
Monitoring synaptic vesicle recycling in frog motor nerve terminals with FM dyes

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Abstract

Ultrastructural observations made in the study of the frog neuromuscular junction (NMJ) almost three decades ago showed that synaptic vesicle cycling functions through a slow pathway, requiring the use of clathrin-coated vesicles and an endosomal compartment. Simultaneously, a conceptually simpler model emerged, postulating rapid retrieval of vesicle membrane through a mechanism similar to a reversal of vesicle fusion. With the advent of fluorescence imaging which allows the investigator to monitor recycling in living nerve-muscle preparations, new data appeared which reconcile at least in part the two models, indicating that both may be important at this synapse. Two different synaptic vesicle pools can be defined, a readily releasable pool (RRP), consisting of quanta that are immediately available for release, and a reserve pool (RP) that is exocytosed only after prolonged stimulation. Vesicles in the RRP recycle through a fast endocytic pathway, which does not rely on an endosomal compartment, while vesicles in the RP cycle more slowly through formation of infoldings and endosomes and their subsequent severance into vesicles. The two pools mix slowly, and their recycling may be regulated by different mechanisms.

Introduction

Neurotransmitter release requires the fusion of synaptic vesicles with the surface membrane at specialized areas of the nerve terminal (active zones), thereby releasing their contents (exocytosis). The vesicles then reform from the plasma membrane (endocytosis), are refilled with neurotransmitter, and travel back to the existing population of vesicles.

When a nerve terminal is stimulated, not all vesicles can be released immediately. The existence of two functionally different stores of neurotransmitter, a rapidly releasable one and a slowly releasable one has been hypothesized for more than 40 years. Early investigations of intact and perfused cat sympathetic ganglia (Birks & MacIntosh, 1961) indicated the existence of two distinct presynaptic pools of transmitter, a “readily releasable” fraction, which was depleted at high frequencies of stimulation, and a “non-readily releasable” fraction. This hypothesis was also in agreement with data obtained from experiments at the neuromuscular junction; for example, the run-down observed in the amplitude of end plate potentials during tetanic stimulation was interpreted as resulting from the depletion of the immediately re-

leasable neurotransmitter pool (Elmqvist & Quastel, 1965).

Different functional vesicle pools—An important concept in a number of synaptic systems

Current research suggests that the readily releasable pool (RRP) is the vesicle sub-population that is immediately available for release upon stimulation (usually being thought of as including vesicles docked at the active zone and primed for release). A much bigger population of vesicles constitutes the reserve pool (RP); these vesicles are thought of residing at some distance away from the active zone, and requiring some form of transport or change in docking state to become fusion-competent (for reviews see Cousin & Robinson, 1999; Rodesch & Broadie, 2000).

The functional definition of the RRP varies somewhat with the system used. At central nervous system synapses, the RRP is hypothesized to be constituted by the vesicles that can be released by a standard stimulus. At mammalian hippocampal synapses, the pool of readily releasable quanta has been defined as “those

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quanta that are released during the transient burst of exocytic activity following application of hypertonic solution" (Rosenmund & Stevens, 1996; also Stevens & Tsujimoto, 1995). At synaptic terminals of goldfish retinal bipolar neurons, the RRP is thought of as the population of vesicles that can be released in a short pulse (approximately 200 ms) of strong depolarization (von Gersdorff *et al.*, 1996; von Gersdorff & Matthews, 1997). The number of vesicles in the RRP, estimated from electrophysiological measurements, correlates with the number of docked vesicles in these systems (Schikorski & Stevens, 2001; von Gersdorff *et al.*, 1996). At the *Drosophila* neuromuscular junction, the RRP is defined as the "endo-exo cycling pool" (Kuromi & Kidokoro, 1998), consisting of all the vesicles that can recycle in an activity-dependent manner (nerve stimulation or depolarization with high concentrations of K^+). A reserve pool of vesicles which cycles only under intense stimulation (30 Hz tetani) was identified using the *shibire* mutant, in which endocytosis is inhibited at non-permissive temperature ($>29^\circ C$), allowing for depletion of all synaptic vesicles and subsequent reformation at permissive temperature (Kuromi & Kidokoro, 2000).

The presence of vesicle pools has also been postulated from electrophysiological experiments at the calyx of Held (reviewed by Schneggenburger *et al.*, 2002). Modulating the levels of synapsins, proteins thought to interact solely with RP vesicles (see below) also revealed different vesicle pools at lamprey reticulospinal nerve terminals (Pieribone *et al.*, 1995), at *Aplysia* cholinergic synapses (Humeau *et al.*, 2002) and at the squid giant synapse (Hilfiker *et al.*, 1998).

At the frog neuromuscular junction vesicle pools have not been described until recently (Richards *et al.*, 2000, discussed in detail below). However, the existence of another parameter differentiating vesicles has been proposed for almost 30 years: two-widely different ul-

trastructural mechanisms for vesicle cycling existing in the same nerve terminals.

