Properties of Synaptic Vesicle Pools in Mature Central Nerve Terminals*

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Readily releasable and reserve pools of synaptic vesicles play different roles in neurotransmission, and it is important to understand their recycling and interchange in mature central synapses. Using adult rat cerebrocortical synaptosomes, we have shown that 100 mosm hypertonic sucrose caused complete exocytosis of only the readily releasable pool (RRP) of synaptic vesicles containing glutamate or \( \gamma \)-aminobutyric acid. Repetitive hypertonic stimulations revealed that this pool recycled (and reloaded the neurotransmitter from the cytosol) fully in \(< 30 \) s and did so independently of the reserve pool. Multiple rounds of exocytosis could occur in the constant absence of extracellular Ca\(^{2+} \). However, although each vesicle cycle includes a Ca\(^{2+} \)-independent exocytotic step, some other stage(s) critically require an elevation of cytosolic [Ca\(^{2+} \)], and this is supplied by intracellular stores. Repetitive recycling also requires energy, but not the activity of phosphatidylinositol 4-kinase 4-kinase, which maintains the normal level of phosphoinositides. By varying the strength of hypertonic stimulations, we found that \( \sim 70\% \) of the RRP vesicles fused completely with the plasmalemma during exocytosis and could then enter silent pools, probably outside active zones. The rest of the RRP vesicles underwent very fast local recycling (possibly by kiss-and-run) and did not leave active zones. Forcing the fully fused RRP vesicles into the silent pool enabled us to measure the transfer of reserve vesicles to the RRP and to show that this process requires intact phosphatidylinositol 4-kinase and actin microfilaments. Our findings also demonstrate that reserve vesicle pools have similar characteristics and requirements in excitatory and inhibitory nerve terminals.

Synaptic vesicles in a nerve terminal that undergo release/recycling consist of the readily releasable pool (RRP) and the reserve pool (1, 2). These vesicle populations play distinct roles in neurotransmission, and their relative sizes change with synapse maturation (3, 4). Therefore, to understand the physiologically relevant properties of the vesicle pools, it is important to study them in mature central nerve terminals. When conducted in acute slices of adult brain, such studies are usually limited to specific regions of the hippocampus and thus do not necessarily reflect the behavior of vesicle pools in the rest of the brain. An alternative, well established system, cerebrocortical synaptosomes, is more universal and has several important advantages: (i) synchronized stimulation of all nerve terminals even by viscous solutions such as sucrose, (ii) direct comparison of terminals containing different transmitters, (iii) high-throughput pharmacological analysis, (iv) evaluation of multiple samples, and (v) uniform neuronal sampling. Finally, synaptosomal studies reveal pathways that are common to most nerve terminals, providing a level of generalization that is difficult to achieve by studying individual synapses.

This simplified model, which lacks axons and cell bodies, is able to take up a radiolabeled neurotransmitter from the medium, accumulate it in the cytosol, and then use it to refill synaptic vesicles that undergo recycling (5–7). Induced exocytosis is determined by measuring the radioactivity released above that in unstimulated controls. Synaptosomes are sensitive to all stimuli used in other neuronal systems, including electric field (8), elevated [K\(^{+} \)] (7), \( \alpha \)-latrotoxin (LTX) (9), and hypertonic sucrose (HS) (9, 10); however, one must clearly establish that any release observed is vesicular.

Previously, we used LTX and HS to define the size of the RRP and reserve pool in mature cortical terminals containing glutamate (Glu) or \( \gamma \)-aminobutyric acid (GABA) (9). Wild-type LTX causes secretion of the amino acids (AAs) in two pharmacologically distinct phases, fast and slow, corresponding to exocytosis of readily releasable and reserve vesicles, respectively (9). HS (known to act on readily releasable vesicles only (11)) competes with LTX for the fast phase, but does not perturb the slow phase. As all vesicles contain neurotransmitters, it is difficult to distinguish vesicle populations by electrophysiology. However, we have discovered that readily releasable vesicles exchange their contents with the cytosolic pool of neurotransmitters much faster than reserve vesicles (9). This made it possible (by using the “short” and “long” labeling procedures (9); see also below) to radiolabel selectively and to differentiate the RRP and reserve pool.

Using these approaches, we analyzed the properties of the RRP in synaptosomes and vesicle mobilization from the reserve pool. Vesicles were labeled by loading with radioactive AAs or by stimulated uptake of fluorescent dye. RRP exocytosis was induced by HS, and the release of reserve vesicles was induced by other methods. We show that, in mature AA-containing terminals, readily releasable vesicles can undergo multiple cycles of release without mobilization of reserve vesicles. Some stages of this cycle critically require an increase in cytosolic [Ca\(^{2+} \)].

**EXPERIMENTAL PROCEDURES**

**Materials**—Radiolabeled AAs were from Amersham Biosciences. Tetanus toxin (TeTX), BAPTA-AM, and thapsigargin were from Calbiochem-Novabiochem. Fluorescent dyes were from Invitrogen. All other reagents, salts, and buffers were from Sigma.

**Release of Amino Acid Neurotransmitters**—Rat cerebrocortical synaptosomes (P2) were prepared as described previously (12). These were initially suspended in basal buffer (145 mM NaCl, 5 mM KCl, 1 mM...
MgCl2, 10 mM glucose, and 20 mM Hepes, pH 7.4) supplemented with 2 mM CaCl2 and 0.1 mM aminoxyacetic acid (used to inhibit metabolism of GABA and present in all buffers when secretion of this AA was measured (e.g. Ref. 13)). Aminooxyacetic acid did not affect the release of Glu, and synaptosomes containing this drug were also employed in Glu studies. All buffers and synaptosomal suspensions were continuously oxygenated.

The short and long (also called “normal”) protocols for loading synaptosomes with radiolabeled AAs were described previously (9), and the principles of these procedures are further explained under "Results." Briefly, for short loading, synaptosomes (1 mg of protein/ml) were preincubated in basal buffer containing Ca2+ at 37 °C for 10 min and then supplemented with [14C]Glu (2–5 μCi/ml) or [3H]GABA (2–5 μCi/ml) and incubated for another 5 min. Terminals were quickly washed (twice with basal buffer containing 1 mM EGTA and once with basal buffer at room temperature), resuspended in basal buffer, and aliquoted (25 μg of protein/sample) for immediate release measurements. For long loading, synaptosomes were similarly incubated at 37 °C for 5 min with [14C]Glu or for 60 min with [3H]GABA, washed, and resuspended as described above and then subjected to “post-loading incubation” for an additional 60 min at 37 °C, washed, and used to measure release.

When TeTX was used, to avoid any effect of the toxin on neurotransmitter uptake, terminals were first loaded with radiolabeled AAs for 10 min and then mixed with TeTX (100 nM final concentration) and incubated for an additional 2 h at 37 °C. Some synaptosomes were treated for 60 min at 37 °C (during the second stage of the long loading protocol) with 1 μM bafilomycin A1 (BAF; to inhibit vesicular H+-ATPase) or 15 μM latrunculin A (to disassemble actin microfilaments).

