, glycine and acetylcholine). Vesicles morphologically similar to SVs are also present in other cells highly specialized in secretion, i.e. the endocrine cells, where they are called synaptic-like microvesicles (SLMVs). Instead, DCVs (or secretory granules) are large vesicles (~ 200 nm) with an electron-dense core at their center. In neurons, they come in two different sizes: small DCVs (SDCVs, ~ 50 nm), which store biogenic amines, and large DCVs (LDCVs, ~ 200 nm), which carry peptide neurotransmitters, biogenic amines and other non-peptide neurotransmitters (12). DCVs are the major secretory organelles in endocrine cells.

Two exocytic processes have been shown to occur at the synapse and in endocrine cells (Fig. 1). In the first process, named 'full-fusion' or 'all-or-none' exocytosis, secretory vesicles fuse completely into the plasma membrane and then are recaptured by clathrin-mediated endocytosis (27). Instead, during 'kiss-and-run'/'kiss-and-stay'/'cavicapture' exocytosis, secretory organelles open a transient pore through which the neurotransmitter diffuses out; then the pore closes and the vesicle is ready to be refilled and reused locally (13). This latter process is best suited to explain not only the speed but also the efficiency of SV turnover since the transient pore assembly is likely to act as a fence, preventing the dispersal of SV and DCV membrane components. However, most of the experimental evidence points to a large prevalence of full-fusion over kiss-and-run secretion in quantitative terms.

In this review, we will discuss the electrophysiological and morphological analysis done on these two forms of exocytosis in the recent past with a special emphasis on kinetic aspects of the field.

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TECHNIQUES TO ANALYZE VESICLE TRAFFIC AT THE PLASMAMEMBRANE

Electrical measurements

The most direct way to measure dynamic changes in cell surface area is membrane capacitance. When a vesicle fuses with a target membrane, it adds its capacitance to that of the acceptor membrane and, therefore, it produces a step increase in membrane capacitance (20, 29). By this technique, exocytosis has been extensively studied in endocrine cells where addition of a single DCV contributes a fairly large increase in membrane capacitance (in the order of few femto Farads [33]). On the contrary, fusion of a single SV increases plasmalemma capacitance of only few tens of ato Farads, a measurement well below the limit of the standard technique. Till few years ago, only special synapses were amenable to membrane capacitance measurements – mainly bipolar ribbons (e.g. [54]) and inner ear hair cells (e.g. [32]), where exocytosis of a massive number of SVs occurs in synchronized rounds and produces large capacitance changes. Very recently, instrumental refinements of the technique and, especially, the extensive use of averaging techniques have made possible the capacitance measurements of single SV and SLMV fusion/retrieval events (28, 51).

However, during prolonged stimulation of secretory cells, addition (exocytosis) and retrieval (endocytosis) of membranes overlap to a large extent, thus confusing capacitance measurements. Amperometry helps to solve this problem by acting as an independent index of release. This technique monitors the oxidation of neurotransmitter molecules at the surface of a carbon fiber. Spike shape in amperometric traces is thought to reflect the dynamics of pore formation, and parameters like rise and decay times and quantal size are analyzed. The development of small-sized carbon fibers has allowed the detection of neurotransmitter release from single cells (57), and, more recently, even from specific region of a cell, by inserting the carbon fiber into a patching pipette (patch amperometry) (1). However, only a limited number of secreted products are readily oxidizable; therefore, amperometric measurements have been limited to secretion of catecholamines (e.g., epinephrine, norepinephrine or dopamine) and indolamines (e.g. serotonin). Notably, patch amperometry is so sensitive that the tiny amount of neurotransmitter released by a single SV is still detectable (10, 11).

Morphological techniques

Optical techniques to visualize vesicle fusion and retrieval are traditionally based on a class of styryl dyes – the FM dyes – that have the interesting property of quickly and reversibly partitioning between membranes and fluids upon concentration changes (reviewed in [15, 41, 46]). FM dyes insert into but do not pass through the outer leaflet of the plasma membrane because of adjacent lipophilic and hydrophilic moieties. When inserted into lipids, the quantum yield of FM-dyes is dramatically boosted (8). Interestingly, FM dyes can be also visualized at the electron microscopy level by diaminobenzidine photoconversion (26). Classical experiments in the field are the FM1-43 uptake and release studies, which probe endocytosis and exocytosis,

respectively. Dissection of specific steps of the exo/endocytic traffic can be obtained by careful control of cell exposure to FM dyes.