RECYCLING AT THE FROG NMJ

The classic studies of synaptic vesicle cycling at the frog neuromuscular junction involved electrophysiology and electron microscopy (Heuser & Reese, 1973; Ceccarelli *et al.*, 1973). Both groups followed the effects of prolonged stimulation on the nerve terminals; exocytosis was assessed by recording the responses of the muscle cell to nerve terminal-released acetylcholine. To investigate vesicle recycling, preparations were stimulated in presence of extracellular markers (horseradish peroxidase or dextran), fixed and examined at the ultrastructural level. The membrane-bound objects that contained extracellular markers were interpreted as being recently endocytosed organelles. Despite using the same preparation and similar techniques, the two groups came to surprisingly different interpretations of the endocytic process (see also the review by Wilkinson & Cole, 2001).

Heuser and Reese developed a model in which synaptic vesicles coalesce with the plasma membrane at specialized release sites, and membrane is retrieved by coated vesicles at regions of the plasma membrane adjacent to the Schwann cell. The coated endosomes lose their coats and fuse, generating cisternae (endosomes), which then divide and form new synaptic vesicles (Fig. 1a). Further studies produced a series of modifications of this model; for example, Miller and Heuser (1984) suggested the possibility that endosomes arise directly from the plasma membrane (infoldings), preceding the appearance of coated vesicles. Coated objects were shown to form on such membrane infoldings (Gennaro *et al.*, 1978). These and other results (for example on synaptosomes and hippocampal nerve terminals, Takei *et al.*, 1996) suggested a variation of the

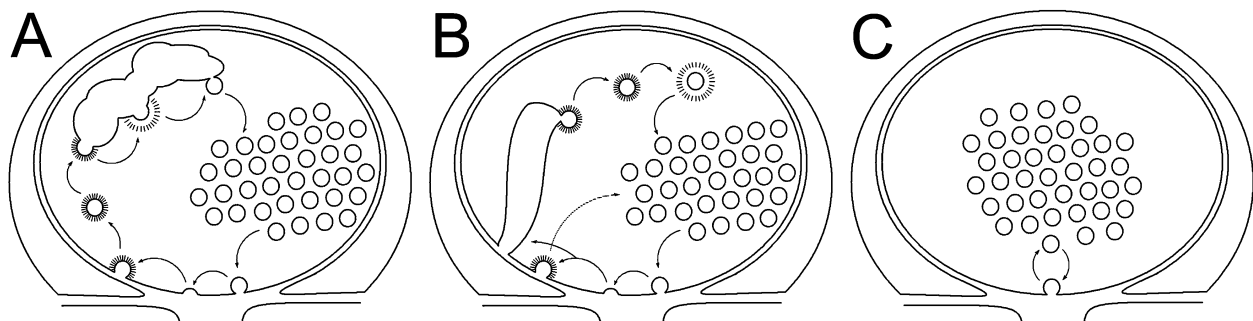


Fig. 1. (A) The "slow endocytic pathway" described by Heuser and Reese (1973). Vesicles fuse with the axoplasm during exocytosis, membrane is retrieved via coated vesicles, which merge and lose their coats, forming endosomes. New synaptic vesicles bud then from the endosomes. (B) A refined "slow endocytic pathway" model (Gennaro *et al.*, 1978). Vesicles fuse with the axoplasm, and membrane is retrieved by infoldings; coated vesicles bud from the infoldings and form new synaptic vesicles. A more rapid retrieval pathway is indicated by the dotted arrow (synaptic vesicles form directly from the plasma membrane through coated vesicle budding). (C) "Kiss-and-run" model. Vesicles transiently fuse with the plasma membrane and reform from it in a direct reversal of fusion.

initial model in which after vesicle fusion membrane is retrieved not only by coated vesicles, but also by infoldings, which can form internal endosomes by pinching off from the plasma membrane. Synaptic vesicles are then directly formed by coat-mediated budding from infoldings/endosomes or from the surface membrane (Fig. 1b). The protein forming the coats has been identified (clathrin), and many aspects of clathrin-coat formation, as well as a number of adapter proteins, have been described more recently (for reviews see Cremona & DeCamilli, 1997; Brodin *et al.*, 2000; Takei & Haucke, 2001).

Ceccarelli and colleagues did not agree with the slow endocytosis interpretation, as they did not observe stimulation-induced change in the numbers of coated vesicles/cisternae after prolonged stimulation (Ceccarelli *et al.*, 1973, see also review by Ceccarelli & Hurlbut, 1980). They suggested a simpler, fast endocytosis model, in which vesicles fuse with the plasma membrane and then reform directly from it, in a reversal of exocytosis (Fig. 1c). Since this model relies on a concept of incomplete, transient fusion between vesicles and the plasma membrane, as opposed to the models presented above, it is usually referred to as "kiss-and-run" endocytosis (Fesce *et al.*, 1994).