For HS stimulation, basal buffer containing concentrated sucrose was added to the synaptosomal suspension (with immediate mixing) to achieve the desired final osmolarity of the solution. The "hypertonicity" was expressed as an increase in osmolarity above that of the isotonic basal buffer (320 mosM). Thus, when samples were stimulated with 100–500 mosM HS, they contained 100–500 mM sucrose in basal buffer and had an absolute osmolarity of 420–820 mosM. Control samples were treated identically, except only basal buffer was added during the stimulation period. After stimulation was terminated by diluting samples and reducing their osmolarity to isotonic 320 mosM, the sample volume was adjusted to 1.5 ml by an appropriate buffer, followed by centrifugation and removal of the supernatant. The radioactive content of the supernatant and solubilized pellet was determined by liquid scintillation counting. The choice of a small amount of synaptosomes (25 μg/sample) and a relatively large sample volume (0.4 ml) helped to prevent any re-uptake of the released neurotransmitter. (Indeed, when inhibitors of AA transporters were added during stimulation, this did not change the amount of release detected (data not shown).) Varying the sample volume from 0.05 to 1 ml did not affect the total release measured, and this was important for the multiple release experiments (see below).

For repetitive stimulations, synaptosomes were preloaded with radiolabeled AAs (by one of the methods above), and radiolabel was never added to synaptosomal suspensions during the stimulations, allowing vesicles to refill with the radioactive tracer from the cytosol. The multiple stimulation protocol consisted of up to six cycles, each including a 3-s incubation with 100 mosM HS, dilution to isotonicity, and a 177-s rest period (see Fig. 4A). In the first cycle, the osmolarity was increased to 420 mosM (by adding sucrose in basal buffer) and then reduced to 320 mosM (by diluting with an appropriate amount of 10 mM Hepes, pH 7.4). This also led to a 24% dilution of basal buffer constituents (NaCl, KCl, and MgCl2). However, this reduction in the ionic composition during the rest periods was not detrimental, as the solution remained isotonic, and agreed with the finding that reduction of [NaCl] by >30% does not affect the properties of vesicle recycling in cultured hippocampal cells (14). During subsequent stimulations, the osmolarity was raised again to 420 mosM, and the normal ionic composition was reinstated by adding appropriately concentrated sucrose and basal buffer constituents. Dilutions again reduced both the osmolarity and ionic concentration. The total sample volume increased with each cycle, and the amounts of stimulation and dilution buffers were adjusted proportionately; the volume difference between successive rounds of stimulation did not affect the induced release (data not shown). Parallel samples were stimulated different numbers of times and, before the final stimulation, were always adjusted to the same volume by a dilution buffer with a final composition equivalent to that during the rest periods (110 mM NaCl, 3.78 mM KCl, 0.76 mM MgCl2, 15.14 mM Hepes, 7.57 mM glucose, and 75.68 mM sucrose). Release in the final round was terminated by adding a final stop buffer, which re-established isotonic conditions and brought the volume to 1.5 ml. The samples were then spun down to measure the amount of released AAs. Release in round n (Rn) was calculated by subtracting the total amount of release in samples stimulated n–1 times with HS and once with basal buffer from that in parallel samples stimulated n times with HS.

For chelation of cytosolic Ca2+L, synaptosomes were loaded with [14C]Glu or [3H]GABA for 5 min and then incubated with 100 μM BAPTA-AM at 37 °C during the 60-min post-loading period. (This represented the long loading protocol.) For similar measurements in short-loaded terminals, synaptosomes were washed to remove extracellular Ca2+ (Ca2+2), incubated for 60 min at 37 °C with 100 μM BAPTA-AM, diluted 2-fold in basal buffer containing 4 mM Ca2+, and short-loaded with [3H]GABA for 5 min at 37 °C. Thus, the effect of cytosolic BAPTA was tested in both short- and long-loaded terminals and appeared to be the same. To investigate the role of Ca2+ stores, synaptosomes were incubated with 10 μM thapsigargin for 25 min at 37 °C prior to a 5-min loading with [14C]Glu or [3H]GABA. In combined BAPTA/thapsigargin experiments, synaptosomes were loaded with radiolabeled AAs for 5 min at 37 °C and then post-incubated for an additional 60 min in the presence of 100 μM BAPTA-AM, with 10 μM thapsigargin added for the last 30 min of this incubation. The ATP requirement was assessed by incubating short-loaded synaptosomes for 5 min at 37 °C in the presence of 10 μM rotenone plus 2 mM iodoacetic acid; these drugs were present in all buffers used in repetitive stimulations. To evaluate the role of phosphatidylinositol (PI) 4-kinase, synaptosomes were incubated with 3 μM phenylarsine oxide (PAO) for 15 min at 37 °C. For short loading, radiolabeled AAs were added for the last 5 min of this period; for long loading, the drug was present during the last 15 min of the 60-min post-loading incubation. To assess the effect of protein kinase C activation on HS-sensitive vesicle pools, terminals were preincubated with 1 μM active phorbol ester (β-phorbol 12,13-dibutyrate) or 1 μM inactive 4α-phorbol at 37 °C for 10 min just prior to measuring release. Control samples were treated identically, except drug-free solvent was applied.

LTX was purified in this laboratory and used in the presence of 1.2 mM Ca2+ (to induce the receptor-mediated mechanism) and 0.1 mM La3+ (to block toxin pores) (9). Electric field stimulation (8) was done for 2 min at 10 Hz and a voltage density of 40 V/cm.

**FM 2-10 Uptake and Release**—A published method of loading synaptosomes with FM 2-10 (15) was modified to facilitate the labeling of both RRP and reserve vesicles (see Ref. 16) and carried out at room temperature. Synaptosomes in basal buffer (0.5 mg of protein/ml) were incubated for 150 s with 100 μM FM 2-10. During the last 90 or 3 s of this
period, terminals were stimulated with sucrose (final hypertonicity of 100 mosM) or with 30 mM K+ and 2.5 mM Ca^{2+} (referred to as K+/Ca^{2+}). This stimulation was terminated by dilution with hypotonic or isotonic buffer, respectively, containing 100 mM FM 2-10, and the samples were centrifuged, resuspended in basal buffer containing the dye, and incubated for an additional 5 min. Synaptosomes were then diluted 5-fold with basal buffer containing 0.1% bovine serum albumin, centrifuged, and washed twice. Finally, terminals were resuspended in basal buffer and placed into 96-well black microtiter plates (100 μg of protein/well). The basal fluorescence of the samples was determined at 485 ± 14 nm excitation and 555 ± 25 nm emission (1-s integration period) in an automated Fluoroskan Ascent FL fluorometer (Thermo Labsystems). A stimulus was then added (see “Results”), and changes in fluorescence were recorded over time. To measure the release of the dye taken up specifically by endocytosed vesicles, the fluorescence of unstimulated samples was subtracted from that of parallel stimulated samples.