An emerging technique to observe plasma membrane dynamics is 'Total Internal Reflection Fluorescence Microscopy' (TIRFM or 'evanescent wave microscopy'). It is based on the optical phenomenon that occurs when light propagating in a dense medium (such as glass) meets an interface with a less dense medium, such as water. If the light reaches the interface at a small angle, some of the light passes through the interface (is refracted) and some is reflected back into the dense medium. At a certain angle, all of the light is reflected. This angle is known as the critical angle, and its value depends on the refractive indices of the media. TIRFM collects the light emitted by fluorophores after they are excited by photons that escape total reflection at the glass/plasma membrane interface. Since the depth of the useful evanescent wave is about the diameter of a DCV (200-300 nm), this technique is ideally suited for studying the recruitment of fluorescently labeled molecules at the plasma membrane. With this technique, exo/endocytosis in endocrine cells has been visualized (reviewed in [50]).

Recently, variants of the green fluorescent protein (GFP) that are sensitive to rapid pH variations (and therefore named pHluorins) have been developed as optical probes to study SV trafficking (30). These new GFPs were targeted to the SV lumen by fusion with the intraluminal domain of synaptobrevin (and therefore named synapto-pHluorin). A strong fluorescent signal is generated when SVs fuse with the plasma membrane and therefore exposes synapto-pHluorin to the neutral pH of extracellular fluids; on the other hand, refilling of the neurotransmitter, which occurs in the recaptured SV, acidifies its interior and quenches synapto-pHluorin (19, 30, 43, 44). This technique holds the intrinsic advantage over FM dye studies to directly assay recycling instead of inferring it from a series of complex chasing experiments.

LDCV EXOCYTOSIS

Chromaffin cells have been the preferred model to monitor exocytosis by electrical measurements (16, 36). Amperometric analysis has shown the existence of at least three types of exocytic events in these cells (14, 18, 55, 56). (i) Simple spikes with large currents in amperometric traces have been interpreted as a fusion pore that fully opens and closes ('full-fusion' exocytosis). (ii) A stand-alone foot not followed by a spike has been considered a pore that closes without dilating ('kiss-and-run' exocytosis). (iii) Sometimes, the spike is preceded by a prespike foot, which is thought to represent an exocytic event that starts as kiss-and-run exocytosis (prespike foot) but then evolves to full fusion (spike). Comparison of membrane capacitance with amperometric recordings has immediately shown that transmitter release from LDCVs follows a somewhat different and delayed kinetics with respect to pore formation and opening, with an initial slow release phase ("foot") that lasts for almost the entire rise of capacitance and that occasionally may return to baseline (10); then amperometric current increases and the bulk of release occurs (2, 14, 16). These data

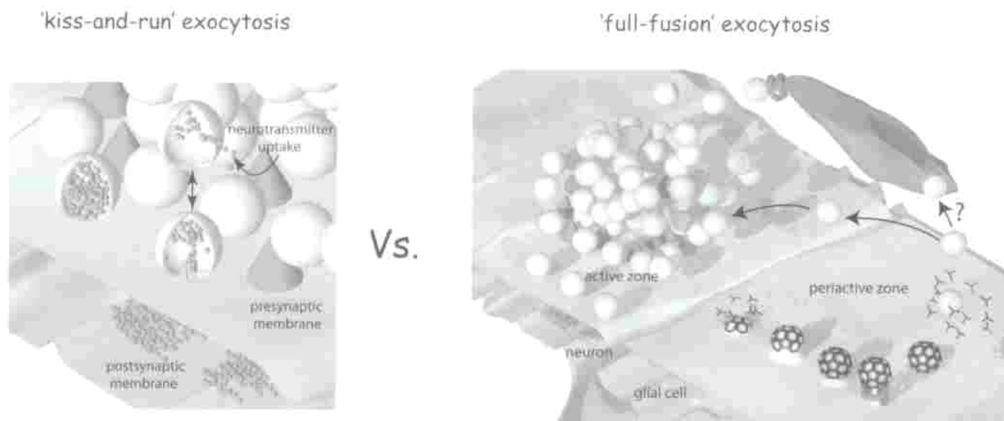

Fig. 1. - *Models of exocytosis: 'kiss-and-run'/'cavicapture' secretion and 'full-fusion' exocytosis in nerve terminals are illustrated*
The diagrams include the recycling pathways currently assigned to these models, i.e. direct vesicle retrieval for 'kiss-and-run' exocytosis and clathrin-mediated endocytosis for 'full-fusion' exocytosis. For schematic purposes only, the fusion pore is represented as a channel of cylindrical subunits spanning the plasmalemma; however, the actual fusion pore structure is unknown. Cones indicate the presynaptic density.

indicate that LDCV secretion happens only when the fusion pore opens wide enough to allow for rapid diffusion of the transmitter, thus implicating a model of complete vesicle collapse into the plasmalemma during regular LDCV exocytosis.