The large differences between the two recycling models were difficult to reconcile initially. However, one explanation was provided by the fact that the two groups used different stimulation paradigms; while both delivered high numbers of electrical stimuli to the preparations they subsequently investigated by electron microscopy, Heuser and Reese used high-frequency (10 Hz) tetanic stimulation, while the Ceccarelli group used low-frequency (2 Hz) stimulation. This observation suggested the hypothesis that nerve terminals employ both recycling methods depending, perhaps, on the amount of compensatory endocytosis necessary (the slow recycling route coming into play when exocytosis surpasses certain limits).

Fast vesicle cycling had few supporters initially, (reflecting the relative lack of quantitative evidence in favor of it) but it was recently revived by data obtained mainly from capacitance electrophysiology (for a brief review, see Valtorta *et al.*, 2001). Capacitance recording, which gives an indication of the membrane area of patch-clamped cells, showed that transient exocytosis/fast endocytosis may be common events in certain cell types. However, little further evidence in favor of the rapid endocytosis model appeared from the system in which it had been originally proposed—the frog NMJ, until the advent of fluorescence imaging of living synapses.

FM dye imaging

The introduction of fluorescent dyes as endocytic markers made it possible to monitor recycling in living

preparations (Lichtman *et al.*, 1985; Betz *et al.*, 1992). Preparations are usually bathed in solutions containing the dye of interest, which stains extracellular membranes. Upon nerve stimulation, vesicles fuse with the plasma membrane, and during compensatory endocytosis membrane stained with the dye is taken up. The excess dye in the extracellular solution is washed away, and internalized membrane can be visualized, thus measuring endocytosis. By further stimulation in the absence of the extracellular dye one can monitor the release of the internalized dye, which provides a measure of exocytosis.

Among the dyes used, styryl dyes (Fig. 2) have proven to be valuable (Cochilla *et al.*, 1999). They have a number of advantages: they reversibly stain membranes; they do not cross membranes, ensuring that the dye molecules do not escape from the membrane-bound organelles formed during endocytosis. Also, they are more fluorescent in a hydrophobic environment (cellular membrane) than in aqueous solutions (Henkel *et al.*, 1996a). Thus, during exocytosis, the dye released from vesicles immediately becomes significantly less fluorescent, ensuring accurate

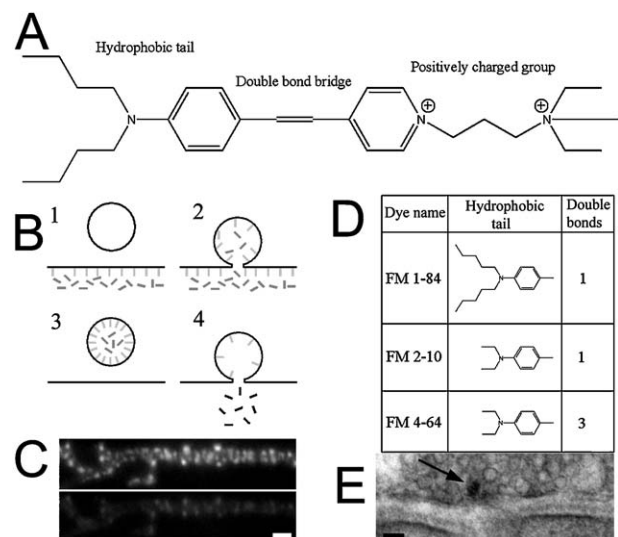


Fig. 2. (A) Structure of FM 1-43. (B) Typical FM dye experiment. Panel 1: the dye is added to a preparation; it does not have access to intracellular organelles. Panel 2: the preparation is stimulated, and vesicles fuse with the plasma membrane allowing entrance of the dye. Panel 3: The dye is washed from the chamber, and imaging can be performed. Panel 4: Preparations are stimulated in absence of the dye (destained); vesicles fuse with the plasma membrane and release the dye (C) Two images of a frog motor nerve terminal loaded with FM 1-43 before (top) and after destaining (bottom). Scale = 2 μ m. (D) Other FM dyes of interest. The size of the hydrophobic tail of the dye affects its membrane washout properties while the spectral characteristics of the dye are influenced by the number of double bonds. (E) An example of FM dye photoconversion. Arrow indicates the only labeled vesicle in this electron microscopic image. Scale = 100 nm.

measurements of the loss of dye from the cells (though, as dye molecules tend to bind other membranes in the preparation, dye wash-off is still necessary). Styryl dye molecules consist of lipophilic tails and positively charged head groups, linked via double bond bridges. The length of the lipophilic tail determines the affinity of the dye for membranes; dyes with longer tails stain membranes more brightly and have longer washout kinetics (Betz *et al.*, 1996; Ryan *et al.*, 1996; Richards *et al.*, 2000). The positively charged group ensures that the dye cannot permeate membranes; thus the dyes are sequestered in membrane bound compartments after endocytosis. The length of the double bond bridge determines the spectral characteristics of the dye; dyes that have one double bond (as FM 1-43, FM 2-10) are typically imaged using fluorescein optics; FM 4-64, which has a bridge formed by three double bonds is imaged using rhodamine optics.