**Intracellular Ca^{2+} (Ca^{2+}) Measurements**—Synaptosomes in basal buffer (1 mg of protein/ml) were incubated for 30 min with 5 μM Fura-2 acetoxymethyl ester (a ratiometric Ca^{2+} indicator) at 37°C, washed two times, and resuspended in basal buffer. Some synaptosomes contained 10 μM thapsigargin for the last 25 min of this incubation and in subsequent wash and stimulation buffers. The synaptosomes (20 μg of protein) were placed into microtiter plates, and fluorescence was recorded before and after the addition of sucrose in basal buffer (to produce a hypertonicity of 100 mosM) against a parallel unstimulated sample. Fluorescence was measured using the Fluoroskan fluorometer with excitation at 340 nm (300–360 nm band) and 380 nm (370–400 band) and emission at 510 ± 10 nm. The maximal F_{340/F_{380}} ratio for each sample was determined by adding 0.3% Triton X-100 and 2.5 mM Ca^{2+} (final concentrations). The minimal F_{340/F_{380}} value was determined by treating samples with 0.3% Triton X-100 and 15 mM EGTA. Fluorescence ratios (F_{340/F_{380}}) were converted to [Ca^{2+}] using the equation from Grynkiewicz et al. (17). To determine the stimulated change in Ca^{2+} ([Ca^{2+}]), [Ca^{2+}], in control samples was subtracted from that in stimulated samples.

It was important to use a ratiometric dye in these experiments because hypertonic solutions cause shrinkage of nerve terminals (see “Results”) and, consequently, an increase in the cytosolic dye concentration. If a non-ratiometric indicator were employed, changes in its concentration would affect its absolute fluorescence and could be misinterpreted as changes in [Ca^{2+}]. Because Fura-2 fluorescence intensities at 340 and 380 nm have opposite responses to Ca^{2+}, measuring the 340/380 nm ratio results in the cancellation of artifactual variations in the fluorescent signal due to fluctuations in dye concentration and instrument efficiency (17).

**Electron Microscopy**—The structure of synaptosomes was examined by electron microscopy essentially as described previously (18). Synaptosomes were exposed for 3 s to basal buffer, 100 mosM HS, or 500 mosM HS, followed by the immediate addition of a cold fixative containing 1.69% glutaraldehyde and 1.35% paraformaldehyde (final concentrations) and the relevant buffer to maintain the appropriate osmolarity. In another set of experiments, similarly stimulated synaptosomes were first diluted 10-fold to isotonic conditions and 3 min later fixed in glutaraldehyde/paraformaldehyde in basal buffer. Similar fixation of cultured hippocampal cells did not induce any further release or perturb the vesicle pools (19). All samples were post-fixed for 60 min in 1% osmium tetroxide, treated for 20 min with 1% aqueous uranyl acetate, dehydrated, and embedded in Epon resin. Ultrathin 80-nm sections were counterstained with uranyl acetate and lead citrate and examined and photographed using a Phillips C100 electron microscope. To estimate hypertonicity-induced changes in synaptosomal volume, the midsection parameters of treated synaptosomes were compared with those of control terminals with a similar perimeter length.

**Assessment of Membrane Damage**—Following stimulation, synaptosomes were centrifuged, and the pellet and supernatant were separated. The pellets were solubilized in 1% Triton X-100, and an equivalent amount of detergent was added to the supernatants. To determine the lactate dehydrogenase activity, 0.02 ml of appropriately diluted samples were mixed with 3 ml of 100 mM phosphate buffer, pH 7.0, 0.1 ml of sodium pyruvate (0.77 mM final concentration), and 0.05 ml of NADH (0.2 mM final concentration), followed by measuring the linear rate of absorbance changes at 340 nm and 37°C.

**Statistical Validation of Data**—All experiments were conducted several times using multiple parallel samples (n is specified in the figure legends). Values in the figure legends are the means ± S.D. Where necessary, the values were compared using a two-tailed Student’s t test; the difference was considered significant when p was ≤0.05.

**RESULTS**

**Hypertonicity and Exocytosis in Synaptosomes**—Hypertonicity is often used to trigger the exocytosis of only the RRP (4). However, too high a hypertonicity might also activate reserve vesicles and even damage nerve terminals. We therefore determined the dose-response curve for HS using synaptosomes loaded with [³⁴Cl]Glu or [³H]GABA. AA secretion correlated with hypertonicity (Fig. 1, A and B). To ascertain whether this release was vesicular, we treated synaptosomes for 2 h with TeTX (protoype of synthetobrevin and blocks vesicle fusion (e.g. Ref. 12)) or for 1 h with BAF (prevents loading of vesicles and leads to eventual depletion of their neurotransmitter content (e.g. Refs. 9 and 20)). Surprisingly, only release triggered by HS concentrations up to 100 mosM was blocked by either drug (Fig. 1, A–D), whereas any further elevation of hypertonicity produced the same amount of exocytosis superimposed with an increasing amount of SNARE-independent and BAF-insensitive release. If all release caused by >100 mosM HS was vesicular, this would provocatively suggest that massive exocytosis might occur without the participation of SNARE proteins and from vesicles insensitive to BAF. Alternatively, some of this release might represent leakage of the transmitter due to HS-induced damage of terminals. We assessed any possible lysis by measuring liberation of the cytosolic enzyme lactate dehydrogenase. As expected, exposure to 100 mosM HS produced negligible lactate dehydrogenase release compared with the amount of Glu and GABA secretion, whereas exposure to >100 mosM HS caused substantial lysis (Fig. 1, E–G).

To approach an understanding of how hypertonicity affects synaptosomes, we studied their ultrastructure by electron microscopy. Synaptosomes were incubated for 3 s under hypertonic conditions and fixed instantly as described (18). The following parameters of individual terminals were used to quantify the changes observed: (i) the presence of vacuoles (reflecting perturbation of internal membrane-delimited structures), (ii) the major to minor axis ratio (as a measure of shape distortions), and (iii) the cross-section area (related to the volume). In basal buffer, only a small proportion of synaptosomes (~20%) contained a vacuole (Fig. 2, A and D), and this fraction did not change significantly in 100 mosM sucrose (Fig. 2, B and D). In contrast, >80% of all terminals exposed to 500 mosM HS displayed large vacuoles, possibly corresponding to vesicles fusing with each other (Fig. 2, C and D). Control terminals had a round appearance (Fig. 2A), with an axis ratio of ~1 (Fig. 2, A and E). This shape only slightly changed to elliptical in 100 mosM HS (Fig. 2, B and E), whereas synaptosomes exposed to 500 mosM sucrose had a...
drastically altered morphology, often assuming tube-like or dumbbell shapes (Fig. 2, C and E). The average parameters of control synaptosomes were as follows: diameter, 0.7 ± 0.2 μm; midsection area, 0.38 ± 0.1 μm²; and approximated volume, 0.165 μm³. Treatment with 100 and 500 mosM sucrose reduced the area by 35 and 72% and the approximated volumes by ~50 and >85%, respectively. (In the latter case, vesicles were still present, but filled up all of the internal space (Fig. 2, A–C and F).)

In most of our experiments, dilution to isotonicity was used to stop the HS-induced release. Dilution itself did not produce any additional AA release (data not shown). However, the intactness of synaptosomes after their re-swelling was essential for repetitive stimulations (described below) and hence was also evaluated by electron microscopy. Therefore, these "long-loaded" synaptosomes contained the radiolabeled AAs in a greater proportion of vesicles, which included both the RRP and reserve pool. If HS stimulation affected not only the RRP but also the reserve pool, then different amounts of AA release would be expected from terminals labeled by these two procedures. However, we found that AA release induced by 100 mosM HS did not depend on the length of time employed for vesicle loading (Fig. 3, A and B) and thus involved only the fast-refilling pool, i.e. the RRP.