Increasing extracellular Ca2+ concentration induces a progressive switch to a form of exocytosis characterized by much faster fusion pore dynamics (2). At 5 mM extracellular Ca2+, prolonged opening (around 500 ms) of the fusion pore prevails, a phenomenon that is interpreted as full vesicle collapse into the plasmalemma. Increasing Ca2+ up to 90 mM results in the progressive increase of 'flickering' phenomena, i.e. of sudden spikes of capacitance (previously described during mastcells degranulation [17]), that indicate shuttering of a fusion pore with a periodicity of ~ 50 ms. Surprisingly, patch amperometry shows a complete release of the SV content even during these fast spikes, as a result of a much faster increase in conductance of the fusion pore. Flickering is strong evidence for kiss-and-run exocytosis of LDCVs. These data are of great relevance for SV exocytosis too, since neurotransmitter release is triggered by massive inward currents of Ca2+ at the active zone, where docked SVs are ready for secretion.

Step increase in cytosolic Ca2+, by photoactivation of caged compounds, elicits synchronized exocytosis of a large number of vesicles and can be used to study the kinetics of concomitant exo/endocytic events. Under these experimental conditions, capacitance recordings show a typical three-phase kinetics (34, 53, 58). Initially, few hundred vesicles are exocytosed at a very fast peak rate (of thousands of vesicles/s). Then, a larger vesicular fraction is released in less than a second. Finally, a persistent, slow secretion (tens of vesicles/s) lasting several seconds often follows. These data have suggested the idea that three functionally different LDCV populations participate to chromaffin cell secretion (39). One population of vesicles – the readily-

releasable pool (RRP see below) – is docked to the plasma membrane and ready to fuse upon Ca2+ increase (first kinetic component of exocytosis); a second population – the slowly releasable pool – is probably docked but has to be primed to become competent to respond to Ca2+ (second kinetic component); finally the third pool – the reserve pool – is recruited only lately (and possibly under intense stimulation) and has to undergo the entire maturation cycle in order to fuse (third kinetic component).

SMALL VESICLE (SV AND SDCV) EXOCYTOSIS

Morphological studies show the existence of different types of SV exocytosis, some of which are compatible with the transient pore hypothesis. (i) Hypertonic sucrose stimulation releases glutamate from hippocampal synapses without enabling FM1-43 to stain vesicles (49). (ii) SVs have different release rates for distinct FM-dyes, with 20% of vesicles showing retention of FM1-43 but not of FM2-10, an FM-dye with a small aliphatic moiety that allows for very fast partitioning rates (37, 40, 49). (iii) In hippocampal neurons, visualization of single exocytic events from vesicles labeled with synapto-pHluorin reveals secretory bursts of less than one second (19). Surprisingly, the duration of these transients can be increased by neutralizing the culturing medium with Tris but not with the HEPES, suggesting the existence of a selectively permeable fusion pore. (iv) Under certain conditions, depolarization of hippocampal neurons triggers only partial release of FM1-43 from SVs as if the fusion pore did not allow for FM1-43 destain (4). These data also show that during exocytosis the dye leave the vesicle only in the aqueous phase but not by lateral diffusion in the plasma membrane, indicating that the fusion pore acts as a fence to prevent exchange of lipid between the cell and vesicle membranes.

Although electrical measurements of exocytosis have been successfully performed in chromaffin cells since more than a decade, secretion from single small vesicles has been successfully resolved only recently and in special experimental setups (28, 51). Capacitance measurements show that 'flickering' (possibly, kiss-and-run) events happen with a rather low frequency (5% of all fusion events) for SDCVs of the posterior hypophysis, while prolonged conductance (possibly, full fusion) appears to be the rule. Furthermore, in contrast to DCVs, the pore conductance of flickering SDCVs is so small (10 fold lower than the 'flickering' fusion pore conductance of DCVs) to represent a substantial barrier to neurotransmitter release (28).

High resolution measurements of dopamine release from SDCVs of cultured midbrain neurons show at least two types of amperometric events, called simple and complex events (48). In contrast to simple events, which represent 80-85% of total events and consist of single peaks, there is a 20% of events that comprise 2-5 flickers sequentially decreasing in amplitude and therefore named 'complex'. Interestingly, the number of molecules released in complex amperometric events was much greater than in simple events and the first flicker within complex events was similar to

simple events in amplitude and number of dopamine molecules released. These data suggest that simple events may generally represent neurotransmitter release through short-lived pores that are not open long enough to release the entire SDCV neurotransmitter content, implying kiss-and-run exocytosis. Complex events appear to be exocytotic events in which the fusion pore either flickers or fluctuates several times in rapid succession, resulting in the release of a larger fraction of neurotransmitter. Surprisingly in these terminals, 'full-fusion' or complete exocytosis is not observed suggesting that some synapses may primarily use kiss-and-run exocytosis.