Another advantage of the FM dyes is that they can be photoconverted and used as markers for ultrastructural studies. FM dye-stained preparations are fixed and illuminated in presence of diaminobenzidine (DAB). The internalized dye molecules emit reactive oxygen species that convert DAB to an electron-dense precipitate that is easily observed in electron micrographs (Henkel *et al.*, 1996a).

Early FM observations supported the hypothesis of a slow recycling route filling one major pool of vesicles

FM dyes, and FM 1-43 in particular have been used extensively to study vesicle cycling at frog motor nerve terminals. Initial studies suggested that newly endocytosed vesicles mix fully with the resting vesicle pool both at a functional and at a morphological level (Betz & Bewick, 1992). However, these experiments did not address the existence of readily releasable/reserve pools, since a much larger fraction of the vesicles had been released than the expected value for the RRP (from experiments in other synaptic systems, the RRP constitutes from approximately 1% of the total vesicle number in goldfish bipolar neurons to ~5–10% at hippocampal synapses, Schikorski & Stevens, 2001). The minimum time interval required for vesicles to recycle was approximately one minute (Betz & Bewick, 1992), more suggestive of a coat-dependent recycling model than a “kiss-and-run” model. Further studies suggested that most vesicles reside in a reserve pool, from which they can be mobilized to become docked and fuse with the plasma membrane (Wu & Betz, 1998). This model relies on a single vesicle-recycling route, which recovers fused vesicles and delivers them to the reserve pool, the main vesicle pool of frog motor nerve terminals. These results were in good agreement with the coat-dependent, slow vesicle cycling pathway proposed originally by Heuser and Reese. This was not an

unexpected result, since most of the endocytosis data was obtained from experimental conditions similar to those employed by Heuser and colleagues—dye uptake and release were investigated mainly using tetanic stimulation.

Different FM dyes used to identify different recycling routes and pools of vesicles

Use of the more hydrophilic dye FM 2-10 suggested a new line of experiments (Richards *et al.*, 2000). In this study, the uptake of different FM dyes was investigated. Frog NMJ preparations were tetanically stimulated in presence of either FM 1-43 or FM 2-10, incubated in presence of the dye for various lengths of time (0–15 minutes), washed in dye-free extracellular saline to remove excess FM dye, and imaged. Most compensatory membrane uptake was completed during stimulation, since the intensity of nerve terminals that were loaded with FM 1-43 changed little with incubation time (they took up similar amounts of dye when it was present in the extracellular solution only during stimulation or when it was present also for 15 minutes after stimulation). However, when FM 2-10 was used, washing immediately after stimulation resulted in dramatically decreased uptake; terminals took up approximately four times more FM 2-10 when incubated in presence of the dye after stimulation. Two interpretations of this result were possible: that nerve terminals continue to take up FM 2-10 after stimulation (unlikely, since they do not take up significant amounts of FM 1-43 after stimulation), and that nerve terminals lose FM 2-10, but not FM 1-43 during pre-imaging washing. This second hypothesis was also supported by experiments directed at investigating washing of the dye from terminals after stimulation. FM 2-10, but not FM 1-43 fluorescence decreases significantly by washing; also, incubating nerve terminals in the presence of FM 2-10 after stimulation reduced the amount of FM 2-10 lost by washing, in an incubation time-dependent manner (Richards *et al.*, 2000).

These results suggest that both dyes are internalized in similar ways, but that approximately 75% of the internalized FM 2-10 can escape from the compartment it is trapped in during endocytosis. A morphological correlate of such an endocytic compartment was suggested by early electron microscopic work (for example, Miller & Heuser, 1984), which described the formation of membrane infoldings during tetanic stimulation, probably as a compensatory mechanism for the massive exocytosis experienced by the terminals. The hydrophilic FM 2-10 dye may be able to escape infoldings open to the extracellular solution, while the more hydrophobic FM 1-43 may be permanently trapped. Photoconversion of FM dyes supports the hypothesis as FM 1-43 labeled significantly more endosomal objects than FM 2-10 (Richards *et al.*, 2000). This interpretation

is also in agreement with the reduction in the loss of FM 2-10 with increasing incubation time, as vesicle reformation eliminates the endosomal objects/infoldings, replacing them with vesicles from which dyes would be unable to escape (Heuser & Reese, 1973).

These experiments supported a “slow endocytosis” model, in which endosomal objects arise from the plasma membrane and are subsequently transformed into vesicles (Takei *et al.*, 1996). However, that model may not apply to all vesicles; while most of the FM 2-10 fluorescence was lost by washing the preparations immediately after stimulation, a significant amount of dye was retained by the terminals. The ability of terminals to release this dye was higher than expected on the basis of random mixing (Richards *et al.*, 2000). Nerve terminals were loaded with FM 1-43 or FM 2-10 by having the dye present only during tetanic stimulation, and then the ability of the terminals to release the dye was monitored by stimulating preparations in absence of the dyes (destaining). Only a small fraction of FM 1-43 fluorescence was released immediately after loading (as expected, since most of the dye probably resides in non-releasable endosomal objects at this time point), but the FM 2-10 fluorescence was almost completely released, indicating that the small fraction of FM 2-10 dye that the terminals retained after wash was contained within fusion-competent synaptic vesicles and not infoldings or endosomes. This result suggested that a fraction of the vesicles could cycle faster than the “slow endocytosis” model predicts. It is difficult to interpret these results as to whether this fraction of the vesicles cycles via a “kiss-and-run” pathway or via a process of rapid clathrin coat budding from the plasma membrane, but it is likely that no endosomal intermediates are necessary for the cycling of this vesicle fraction.