Did this exocytosis involve the whole RRP or only part of it? The vesicular component of release reached a maximum at 100 mosM HS and did not change further with increasing sucrose concentration, suggesting that the whole pool of hypertonicity-sensitive vesicles was already released by 100 mosM sucrose (Fig. 1, A–D). This was further confirmed by using LTX under the conditions in which it acts through the receptor transduction pathway (9, 21). Although this action of LTX involves a mechanism (9) distinct from that of HS, the two stimuli induced the same maximal amount of AA exocytosis (Fig. 3D). Electric field (10 Hz) stimulation produced similar Glu secretion (6.26 ± 1.2%, n = 4). Furthermore, although high doses of HS and LTX were additive (Fig. 3C), high doses of the two stimuli together caused the same secretion as was maximally achieved by each stimulus alone (Fig. 3D), indicating that they competed for the same pool of vesicles. Finally, if these stimuli caused secretion of only a specific vesicle subspool of finite size (rather than the whole RRP), then increasing the total RRP size would have no effect on the maximal AA release. However, treating synaptosomes with an active phorbol ester (which is known to increase the size of the HS-sensitive RRP in excitatory neurons (22)) clearly augmented Glu exocytosis induced by 100 mosM HS, whereas inactive phorbol was ineffective (Fig. 3E). Again, this effect did not involve the reserve vesicles because it was not affected by the labeling of the slow-loading vesicles (Fig. 3E). Phorbol ester may also enhance secretion by increasing the sensitivity of vesicle fusion to Ca²⁺; however, in our system, hypertonicity-induced exocytosis was independent of both intracellular and extracellular Ca²⁺ (see below).

Taken together, these results indicate that 100 mosM HS acts on the whole RRP rather than a subpopulation. Clearly, synaptosomes are more sensitive to hypertonicity than hippocampal autapses, for which puffs of 500 mosM sucrose were required to cause maximal RRP release (11). However, in the latter work, the exact sucrose concentration around terminals was not defined and was probably <500 mosM. In any case, different synaptic preparations exhibit distinct sensitivities to

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**Synaptic Vesicle Pools in Mature Central Terminals**

**The RRP in Synaptosomes**—Although HS is known to induce only the RRP, it was possible that, in isolated nerve terminals, the reserve pool was also affected. To address this question, we took advantage of our recent discovery that nerve terminals contain two vesicle pools with different rates of neurotransmitter uptake from the cytosol (9). Based on their differential sensitivities to HS and to the two modes of LTX action, the fast- and slow-refilling vesicle populations have been identified as the RRP and reserve pool, respectively (9).

The distinct refilling rates of these pools allowed us to devise the short and long loading protocols, which labeled the RRP alone or the two pools together, respectively. In both procedures, the terminals were first incubated with radiolabeled AAs for a short time and then washed. This resulted in uptake of the neurotransmitter into the cytosol, from where some of it quickly entered the fast-refilling pool (RRP). If such synaptosomes were immediately stimulated for release, they were considered "short-loaded." However, if these terminals were incubated for an additional 1–2 h in the absence of extracellular radioactivity, the cytosolic tracer started accumulating also in slow-refilling vesicles (reserve pool). Therefore, these "long-loaded" synaptosomes contained the radiolabeled AAs in a greater proportion of vesicles, which included both the RRP and reserve pool. If HS stimulation affected not only the RRP but also the reserve pool, then different amounts of AA release would be expected from terminals labeled by these two procedures. However, we found that AA release induced by 100 mosM HS did not depend on the length of time employed for vesicle loading (Fig. 3, A and B) and thus involved only the fast-refilling pool, i.e. the RRP.
hypertonicity (23–25), due in part to differences in synapse stabilization by the substrate in cell culture or by surrounding cells in a tissue (see also "Discussion").

Recovery of the RRP—The total hypertonicity-sensitive vesicle pool containing [14C]Glu or [3H]GABA was released within 3 s of the addition of 100 mosM HS (Fig. 4, A–C), and longer exposures produced no further release. The size and kinetics of this secretion were identical in synaptosomes labeled by the short or long loading procedure (Fig. 4, B and C), further indicating that this stimulus acted only on one pool, the RRP.

We decided to study the recovery of this pool. One way to do this was to resuspend the pellet (obtained after initial stimulation) in basal buffer, allow the synaptosomes to recover for different periods of time, and then subject them to another round of HS stimulation (Fig. 4, A), followed by dilution and pelleting. The amount of HS-induced AA release in the second period (R2) was determined by subtracting the release in samples stimulated once with hypertonicity (R1) and once with basal buffer from that in samples stimulated with hypertonicity both times (R1/H1001 R2).

At first we looked at the effect of the length of the first stimulation (ΔTstim) on the release in the second round (R2) while maintaining the same resting period (240 s). We found that, if the initial stimulation lasted for 3 s, then R2 was equal to R1 (Fig. 4, D and E). However, when ΔTstim was increased from 3 to 90 s, R2 was gradually reduced (Fig. 4, D and E), suggesting that longer initial hypertonic exposures either decreased the size of the RRP or inhibited its subsequent exocytosis (R2).

Therefore, by applying a long first stimulation and then varying the resting period (ΔTrest) before the second stimulation, we were able to study the time course of the RRP recovery. In both short- and long-loaded synaptosomes, when ΔTstim was 3 s, a full RRP was released by a second stimulation 30 s later (the shortest rest time tested) (Fig. 4, F–I). Then ΔTstim was increased to 90 s. As a result, in short-loaded terminals, only 25–30% of the RRP was released by a second stimulation, and surprisingly, no recovery of this pool was observed even after 900 s (the longest rest time tested) (Fig. 4, F and G). In contrast, in long-loaded synaptosomes, the sucrose-sensitive RRP recovered gradually with time (Fig. 4, H and I) and was fully available for HS-induced release after a 15-min rest period. Because both the RRP and reserve pool are labeled in long-loaded terminals, the recovery of the RRP under these stimulation conditions must involve mobilization of reserve vesicles.

Repetitive Exocytosis of the RRP—To study the recycling of vesicle pools, we developed a protocol for multiple repetitive stimulations of synaptosomes (see "Experimental Procedures" for details) (Fig. 5A).
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Terminals were first preloaded with a radioactive neurotransmitter, washed, and subjected to one to six cycles of alternating stimulation and rest. During each cycle, the osmolarity of the synaptosomal suspension was raised to 420 mosM (by adding sucrose) and 3 s later returned to isotonic 320 mosM (by dilution); the terminals were allowed to rest for 3 min before the next stimulation. (This resting time guaranteed complete recovery of the RRP (26).) No radiolabeled AAs were added to the experiment. (It takes up to 2 h to equilibrate reserve vesicles with radioactive AA transmitters from the cytosol (9).) An alternative possibility was that non-labeled reserve vesicles were converted into the RRP during the experiment and then quickly took up the radioactive neurotransmitter with PAO did not affect the RRP recovery (see below). Therefore, repetitive release must occur from RRP vesicles that are quickly refilled with radioactive AAs present in the cytosol rather than by mobilization of reserve vesicles. Thus, our method provided a convenient new system to follow specifically the recycling of RRP vesicles.