Exocytosis of multiple SVs has been measured with a variety of techniques, and, as expected, is faster (τ 0.1-1 ms [9, 42, 45]) than multiple DCV secretion. Classical electrophysiological studies of EPSC at the frog neuromuscular junction (NMJ) (7) and at mammalian central synapses (5, 21) as well as synapto-pHluorin trafficking experiments in cultured hippocampal neurons (19) have shown that the rate of neurotransmitter release decays biphasically after an initial rapid rise. The fast phase of decay (in which the majority of release occurs) is synchronized with Ca^{2+} influx and prevails at low frequencies of stimulation. Instead, the slow phase follows Ca^{2+} entry with some delay (therefore called asynchronous release) and persists for tens of ms; this latter phase predominates at high frequency of stimulation (25). A three-phase kinetics closely resembling multiple DCV secretion has been described for exocytosis at ribbon synapses after prolonged stimulation (35).

Taken these data together, speed is the most evident and major difference between SV (SLMV) and DCV rates of exocytosis. Even the fastest component of DCV secretion ($\tau \sim$ 7-27 ms) is about ten times slower than SV exocytosis at nerve terminals ($\tau \sim$ 0.1-1 ms): this is not surprisimg since neurons have to cope with high frequencies of firing/secretion. Direct evidence of fast exocytosis compatible with the kiss-and-run model has been obtained for both DCVs and SVs but its impact on synaptic transmission is still a matter of discussion.

FUSION PORE STRUCTURE

Although there are little doubts about the existence of multiple forms of exocytosis, it still remains to be solved the intimate structure of the fusion pore. This information is key to understand the molecular basis of the kinetic differences observed in secretion. As a first step toward this direction, effort has been recently put in identifying proteins that, when overexpressed or depleted, can affect amperometric recordings of single exocytic events. Due to technical constraints, these studies have been only performed on non-neuronal cells to measure LDCV fusion.

According to the effect on amperometric traces, proteins regulating secretion can be classified in two major groups: (i) factors that change spike shape and (ii) factors that affect prespikes and/or stand-alone foot (see also [3]).

Factors that are considered to be *bona fide* regulators of 'full-fusion' exocytosis belong to the first group, since spikes of large amplitude are considered to be exocytic events in which vesicles flattens on the plasma membrane (see above). According

to the effect on spike amplitude, factors can be further divided in two subgroups: (i) proteins that reduce the width of the spike, and, therefore, seem to close the pore, like munc18 (a protein essential for neurotransmitter release and LDCV docking) (6), complexin II (which functions at a late step in Ca2+ -triggered neurotransmitter release) [38], CAPS (a Ca2+ - and phospholipid-binding protein required for LDCV but not SV exocytosis) (52), dynamin (whose GTPase activity correlates with the pinching off of the vesicle from the plasma membrane) (23) and (ii) proteins that increase the spike width, i.e. by increasing quantal size when overexpressed, like cysteine string proteins (which are implicated in Ca2+ -dependent exocytosis) (22) and a mutant syntaxin (an essential t-SNARE of the plasma membrane) lacking munc18 interaction (24).

Factors that are thought to regulate 'kiss-and-run' exocytosis belong to the second group, since prespikes and 'stand-alone feet' are considered to be 'incomplete' exocytotic events. These factors include synaptotagmin I (the Ca2+ sensor for the fast component of SV exocytosis whose overexpression increases prespike amplitude) (55), synaptotagmin IV (that decreases prespike duration but, surprisingly, increases the 'stand-alone foot' duration) (55, 56) and SNAP-25 (another essential t-SNARE of the plasma membrane which increases prespike duration) (47).

Taken together, these data indicate that SNAREs (and especially t-SNAREs) and their major interactors acts as key components of the fusion pore. However, lipids are supposed to contribute as well, if not more, to pore assembly and function (31, 59), but precise data are still missing on this topic.

CONCLUDING REMARKS

It is well established that secretory vesicles from neurons and endocrine cells are capable of undergoing full fusion with the plasma membrane. This form of exocytosis most probably plays a major role in SV recycling in neurons. However, definite evidence of other forms of endocytosis with incomplete fusion ('kiss-and-run' exocytosis) has been obtained as well.

Notably, estimates of transient pore kinetics, especially during the fastest forms of exocytosis, vary widely in the literature. Although some of these discrepancies might arise from differences between synapses, it is likely that most of the controversies come from the experimental approaches used to analyze pore dynamics. A careful comparison of different techniques within a single experimental set-up is clearly needed.

Although much effort has been put in the analysis of 'kiss-an-run' kinetics, still the nature of the fusion pore is largely unknown. However, the convergence of electrical techniques with classical cell biology, molecular biology and genetic approaches has now given an incredible boost to the field, promising to solve pore structure and dynamics in molecular terms in the near future.

SUMMARY

In this short review, kinetic aspects of exocytosis are discussed. A special emphasis is put on recent data that highlight dynamic differences between neurotransmission and other forms of secretion.

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