The Structural Organization of the Readily Releasable Pool of Synaptic Vesicles
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The defining morphological feature of chemical synapses is the vesicle cluster in the presynaptic nerve terminal. It has generally been assumed that vesicles closest to release sites are recruited first during nerve activity. We tested this by selectively labeling the "readily releasable" pool, those vesicles released first during physiological stimulation. The readily releasable vesicles were not clustered close to the presynaptic membrane but instead were dispersed almost randomly throughout the vesicle cluster. Thus, vesicles are not recruited according to proximity to release sites but are mobilized differently, perhaps by being peeled from the surface of the cluster.

Synaptic vesicles are virtually identical in ultrastructural appearance. However, physiological studies provide abundant evidence for the existence of different functional "poools" of vesicles (1–5). For example, during repetitive nerve stimulation, the amplitudes of synaptic signals typically decline from an initial high value, as the readily releasable pool (RRP) is depleted, to lower values, as vesicles are mobilized from a "reserve" pool (6, 7). The natural assumption is that the RRP comprises those vesicles lying closest to the release sites ("active zones"), and the reserve pool, those vesicles farther away.

At the frog neuromuscular junction, the RRP is depleted in ~10 s of stimulation at 30 Hz, and the endocytosed vesicles do not mix randomly with other vesicles as they recycle but return selectively to the RRP (4, 7). Here we labeled RRP vesicles by nerve stimulation (10 s, 30 Hz) in presence of FM1-43, a fluorescent dye that is taken up by recycling vesicles. We allowed the preparations to recover for 10 min in presence of the dye, then fixed, photoconverted, and prepared them for electron microscopy (EM) (8). Labeled vesicles appeared dark, and were easily distinguishable from unlabeled vesicles (Fig. 1A; fig. S1). Generally, 12 to 17% of the vesicles were labeled, in agreement with optical and electrophysiological estimates of the size of the RRP (~15%) (4, 7). The labeled vesicles were distributed throughout the nerve terminal, except for the core of the vesicle cluster. A three-dimensional reconstruction of 24 serial sections from a typical terminal segment was produced (Fig. 1, B to D) (1 of 12 serial reconstructions, averaging 1.64 μm in length; ~4200 labeled and ~36,000 unlabeled vesicles total, with 12 ± 1.5% labeled vesicles).

We measured the positions of vesicles, active zones, and surface membrane and assigned each vesicle to one of four locations (Fig. 1E): active zone (vesicles lying within 100 nm of active zones); surface membrane (vesicles located more than 100 nm from active zones, but within 100 nm of the surface membrane); cluster edge (vesicles more than 100 nm from the membrane lying within 50 nm of the vesicle cluster edge); and cluster core. More than 99% of all vesicles fell into one of these categories. The relative distribution of vesicles in the different locations is shown (Fig. 1F). The major difference between the labeled and unlabeled populations was that labeled vesicles were relatively excluded from the cluster core (52 ± 2% unlabeled versus 38 ± 1.6% labeled, P < 0.001, t test) (Fig. 1A). Otherwise, the labeled vesicles were distributed approximately randomly throughout the other three regions.

We obtained similar results using two other stimulation paradigms proposed to release selectively the RRP [low frequency stimulation (7) and hypertonic shock (1)] (Fig. 2, A and B). To check whether a significant fraction of loaded vesicles released their dye during chemical fixation and thus modified the labeled vesicle distribution [unlikely according to (9, 10)], we used rapid freezing (instead of chemical fixation) in conjunction with dextran particle uptake (Fig. 2, B to C) (11). This procedure resulted in a similar vesicle distribution, though fewer vesicles were labeled (~3%, reflecting the less reliable uptake of dextran particles (8)).
In these experiments, fixation occurred after a delay following stimulation, during which time vesicles might have moved to new locations inside the terminal. Therefore, we plunged preparations into ice-cold fixative immediately after stimulation (30 Hz for 10 s; Fig. 3A). This resulted in a large increase in the fraction of labeled vesicles at the surface membrane but not at active zones (Fig. 3B). This suggests that vesicles were endocytosed at sites away from the active zone, and then migrated to other locations during the next several minutes. A closer examination of vesicle neighbors revealed that labeled vesicles tended to cluster together (fig. S2).

An additional explanation for the scattered appearance of vesicles was that the dye-loading paradigm partially loaded the reserve pool, which produced labeling of vesicles far from the active zone. To test this, we shortened the dye-loading stimulus train by 20-fold (30 Hz/0.5 s). This procedure labeled 1.9% of vesicles (~0.32% in immediately fixed terminals). Labeled vesicles were localized at non–active zone surface membrane (immediate fixation) and scattered widely in preparations fixed 2 min after stimulation (Fig. 3C), as with our longer, RRP-releasing stimuli.

These results do not favor a “kiss-and-run” (12) mechanism, which predicts an accumulation of labeled vesicles near the release sites. Because kiss-and-run events that release neurotransmitter but take up no dye have been postulated under certain conditions (13), we compared estimates of release obtained electrophysiologically with vesicle labeling (Fig. 3D). The good agreement under all conditions does not suggest a role for a kiss-and-run scenario, unless it occurred to the same extent under all conditions, which is unlikely (13, 14).