ATP Requirements for RRP Recycling in Synaptosomes—Although isolated nerve terminals stably supported at least six rounds of repetitive vesicular release (Fig. 5), their metabolism could differ from that in intact neurons. Normally, each cycle of exocytosis includes ATP-requiring steps such as the acidification of vesicles by $\text{H}^+\text{-ATPase}$ (for subsequent loading of neurotransmitters) and the disassembly of cis-SNARE

![Figure 3. RRP size in isolated nerve terminals.](image)

![Figure 4. Time course of hypertonicity-induced RRP exocytosis and inhibition of RRP recovery by prolonged stimulation.](image)
The Role of Ca\textsuperscript{2+}—Release elicited by single HS applications does not require Ca\textsuperscript{2+}, e.g., Refs. 10 and 11). Although the presence of Ca\textsuperscript{2+} had no effect on release triggered by exposure to 100 mosm HS (5.33 ± 1.3% of the total \[^{14}\text{C}\]Glu content in the absence of Ca\textsuperscript{2+}, and 5.42 ± 1.61% in its presence, \(n = 8\)), we conducted all of our experiments in Ca\textsuperscript{2+}-free buffers to exclude any indirect Ca\textsuperscript{2+}-dependent effects (cf. Ref. 28).

Because repetitive exocytosis of the RRP occurred in the continuous absence of Ca\textsuperscript{2+}, it could be possible that Ca\textsuperscript{2+} was also unnecessary for vesicle recycling. Indeed, synaptosomes loaded with BAPTA-AM exhibited a normal first round of HS-induced \[^{14}\text{C}\]Glu or \[^{3}\text{H}\]GABA release (Fig. 5, F and G) (cf. Ref. 11). Thus, if there were a Ca\textsuperscript{2+}-requiring stage in the vesicle cycle, the first-round vesicles must have already passed it, but then the second round of release should be inhibited. In fact, BAPTA did not significantly affect even the second round of HS stimulation (Fig. 5, F and G). However, surprisingly, the subsequent (third to sixth) stimulations produced essentially no exocytosis from BAPTA-loaded terminals (Fig. 5, F and G). This meant that (i) chelation of cytosolic Ca\textsuperscript{2+} eventually perturbed some stage(s) of the RRP cycle (endocytosis, refilling, or repriming), and (ii) Ca\textsuperscript{2+} was initially protected from BAPTA, suggesting that it came from Ca\textsuperscript{2+} stores. Indeed, HS can induce the release of stored Ca\textsuperscript{2+} (29, 30). To test the hypothesis outlined in point ii, we used thapsigargin, which empties certain Ca\textsuperscript{2+} stores. This drug did not deplete RRP or inhibit the first round of HS-induced AA release (Fig. 5, H and I) (9). However, thapsigargin abolished release in all subsequent rounds of sucrose application (Fig. 5, H and I), indicating that the depletion of Ca\textsuperscript{2+} stores completely prevented RRP recycling in the absence of Ca\textsuperscript{2+}.

Using Fura-2-loaded synaptosomes, we directly demonstrated that hypertonicity induced a thapsigargin-sensitive increase in [Ca\textsuperscript{2+}] (Fig. 6, A and B). The use of this ratiometric dye compensated for any sucrose-induced changes in the volume of individual synaptosomes and thus cytosolic dye concentration (see “Experimental Procedures” and Ref. 17). Additionally, the sensitivity to thapsigargin confirmed the specificity of the effects observed. Although the HS-induced increase in complexes. Therefore, to prove the physiological character of the secretion observed in synaptosomes, we assessed its dependence on ATP. A 10-fold reduction in synaptosomal ATP levels (using rotenone and iodoacetic acid (27)) only marginally affected the first round of HS-induced AA release, but progressively inhibited secretion during additional rounds of stimulation such that all release ceased after four to five rounds (Fig. 5, D and E). This inhibitory effect of ATP depletion was activity-dependent because the first round of stimulation at the beginning of the experiment (R1) and at the end (R1 control) triggered the same release (Fig. 5, A, D, and E), indicating that ATP was specifically required for RRP recycling.
[Ca\(^{2+}\)]

was apparently small (Fig. 6A), it could represent a substantial local increase in [Ca\(^{2+}\)]. Furthermore, after its release, cytosolic Ca\(^{2+}\) must be quickly taken up by the same stores (possibly via the rapid release-activated Ca\(^{2+}\) transport mechanism (31)) because subsequent stimulations caused similar elevations of [Ca\(^{2+}\)] (Fig. 6B). These results led us to test whether the exocytosis triggered in our experiments was actually induced by the release of Ca\(^{2+}\) from intracellular stores. However, when synaptosomes were incubated with both BAPTA-AM and thapsigargin, the first round of HS-induced release was still not perturbed (Fig. 6, C and D). Thus, hypertonicity-induced membrane fusion is truly Ca\(^{2+}\)-independent, whereas some other step(s) in the RRP cycle require Ca\(^{2+}\) that is provided by intracellular stores.

_Vesicle Pools Differ in Their Requirement of Phosphoinositides—_Continuous phosphorylation of PIs by PI 4-kinase is necessary for Ca\(^{2+}\)-dependent secretion of norepinephrine, but not for AA release induced by a single application of HS (9, 32). Is this reaction required for the recycling of vesicle pools? Treatment of short-loaded synaptosomes with PAO was previously shown to decrease the resting levels of phosphoinositides by 75–90% due to PI 4-kinase inhibition (32); application of exactly the same conditions to our system did not inhibit six rounds of release (Fig. 7, A and B). Consequently, the recurring release/recycling of RRP vesicles does not require constant phosphorylation of PIs by this lipid kinase. However, we found previously that PI 4-kinase was essential for Ca\(^{2+}\)-dependent exocytosis of AA-containing reserve vesicles (9). To confirm the involvement of this enzyme in mobilization of reserve vesicles, we used the approach shown in Fig. 4 (F–I), in which a 90-s hypertonic stimulation induced the first round of RRP exocytosis, but inhibited the second round unless reserve vesicles had been labeled and given time to convert to the RRP. In the new experiment (shown schematically in Fig. 7C), long-loaded synaptosomes (containing labeled reserve vesicles) were stimulated for 90 s by HS, allowed to recover for 15 min, and then stimulated again. To inhibit PI 4-kinase, some terminals were treated with PAO 15 min prior to the initial HS stimulation. Fig. 7 (D and E) clearly shows that the second round of release elicited by 100 mosM HS was PI 4-kinase-dependent, suggesting that reserve vesicles need a physiological level of PIs to enter the RRP (see also “Discussion”).

_Vesicle Pools Differ in Their Requirement of the Actin Cytoskeleton—_PI 4,5-bisphosphate interacts with the cytoskeleton (e.g. Ref. 33), and the requirement of PIs for mobilization of reserve vesicles might reflect a role for actin. To test whether intact actin microfilaments are necessary for the transfer of reserve vesicles, we employed a protocol similar to that used in the PAO experiment (Fig. 7C), except that an actin-depolymerizing agent (latrunculin A) was added 60 min prior to the first stimulus. It is clear from Fig. 7F that the first round of release (R1) induced by HS was not perturbed by the disassembly of actin microfilaments. However, release in the second round (R2) was blocked by latrunculin A (Fig. 7F). In accordance with previous findings (34–36), this indicates that intact actin microfilaments are required for mobilization of reserve vesicles, but not for RRP exocytosis, further illustrating the distinction between the two pools of vesicles.