These results suggest the existence of two vesicle pools—one that can cycle rapidly, independent of endosomal intermediates, and a larger one that cycles more slowly, requiring the formation of infoldings and endosomes. The rapidly cycling pool was also shown to be more rapidly releasable—tetani that induced the exocytosis of approximately 50% of all vesicles released this pool almost completely (Richards *et al.*, 2000), indicating a strong similarity between these vesicles and RRP vesicles from other systems. However, these experiments did not address the timing of the release of the rapidly cycling pool. As RRP vesicles are expected to be the first vesicles to undergo exocytosis upon stimulation, it was important to test whether the rapidly cycling pool vesicles also are rapidly released at the start of stimulation. To test this, rapidly cycling vesicles were left unlabeled in nerve terminals where the slowly recycling pool was labeled with FM 2-10 (by having the dye present during recovery from tetanic stimulation, but not during actual stimulation). The destaining kinetics of these terminals were then measured; their destaining curve closely paralleled that of

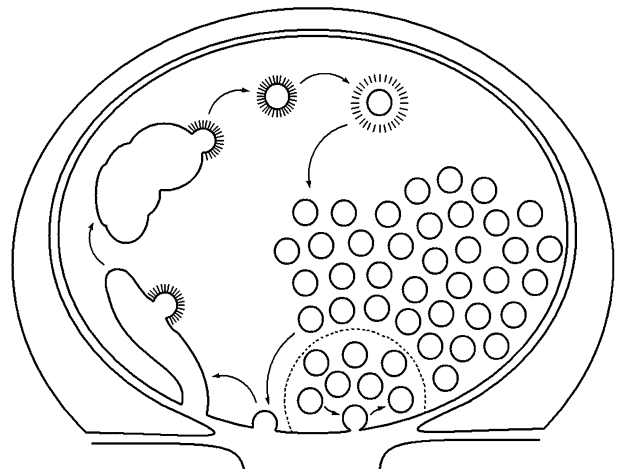


Fig. 3. Current model of vesicle cycling at the frog NMJ. Vesicles in the RRP (delimited by the dotted line) cycle rapidly, by direct retrieval from the plasma membrane (possibly via “kiss-and-run” or by clathrin-coat dependent processes). Cycling of vesicles from the reserve pool requires formation of infoldings and endosomes that are broken up by budding of clathrin-coated vesicles, which later shed their coats and reform RRP vesicles.

fully loaded terminals, but it presented a short (10–15 second) lag after the start of stimulation; these terminals lost only approximately 5% of their fluorescence during this interval, while the control (fully loaded) terminals lost approximately 25–30%, indicating that unlabeled, rapidly cycling vesicles were released primarily during the first seconds of tetanic stimulation. These results suggest that the two vesicle pools are released in a sequential manner, the rapidly cycling pool being immediately released upon stimulation, followed by the more slowly recycling pool. Also, these results suggest that the rapidly recycling pool is identical with a “readily releasable pool” and that the slowly releasable pool may be identified with the “reserve pool”.

In summary, these experiments suggest that both rapid recycling and slow recycling pathways play roles at the frog NMJ (Fig. 3).

Does the retrieval of vesicles from the RRP involve “kiss-and-run” or a different process of rapid cycling?

“Kiss-and-run” predicts fusion and recovery of synaptic vesicles without collapse into the plasma membrane. As FM dyes stain membranes with time constants of a few milliseconds (Neves & Lagnado, 1999) but are washed off much slower (time constants of seconds or tens of seconds, Klingauf *et al.*, 1998; Richards *et al.*, 2000), a simple prediction would be for vesicles to take up dye but not be able to fully release it during “kiss-and-run” events (and this would be all the more evident for more hydrophobic dyes, which have longer

washing times). Limited release of hydrophobic dyes when compared to more hydrophilic ones from synaptic vesicles has been regarded as an indication of possible "kiss-and-run" (Klingauf *et al.*, 1998). However, Stevens and Williams (2000) argued that vesicles undergoing "kiss-and-run" exocytosis do not take up or release any FM dyes, because of either very brief fusion (under 6 milliseconds) or a special configuration of the fusion pore.