It was of interest to examine the release of labeled vesicles. Nerve terminals were loaded with FM1-43 as before, but then they were partially destained by further stimulation before being fixed and photoconverted. Preparations loaded with a 60-s stimulus were ~2.5 times brighter than those loaded with a 10-s train (Fig. 4A). During continuous stimulation at 30 Hz, the fluorescence signals decreased by ~50%. Residual fluorescence represents both labeled vesicles that are not releasable and fluorescence trapped elsewhere (as in postsynaptic folds and Schwann cells). We measured the fraction of photoconverted vesicles before and after stimulation, and used it to calculate nonvesicular background fluorescence (8). We found that labeled vesicles account for about one-half of the residual fluorescence for both paradigms. Thus, RRP vesicles did not recycle with perfect fidelity; ~30% of them were not released during subsequent stimulation. This experiment provided a vesicle-based calibration of FM dye signals and permitted the fluorescence destaining rates of terminals loaded with different paradigms to be compared. The fluorescence curves from Fig. 4A were corrected for nonvesicular fluorescence, and normalized...
to their initial sizes (Fig. 4B, filled circles). The more rapid destaining rate of the terminals loaded for 10 s was clear and confirms previous conclusions that RRP vesicles recycle selectively to the RRP (4, 7). The open circles in Fig. 4B show the absolute difference between the 10-s (RRP vesicles only) and 60-s (RRP and reserve pool vesicles) loaded terminals during destaining. During the first 10 s the difference curve is flat (Fig. 4B, arrow), indicating that no release of reserve pool vesicles occurred during this interval, which confirms previous observations that release from the RRP and reserve pools occurs mostly sequentially, not simultaneously (4, 7).

We also photoconverted terminals (loaded for 10 s at 30 Hz) that were destained for different durations, enabling a direct comparison of the time course of loss of labeled vesicles and loss of fluorescence (Fig. 4C). The vesicle loss is slightly faster than the fluorescence loss, which probably reflects the time required for FM1-43 to be washed from surface membranes in the synaptic region after exocytosis (4). Labeled vesicles from the core of the cluster lagged in their release in comparison to other regions, especially during the first 10 s after stimulation began (Fig. 4D).

When histograms of distances of labeled vesicles from the nearest active zone were plotted (in control terminals and after 10 s of destaining), the data were fitted by a simple model in which RRP vesicles move to the active zone with constant velocity during stimulation (Fig. 4E). With a destaining time constant of ~10 s (Fig. 4C, photoconversion data), the velocity would be ~50 nm/sec (3 µm/min), in agreement with the rates of movement observed in okadaic acid–treated preparations (15).

Our results, while unexpected, are not difficult to reconcile with general ideas of synaptic vesicle mobilization and recycling. Vesicles in the RRP have special identities, either as a result of unique molecular markers, of privileged positions, or of both. The privileged status of recycling RRP vesicles is not immutable, however; about 30% of recycled vesicles were lost to the reserve pool. Also, stronger, nonphysiological stimulus paradigms probably recruit other vesicles first (16–19). Perhaps RRP vesicles at the edge of vesicle clusters are glued less tightly to their neighbors than are vesicles in the core of the cluster and are attached to cytoskeletal elements like actin, which is more abundant outside than inside the cluster (20, 21) and could affect their preferential transport to sites of exocytosis.

Fig. 4. Labeled vesicle release. (A) Preparations were loaded with FM1-43 [30 Hz, for 10 s (red) or 60 s (black)] and washed, and then the loss of fluorescence was followed during 30 Hz stimulation (n = 8 to 10 nerve terminals). Numbers on the graph show the percent of labeled vesicles (measured after photoconversion). (B) The traces from (A) were corrected for nonvesicular background (8) and scaled to their initial values. Open circles show the absolute difference between the 10- and 60-s loaded terminals. (C) Loss of FM fluorescence (red) and loss of labeled vesicles at the ultrastructural level (black) for 10-s loaded terminals (best-fit exponential time constant, 10.1 s). The two traces are entirely independent. Background fluorescence in the red trace was eliminated using a fluorescence-only method (8). (D) Release of labeled vesicles from the four subpopulations (Fig. 1). Ten-second labeled terminals were stimulated for 0, 10, 30, or 120 s, and the vesicle distributions were plotted (normalized to initial values for each subpopulation (at least 3 preparations/40 terminal profiles analyzed). The release from the cluster core was significantly slower during the first 10 s of stimulation (P < 0.01, t test). (E) Histograms of labeled vesicle distances from nearest active zone (50-nm bins; 10-s loaded terminals). Control (solid circles) distribution was well fit by a single exponential (length constant = 514 nm). Ten-second destained preparations (open circles) showed a loss of ~50% of the labeled vesicles. The length constant of the remaining vesicles distribution (586 nm) is in agreement with controls, consistent with vesicles moving with constant velocity (~50 nm/s) toward the active zone during stimulation.

References and Notes
8. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material
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Materials and Methods
Figs. S1 and S2
Movie S1
References
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