**RRP Inhibition by Prolonged Hypertonic Stimulation**—Although 3- and 90-s HS exposures stimulated the RRP equally (Fig. 4, B and C), this pool regenerated in <30 s following the short stimulation, but did not regenerate even 15 min after the longer treatment. One reason could be that, in the latter case, exocytosed vesicles were retrieved into a non-recycling pool. Alternatively, the long exposure could completely prevent endocytosis. Indeed, hypertonicity is known to inhibit both clathrin-dependent (e.g. Ref. 37) and clathrin-independent (e.g. Refs. 38 and 39) endocytosis, including the internalization of exocytosed secretory vesicles (e.g. Ref. 40). However, reversal to isotonic conditions should restart endocytosis (37). We tested this by measuring FM 2-10 uptake by terminals exposed to 100 mosM HS for 3 or 90 s and then diluted to isotonicity (Fig. 8A). Although the dye can be endocytosed with vesicles of any type, the majority of cerebrocortical synaptosomes are glutamatergic or GABAergic (e.g. Refs. 7 and 41), allowing one to correlate the dye uptake with vesicle retrieval after AA release. As shown in Fig. 8B, 3- and 90-s exposures to HS induced equal uptake of FM 2-10, consistent with identical amounts of exocytosis occurring during these stimulation times (Fig. 4, B and C). Thus, the prolongation of hypertonic exposure did not block the eventual retrieval of exocytosed RRP vesicles, but probably directed them into a separate pool.

To compare the pathways of endocytosis and recovery of the RRP and reserve pool, some terminals were also exposed for 90 s to K\(^+\)/Ca\(^{2+}\) (see “Experimental Procedures”), which causes the release of both pools (e.g.}
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FIGURE 8. Different endocytic pathways revealed by FM 2-10 uptake and release. A, experimental protocol. Shown is the loading of synaptosomes with FM 2-10 optimized for maximal dye uptake into vesicle pools sensitive to all stimuli used. In specific dye uptake into synaptosomes induced by 3- and 90-s HS stimulations and by K+/-Ca2+/-C and D, release of FM 2-10 from terminals that were stimulated for uptake by K+/-Ca2+/-C or various stimuli (D) and then induced for release by various stimuli (C) or by HS (D). AFU, arbitrary fluorescence units.

Ref. 9). This should lead to subsequent endocytosis and labeling with the dye of both the RRP and reserve pool. As expected, K+/-Ca2+/- induced a larger dye uptake than did 100 mosM HS (Fig. 8B). Synaptosomes loaded with FM 2-10 by these methods were then stimulated for release by (i) K+/-Ca2+/-, (ii) 100 mosM HS, or (iii) 5 nM LTX (via its receptor-mediated mechanism (9)).

In K+/-Ca2+/--loaded synaptosomes (Fig. 8C), HS and LTX produced quantitatively and kinetically identical FM 2-10 destaining, achieving a maximal dye release within 15–30 s of the stimulus onset. No further exocytosis occurred over the following 2 min of stimulation, providing further evidence that these two stimuli act upon the RRP only (9) and that K+/-Ca2+/- also labels this pool. Exocytosis triggered by K+/-Ca2+/- was greater and consisted of two components: fast initial release phase “1,” followed by a slow but prolonged phase “2.” The two components had properties characteristic of the RRP and reserve pool, respectively (also seen in other systems (2, 42)).

It should be emphasized that not all dye taken up was released by stimulation (Fig. 8) (e.g. Refs. 2, 42, and 43), indicating that some FM dye may bind to membrane structures distinct from synaptic vesicles. However, only the specific dye uptake and release were used here to determine the parameters of vesicle recycling, fully justifying the interpretation of our results. Another noteworthy point is that because the FM dye is bound to the vesicle membrane, it requires more time to depolarize and leave the vesicle than the freely dissolved AA (e.g. Ref. 44), enabling one to use changes in FM fluorescence to detect exocytosis/endocytosis, but giving only a limited measure of the kinetics of neurotransmitter release.

In a reverse experiment (Fig. 8D), FM 2-10 loading was induced by various stimuli, whereas release was triggered by HS (as a measure of the RRP recovery). In synaptosomes loaded by a 3-s HS exposure in which only the RRP had been released and then labeled, a similar amount of dye release was induced by HS and K+/-Ca2+/- (data not shown). In terminals in which both pools had been labeled using K+/-Ca2+/-, hypertonicity also caused the same destaining (quantitatively and kinetically) as in terminals loaded by a 3-s HS exposure (Fig. 8D). This was consistent with hypertonicity affecting only the RRP and not the reserve pool (both when loading and releasing the dye). These data together indicate that, when RRP exocytosis is induced by a short hypertonic exposure or by membrane depolarization (K+/-Ca2+/-), most endocytosed vesicles return to the RRP.

Although 90-s HS stimulations induced the same AA exocytosis and dye endocytosis as 3-s HS exposures (Figs. 4, B and C; and 8B), vesicles that took up FM 2-10 after the longer hypertonic period were insensitive both to hypertonicity (Fig. 8D) and to K+/-Ca2+/- (data not shown). This was consistent with only fractional RRP recovery after a 90-s hypertonic stimulation (Fig. 4, F and G). Thus, long HS exposures directed most vesicles to an alternative endocytotic pathway that did not lead to the RRP or reserve pool. Furthermore, those few RRP vesicles that remained unperturbed by the first 90-s HS exposure and contributed to the residual AA release (Fig. 4, F and G) did not contain/release the dye (Fig. 8D). These vesicles were thus internalized by a mechanism that prevented dye uptake via the fusion pore, but not AA loading from the cytosol (see “Discussion”). This is reminiscent of a kiss-and-run mode in which the fusion pore is too small or short-lived for the dye to enter/exit the vesicle freely (see Ref. 45).

DISCUSSION

When the complete release of the RRP is explored, biochemical measurements in isolated nerve terminals give results very similar to those obtained by electrophysiological studies in neuronal cultures, despite the difference in temporal resolution of these techniques. Our measurements have shown that exocytosis of the whole RRP of Glu- and GABA-containing vesicles occurs within 3 s after HS stimulation (Fig. 2), and the same time (3–4 s) has been determined electrophysiologically in autapses (e.g. Ref. 46). Likewise, synaptosomes faithfully reproduce the slower kinetics of FM 2-10 release (20–30 s) (Fig. 8), as also observed in cultured neurons (e.g. Ref. 47). The RRP in synaptosomes recovers in <30 s (probably much faster) after an initial first 3-s hypertonic stimulation (Fig. 4); similarly, the recovery time for this pool in autapses is ~10 s (11, 46). Hypertonicity-induced exocytosis occurs in the absence of Ca2+/-, in both autapses and synaptosomes. Furthermore, in both systems, depletion of Ca2+/- stores by thapsigargin and/or chelation of cytosolic Ca2+/- by BAPTA does not affect the first round of RRP exocytosis (Figs. 5 and 6) (11). This hypertonicity-sensitive pool can also be released in a Ca2+/--dependent manner by electrical stimulation (11) or by LTX or high [K+/-] (Fig. 8) (9).