According to the data of Stevens and Williams, none of the frog RRP vesicles used "kiss-and-run", since they loaded with both FM 1-43 and FM 2-10. However, if putative "kiss-and-run" events at the frog NMJ have longer fusion times (or larger pores) than their CNS counterparts, FM dye loading (and possibly unloading) would be observed. This is apparent in some systems, as PC-12 cells, where large molecules like neuropeptide Y bound to GFP escape from secretory granules even in the absence of full fusion (Taraska *et al.*, 2003).

Do RRP vesicles release more of the less hydrophobic FM 2-10, as would be expected from CNS experiments? If both the RP and the RRP are loaded with FM 2-10 or FM 1-43 and destaining is monitored, identical curves are obtained (Richards *et al.*, 2000). This result is in good agreement with original electron microscopic observations (Ceccarelli *et al.*, 1973), where in conditions that did not seem to trigger the slow endocytic pathway, vesicular uptake of large markers such as horseradish peroxidase and dextran was still observed. These data seemingly suggest full fusion of RRP vesicles, but they may also be reporting longer lasting "kiss-and-run".

Another possibility for RRP recovery is clathrin-mediated retrieval of vesicles directly from the terminal membrane, as the recovery of the RRP has a time constant of around 20 seconds (Richards & Betz, unpublished observations). However, recovery times lasting for seconds would be expected even for "kiss-and-run" events, if vesicle repriming were necessary.

In conclusion, it is still difficult to assess what the mechanism of RRP recycling is, other than it does not require processing of endosomal intermediates (as it was envisioned by Ceccarelli & colleagues in 1973).

Do synaptic vesicles from different pools intermix?

Movement of vesicles within resting or active nerve terminals seems to be limited, both at the neuromuscular junction (Henkel *et al.*, 1996b) and at central nervous system synapses (Kraszewski *et al.*, 1996). After strong nerve stimulation, newly endocytosed vesicles are able to mix freely with the unreleased vesicles in the nerve terminals (Betz & Bewick, 1992; Kraszewski *et al.*, 1996). However, such experiments give little indication of the behavior of the pools of vesicles, since the stimulation paradigms used affected both the RRP and the RP.

At central nervous system synapses the probability of release for recently recycled vesicles is similar to that of vesicles that resided in the terminals for longer periods of time (Ryan & Smith, 1995). Also, to completely release dye taken up at low frequencies of stimulation (1 Hz for 5 seconds) long trains of stimuli at high frequency are required (20 Hz for 50 seconds), indicating that the RRP vesicles mix with the RP during cycling (Murthy & Stevens, 1998). Pyle *et al.* (2000) demonstrated that vesicles in the RRP can be rapidly retrieved and reused after exocytosis (within 5 seconds for electrically stimulated release), but that the process of RP vesicles mixing with RRP vesicles is also rapid (20 seconds are sufficient for RP vesicles to mix almost completely with RRP vesicles). These results suggest that the two vesicle populations have high mixing rates at CNS synapses.

At the neuromuscular junction mixing appears to be much slower. The exo-endo cycling pool of vesicles from the *Drosophila* NMJ does not back-mix significantly with the reserve pool (at a morphological level) under conditions of low stimulation (Kuromi & Kidokoro, 1998, 2000). At the garter snake NMJ, a pool of vesicles released by brief nerve stimulation at reduced temperature (investigated by high resolution confocal imaging experiments) was shown to cycle locally, in the vicinity of post-synaptic active zones, and to mix slowly (hours) with the rest of the vesicles (Teng *et al.*, 1999). At the frog NMJ, RRP vesicles seem to be preferentially released at the start of tetanic stimulation, as discussed above, suggesting low mixing between the two pools. Also, monitoring the release of RRP vesicles during tetanic stimulation immediately after loading and at various lengths of time after loading indicated that they mix only slowly with the RP vesicles to become less readily releasable (a mixing rate of approximately 10%/hour, Richards *et al.*, 2000).

These results suggest the presence of two independent pools of vesicles at NMJ terminals that mix significantly less than their CNS counterparts. One hypothesis that serves to explain this difference is that the RP and RRP vesicles at neuromuscular junctions arise from independent recycling pathways, and may thus be morphologically and functionally different (as discussed above for the frog NMJ). Also, two endocytic pathways have been described at the *Drosophila* NMJ from morphological experiments (Koenig & Ikeda, 1996, 1999). In a remarkably similar pattern to the frog NMJ, one of the pathways has a fast time course, requires no endosomal intermediates and recycles a small vesicle fraction in the vicinity of the active zone (where RRP vesicles would be located). A slower pathway, requiring endosomal intermediates, recycles a larger fraction of the vesicles. The slower vesicle recycling pathway arises from sites away from the active zone, (which has also been proposed for the slow endocytic pathway at the frog NMJ, Heuser & Reese, 1973).

Vesicle pools are also differentiated at the biochemical level

Most experiments on the regulation of different vesicle pools come from other systems than the frog NMJ, mostly because it is not advantageous to use for biochemical analysis.