On the other hand, synaptosomes are more sensitive to hypertonicity (Fig. 1) compared with neurons in tissues and cell cultures (11). It has been proposed that, by shrinking terminals, hypertonic stimulation brings RRP vesicles close to the plasma membrane (e.g. Ref. 11) and/or increases intracellular concentrations of cations, leading to massive spontaneous vesicle fusion. In agreement with this view, electron microscopy analysis demonstrates that even low hypertonicity causes some shrinkage of synaptosomes (Fig. 2). In tissues and cell cultures, in which synaptic connections, surrounding cells, and substrate lend mechanical support to nerve terminals, relatively high sucrose concentrations may be required to produce deformation sufficient for RRP exhaustion.

Thus, experiments with isolated nerve terminals from mature brain can effectively complement electrophysiological and imaging techniques, provided care is taken to ascertain that the observed release is
vesicular. Being highly reproducible and routinely successful, synapto
tosomal experiments have led us to several novel fundamental
observations.

We have found that the recycling of the RRP and mobilization of
reserve vesicles were almost identical in glutamatergic and GABAergic
synaptosomes. The RRP in mature cerebrocortical terminals underwent
multiple cycles of release without any change in pool size (Fig. 5, B and C). Notably, similar to vesicles in frog peripheral nerves (43), this did not
require mobilization of reserve vesicles (Figs. 4, F and G; and 7, A and B).

We have also discovered that the recurrent RRP recycling in synap-
tosomes required Ca$^{2+}$ (Fig. 5, F and G). (RRP recycling was not previ-
ously tested in the presence of an intracellular Ca$^{2+}$ chelator (11).) Our
results show that, in the constant absence of Ca$^{2+}$, this cation can be
provided in terminals by thapsigargin-sensitive Ca$^{2+}$ stores. Similarly,
*Drosophila* larval neuromuscular junctions contain a vesicle pool that
depends on Ca$^{2+}$, supplied by thapsigargin-sensitive stores (48). Such
stores may play a vital role in vesicle recycling even in the presence of
Ca$^{2+}$. Our experiments directly demonstrate that hypertonicity raises
cytosolic [Ca$^{2+}$] (Fig. 6). Ca$^{2+}$ may briefly exit the storage compartment
and prime vesicles for exocytosis/endocytosis, as happens with fusion of
yeast vacuoles (49). Interestingly, under non-chelating conditions, the
removal of a hypertonic stimulus apparently allows a very fast re-entry
of released Ca$^{2+}$ into the same stores; indeed, subsequent hypertonic
stimulations induce similar increases in cytosolic [Ca$^{2+}$] (Fig. 6).

As the RRP recycles very quickly after a short hypertonic stimulation
in both synaptosomes and autapses (Fig. 4, B and C) (11, 26), it must
undergo rapid endocytosis. Such a mechanism has been detected in
chromaffin cells (50) and may be present in nerve terminals (e.g. Ref. 2).
This recycling is unlikely to involve the clathrin-dependent pathway
because it is insensitive to PAO, which inhibits endocytosis of clathrin-
coated vesicles (see Refs. 42 and 51–53). Furthermore, clathrin-depend-
ent (but not clathrin-independent) internalization requires actin (e.g.
Ref. 54), and the insensitivity of the RRP to latrunculin suggests that its
recycling may not involve clathrin.

Interestingly, although long HS exposures do not block endocytosis
permanently, they prevent ~70% of vesicles from recycling into the RRP
(Figs. 4 and 8). The likely explanation is that, under hypertonic condi-
tions known to inhibit endocytosis (37–40), components of exocytosed
vesicles can migrate laterally in the plasma membrane (e.g. Ref. 55) and,
upon return to isotonicity, become endocytosed by a distinct pathway
that does not lead directly to the reserve pool (Fig. 8). A 3-s sucrose
application is apparently too short for such lateral diffusion to occur,
permitting subsequent endocytosis at the active zones and full RRP
recovery; conversely, a 90-s sucrose exposure may be sufficient for ves-
icle components to diffuse away, resulting in RRP depletion. Obviously,
this mechanism can affect only those vesicles that fuse completely with
the plasma membrane.

Once these fully fused RRP vesicles are removed from their normal
position, reserve vesicles can be mobilized to the RRP (Fig. 4). Because
this process is PI 4-kinase-dependent (PAO-sensitive) (Fig. 7, D and E),
phosphoinositides produced by this kinase may be essential for mobiliza-
tion of reserve vesicles. In agreement with these data, PI 4-kinase
activity has also been implicated in the priming of secretory granules
and their transfer to the RRP (56). This may involve the interaction of
phosphoinositides with the actin cytoskeleton (e.g. Ref. 33). The impor-
tant role of actin microfilaments in mobilization of reserve vesicles (but
not the RRP) is supported by our results with latrunculin A (Fig. 7F).
A previous study has claimed that hypertonicity does not block the
recycling of RRP vesicles because low-level exocytotic activity continues
for 5 min in cultured hippocampal neurons exposed to 500 mosm
sucrose (4). However, vesicle recycling in shrunken nerve terminals
could hardly be normal, and hypertonic overstimulation for extended
time periods could lead to persistent recruitment of reserve vesicles.
This notion was corroborated by the continuous release in those exper-
iments of FM dye that had been reloaded into both the RRP and reserve
vesicles. Indeed, high frequency electrical stimulation of hippocampal
cells (a method normally used to trigger the RRP) leads to gradual mobi-
ization of reserve vesicles (57).

In our experiments, prolonged hypertonic exposure also reveals a second,
distinct mode of RRP exocytosis. Although a 90-s sucrose stimula-
tion of short-loaded synaptosomes inhibits the RRP recovery, ~30% of
the normal AA release still occurs upon further stimulation (Fig. 4, F
and G), even when mobilization of reserve vesicles is prevented by PAO
(Fig. 7, D and E). Because these persistent RRP vesicles do not take up
the FM dye (Fig. 8D), they must undergo only a momentary exocytotic
process that does not lead to their complete fusion with the plasma
membrane (see Ref. 45), and this may explain why such vesicles are
resistant to lateral diffusion during sucrose exposure. These features are
suggestive of a kiss-and-run mechanism (see Ref. 14). If so, then the
complete kiss-and-run cycle does not need the normal level of phos-
phoinositides, but requires an elevation of [Ca$^{2+}$], because cytosolic
BAPTA and thapsigargin completely block all recycling (Fig. 5, F–I).
Thus, our data indicate that, upon hypertonic stimulation, ~30% of the
vesicles in cortical synapses participate in the kiss-and-run type of
exocytosis.

Future studies using the model described here can now address those
stages of the vesicle cycle that require Ca$^{2+}$. It would also be interesting
to characterize the different modes of endocytosis induced by hyper-
tonic sucrose and other stimuli and to determine the role of clathrin
in this process. The involvement of actin microfilaments and PI 4,5-
bisphosphate in the interchange between vesicle pools must be thor-
oughly investigated. Finally, additional studies will be necessary to
explain the differential loading of the readily releasable and reserve ves-
icles with cytosolic neurotransmitters and to compare the biochemical
properties of vesicles that belong to distinct subpopulations.

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REFERENCES

1. Elmqvist, D., and Quastel, D. M. (1965) J. Physiol. (Lond.) 178, 505–529
2. Pyle, J. L., Kavalali, E. T., Piedras-Renteria, E. S., and Tsien, R. W. (2000) Neuron 28, 221–231
3. Harata, N., Pyle, J. L., Aravanis, A. M., Mozhayeva, M., Kavalali, E. T., and Tsien, R. W. (2001) Trends Neurosci. 24, 637–643
4. Sara, Y., Mozhayeva, M. G., Liu, X., and Kavalali, E. T. (2002) J. Neurosci. 22, 1608–1617
5. Bradford, H. F. (1986) *Chemical Neurobiology: An Introduction to Neurochemistry*, W. H. Freeman & Co., New York
6. Whittaker, V. P. (1993) *J. Neurocytol.* 22, 735–742
7. Nicholls, D. G. (1993) *Eur. J. Biochem.* 212, 613–631
8. Hano, T., Jeng, Y., and Rho, J. (1989) *Hypertension* 13, 250–255
9. Astton, A. C., Volynski, K. E., Lelianova, V. G., Orlova, E. V., Yan Renterghem, C., Canepari, M., Seagar, M., and Uszkaryov, Y. A. (2001) *J. Biol. Chem.* 276, 44695–44703
10. Khvotchev, M., Lonart, G., and Sudhof, T. C. (2000) *Neuroscience* 101, 793–802
11. Rosenmund, C., and Stevens, C. F. (1996) Neuron 16, 1197–1207
12. Ashton, A. C., and Dolly, J. O. (2000) *J. Neurochem.* 74, 1979–1988
13. Oja, S. S., and Kontro, P. F. (1988) *Neurochem. Res.* 13, 923–928
14. Gandhi, S. P., and Stevens, C. F. (2003) *Nature* 423, 607–613
15. Cousin, M. A., and Robinson, P. J. (2000) *J. Neurosci.* 75, 1653–1653
16. Richards, D. A., Guatimosim, C., and Betz, W. J. (2000) *Neuron* 27, 551–559
17. Grynkiewicz, G., Pienoe, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* 260, 3440–3450
18. Leenders, A. G., Scholten, G., de Lange, R. P., Lopes da Silva, F. H., and Ghijsen, W. E. (2002) *Neuroscience* 109, 195–206
Synaptic Vesicle Pools in Mature Central Terminals

19. Rosenmund, C., and Stevens, C. F. (1997) J. Neurosci. Methods 76, 1–5
20. Zhou, Q., Petersen, C. C., and Nicoll, R. A. (2000) J. Physiol. (Lond.) 525, 195–206
21. Capogna, M., Volynski, K. E., Emptage, N. J., and Ushkaryov, Y. A. (2003) J. Neurosci. 23, 4044–4053
22. Stevens, C. F., and Sullivan, J. M. (1998) Neuron 21, 885–893
23. Bykhovskaia, M., Polagaeva, E., and Hackett, J. T. (2001) J. Physiol. (Lond.) 537, 179–190
24. Wu, X. S., and Wu, L. G. (2001) J. Neurosci. 21, 7928–7936
25. Suzuki, K., Grinnell, A. D., and Kidokoro, Y. (2002) J. Physiol. (Lond.) 538, 103–119
26. Stevens, C. F., and Wesseling, J. F. (1999) Neuron 24, 1017–1028
27. Kauppinen, R. A., McMahon, H. T., and Nicholls, D. G. (1988) Neuroscience 27, 175–182
28. Lonart, G., and Sudhof, T. C. (2000) J. Biol. Chem. 275, 27703–27707
29. Brosius, D. C., Hackett, J. T., and Tuttle, J. B. (1992) J. Neurophysiol. (Bethesda) 68, 1229–1234
30. Chawla, S., Skepper, J. N., Hockaday, A. R., and Huang, C. L. (2001) J. Physiol. (Lond.) 536, 351–359
31. Cseresnyes, Z., Bustamante, A. I., Klein, M. G., and Schneider, M. F. (1997) Neuron 19, 403–419
32. Khvotchev, M., and Sudhof, T. C. (1998) J. Physiol. (Lond.) 525, 579–586
33. Richards, D. A., Rizzoli, C., and Betz, W. J. (2003) J. Biol. Chem. 278, 403–404
34. Chawla, S., Skepper, J. N., Hockaday, A. R., and Huang, C. L. (2001) J. Physiol. (Lond.) 536, 351–359
35. Bitsch, M., and Sudhof, T. C. (2000) J. Biol. Chem. 275, 27703–27707
36. Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G., and Shetzline, M. A. (1999) J. Biol. Chem. 274, 31515–31523
37. Werbonat, Y., Kleutges, N., Jakobs, K. H., and van Koppen, C. J. (2000) J. Biol. Chem. 275, 21969–21974
38. Troyer, K. P., and Wightman, R. M. (2002) J. Biol. Chem. 277, 29101–29107
39. Wilkinson, R. J., and Nicholls, D. G. (1989) Neurochem. Int. 15, 191–197
40. Di Paolo, G., Sankaranarayanan, S., Wenk, M. R., Daniell, L., Perucco, E., Caldarone, B. J., Flavell, R., Picciotto, M. R., Ryan, T. A., Cremona, O., and De Camilli, P. (2002) Neuron 33, 789–804
41. Richards, D. A., Gnutimosim, C., Rizzoli, S. O., and Betz, W. J. (2003) Neuron 39, 529–541
42. Smith, S. M., Bergman, J. B., Harata, N. C., Scheller, R. H., and Tsien, R. W. (2004) Neuron 41, 243–256
43. Richards, D. A., Bai, J., and Chapman, E. R. (2005) J. Cell Biol. 168, 929–939
44. Stevens, C. F., and Wesseling, J. F. (1998) J. Neurosci. 21, 415–424
45. Schoch, S., Deak, F., Konigstorfer, A., Mozhaeva, M., Sara, Y., Sudhof, T. C., and Kavalali, E. T. (2001) Science 294, 1117–1122
46. Richards, D. A., Guatimosim, C., Rizzoli, S. O., and Betz, W. J. (2003) Neuron 39, 529–541
47. Kuromi, H., and Kidokoro, Y. (2002) Neuron 35, 333–343
48. Peters, C., and Mayer, A. (1998) Nature 396, 575–580
49. Artalejo, C. R., Henley, J. R., McNiven, M. A., and Palfrey, H. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8328–8332
50. Cremona, O., and De Camilli, P. (2001) J. Cell Sci. 114, 1041–1052
51. Gibson, A. E., Noel, R. J., Herlihy, J. T., and Ward, W. F. (1989) Am. J. Physiol. 257, C182–C184
52. Lee, M. C., Cahill, C. M., Vincent, J. P., and Beaudet, A. (2002) Synapse 43, 102–111
53. Moskowitz, H. S., Heuser, J., McGraw, T. E., and Ryan, T. A. (2003) Mol. Biol. Cell 14, 4437–4447
54. Gad, H., Low, P., Zottova, E., Brodin, L., and Shupliakov, O. (1998) Neuron 21, 607–616
55. Olsen, H. L., Hoy, M., Zhang, W., Bertorello, A. M., Bokvist, K., Capito, K., Efanson, A. M., Meister, B., Thams, P., Yang S. N., Rorsman, P., Beggren, P. O., and Gromada, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5187–5192
56. Otus, Y., and Murphy, T. H. (2004) J. Neurosci. 24, 9076–9086