A number of experiments focused on the ability of the reserve pool of vesicles to exocytose. The release of RP vesicles depends upon their ability to reach the active zone and fuse. Vesicle movement within nerve terminals has been suggested to be dependent upon actin-myosin interactions (Mochida *et al.*, 1994). Ryan (1999) suggested that in the case of cultured hippocampal synaptic boutons the cycling of RP vesicles is blocked by inhibition of myosin light chain kinase (MLCK), but that the turnover of the readily releasable pool is unaffected, indicating that the two pools may have different dependencies upon movement within the terminal. However, the results of this study need further confirmation in what the target of inhibition was, since the two MLCK inhibitors (ML-9 and ML-7), that it relied on have recently been shown to have poor selectivity (Bain *et al.*, 2003).

The synapsins are a protein family believed to regulate neurotransmitter release via interactions with synaptic vesicles and cytoskeletal elements (Greengard *et al.*, 1993; Hilfiker *et al.*, 1999). They have been investigated in relation to the vesicle pools in different systems. At lamprey reticulospinal axons, antibodies that recognized a synapsin-I-like protein caused a dramatic alteration of vesicle clusters. A majority of vesicles were lost from the clusters, but the part of the clusters proximal to the active zone (presumably composed mainly of RRP vesicles) remained unchanged (Pieribone *et al.*, 1995). A similar phenotype was observed in synapsin-I deficient mice (densely packed vesicles were only seen in a narrow rim at active zones, with most vesicles dispersed throughout the terminals, Li *et al.*, 1995). The hypothesis that synapsin molecules interact predominantly with vesicles from the reserve pool is also supported by work in two other systems, the squid giant synapse (Hilfiker *et al.*, 1998) and cholinergic synapses of *Aplysia* (Humeau *et al.*, 2001). At the frog neuromuscular junction, a connection between the reserve pool of vesicles and synapsin molecules is yet to be firmly established, though a number of experiments suggest that synapsins have important roles in amphibian synapse function. Synapsin I has been shown to markedly increase in concentration during synaptic maturation (Lu *et al.*, 1996) and to induce functional maturation of the synapses when injected into *Xenopus* embryos (Lu *et al.*, 1992). It dissociates from vesicles upon electrical stimulation (and re-associates during recovery, Torri Tarelli *et al.*, 1992) and it also seems to induce formation of vesicle clusters (Valtorta *et al.*, 1995).

Different regulation of vesicle pools—A role for PI 3-kinase at the frog NMJ?

Despite the numerous recent advances in understanding the regulation of vesicle fusion, other aspects of vesicle cycling are less well characterized. The dynamics of vesicle pools have been investigated for a number of years in a variety of systems; however, only a limited number of reports describing modulation of the pools appeared. At hippocampal glutamatergic synapses, activation of Protein Kinase C (PKC) has been shown to increase the size of the RRP, and to enhance the rate of recovery of the RRP after depletion (Stevens & Sullivan, 1998). At the *Drosophila* neuromuscular junction, the cAMP/PKA cascade has been proposed to regulate the translocation of RP vesicles to the releasable pool (Kuromi & Kidokoro, 2000); also, the replenishment of the two pools may depend on different sources of Ca^{2+} (Kuromi & Kidokoro, 2002).

At the frog NMJ, the two pools of vesicles rely on different recycling routes, which suggests that conditions modulating only one of the pathways may have limited effects on the vesicles endocytosed by a different mechanism. Agents affecting clathrin-coat endocytosis or “kiss-and-run” endocytosis may thus indirectly modulate the two pools. Recent work suggests that phosphoinositides (PI) play important roles in synaptic vesicle cycling, especially in clathrin-coat endocytosis (reviewed by Cremona & De Camilli, 2001). 3-phosphorylated phosphoinositides have been shown to interact with components of the clathrin-mediated recycling pathway (the AP-2, AP180 adaptor proteins, Gaidarov *et al.*, 1996; Norris *et al.*, 1995; Hao *et al.*, 1997). Also, a PI-3 kinase isoform has been shown to be associated with clathrin coats (Domin *et al.*, 2000) and to have its enzymatic activity enhanced by clathrin (Gaidarov *et al.*, 2001). These results suggest the possibility of a 3-phosphorylated PI modulation of the reserve pool cycling, but not necessarily of the RRP cycling.

We have recently examined the effects of a PI-3 kinase inhibitor (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, LY294002, Vlahos *et al.*, 1994) at the frog NMJ (Rizzoli & Betz, 2002). The compound reversibly inhibited vesicle reformation and nerve terminals lost most of their vesicles during treatment. Vesicles were replaced by endosomal objects, which slowly disappeared during drug wash-off, (possibly via clathrin-coat dependent vesicle reformation since a large increase in the number clathrin-coated objects was observed during wash-off). These results suggest a model similar to that proposed by Takei *et al.* (1996) (see above)—endosomal objects form via bulk endocytosis from the plasma membrane and vesicles are then formed from these objects via clathrin-coated mechanisms. PI-3 kinase inhibition blocks recycling at an intermediate step between endocytosis and vesicle reformation, possibly via block of clathrin-coat formation

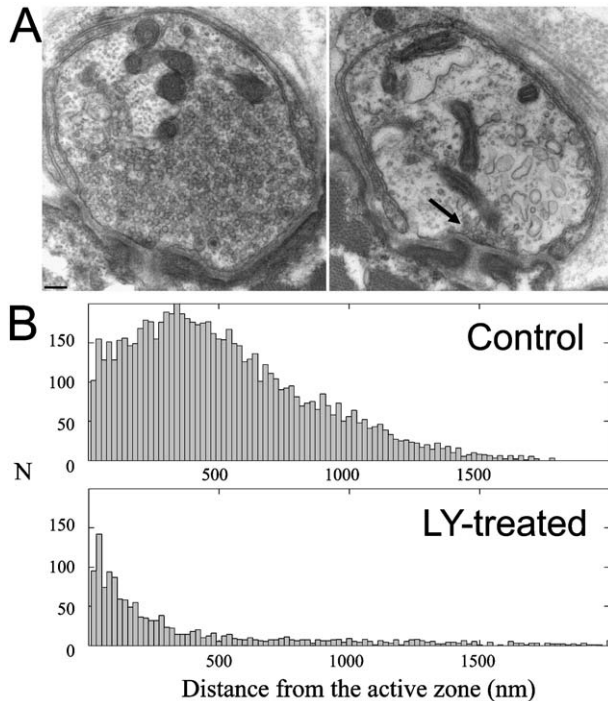


Fig. 4. (A) Electron micrographs of nerve terminals before (left) and after (right) LY treatment. Arrow indicates a cluster of vesicles near an active zone. Scale = 250 nm. (B) Histograms of vesicle positions in control and LY-treated nerve terminals; 32 control and 29 LY-treated terminals were analyzed.

or function. Initially it seemed that these results support only the slow-endocytosis mechanism, but, interestingly, a small proportion of the vesicles persisted after prolonged drug treatments. The size of this population correlated well with the size of the RRP (approximately 20% of the total number of vesicles, Richards *et al.*, 2000), implying the possibility that PI-3 Kinase inhibition affected only the RP. As RRP vesicles are expected to be closer to the active zone than RP vesicles (see above), an expected result was for the vesicles that persisted after LY294002 treatment to be positioned in the vicinity of release sites. We analyzed the electron micrographs of LY294002-treated nerve terminals obtained in our study of LY294002 (Rizzoli & Betz, 2002), (Fig. 4). The population of vesicles remaining after drug treatment has a significantly different distribution from the control solvent-only treated vesicle population (χ^2 test, p value < 0.00001). The vesicles are distributed closer to the active zone (Fig. 4b); also, only small differences are observed between the numbers of docked vesicles and of vesicles within 80 ± 20 nm from the active zone in control or drug-treated terminals.

These results show that a population of vesicles comparable in size with the RRP and located near release sites is not affected by PI 3-kinase inhibition; however, is this population also functionally similar to the RRP?

Another inhibitor of PI 3-kinase, wortmannin, blocks the cycling of more than 70% of the vesicles, as observed from FM dye loading in presence or absence of the drug (Richards & Betz, 2000), but most vesicles that do cycle can be re-released by another round of tetanic stimulation, similar to a situation in which the RRP is selectively released (D.A. Richards & W.J.B., manuscript in preparation), suggesting that the RRP may be unaffected by PI 3-kinase inhibition.

Conclusion and perspectives

At the frog NMJ, both rapid and slow recycling routes participate in endocytosis. The slow recycling pathway is triggered by high frequency stimulation, as observed from the classical ultrastructural studies; also, it appears that the release of the corresponding pool of vesicles (the RP) requires persistent high frequency stimulation as well (the first 300–400 shocks delivered at 30 Hz fail to release a significant number of RP vesicles, Richards *et al.*, 2000). These results indicate that RP vesicles participate in exocytosis after the exhaustion of the RRP by frequencies of stimulation that surpass the ability to recycle of the rapid endocytic pathway. At low frequencies, it is possible that release of only RRP vesicles is triggered repeatedly, as no endosomal/coated compartments form despite the large amounts of neurotransmitter released during long periods of time (Ceccarelli *et al.*, 1973). This hypothesis may be confirmed by further studies involving FM dye imaging—one can envisage experiments relating the pool of vesicles that cycle at low frequencies of stimulation to the RRP/rapid cycling pool. Also, an interesting problem is that of the signal triggering the mobilization of each of the two pools—do RP vesicles require high calcium concentrations (as those seen during tetanic stimulation, Wu & Betz, 1996) to move and fuse with the plasma membrane? Does the release of the RRP trigger the movement of RP vesicles towards the active zone and their subsequent docking and fusion? What are the molecules required for slow endocytosis but not rapid endocytosis? These questions may be answered in the future by approaches integrating electrophysiology, fluorescence imaging, pharmacology and ultrastructural studies at a preparation that has been scrutinized almost since the beginning of modern research, but has still holds many secrets.

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