Synaptic Vesicle Distribution and Release at Rat Diaphragm Neuromuscular Juncitons

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Rowley KL, Mantilla CB, Ermilov LG, Sieck GC. Synaptic vesicle distribution and release at rat diaphragm neuromuscular junctions. J Neurophysiol 98: 478–487, 2007. First published May 9, 2007; doi:10.1152/jn.00251.2006. Synaptic vesicle release at the neuromuscular junction (NMJ) is highly reliable and is vital to the success of synaptic transmission. We examined synaptic vesicle number, distribution, and release at individual type-identified rat diaphragm NMJ. Three-dimensional reconstructions of electron microscopy images were used to obtain novel measurements of active zone distribution and the number of docked synaptic vesicles. Diaphragm muscular nerve preparations were used to perform electrophysiological recordings. We measured the rate of decline in quantal content (QC) during repetitive phrenic nerve stimulation. The number of synaptic vesicles available for release vastly exceeds those released with a single stimulus, thus reflecting a relatively low probability of release for individual docked vesicles and at each active zone. There are two components that describe the decline in QC resulting from repetitive stimulation: a rapid phase (<0.5 s) and a delayed phase (<2.5 s). Differences in the initial rapid decline in QC were evident across type-identified presynaptic terminals (fiber type classification based on myosin heavy chain composition). At terminals innervating type IIX and/or IIB fibers, the initial decline in QC during repetitive stimulation matched the predicted depletion of docked synaptic vesicles. In contrast, at terminals innervating type I or IIA fibers, a faster than predicted decline in QC with repetitive stimulation suggests that a decrease in the probability of release at these terminals plays a role in addition to depletion of docked vesicles. Differences in QC decline likely reflect fiber-type specific differences in activation history and correspond with well-described differences in neuromuscular transmission across muscle fiber types.

INTRODUCTION

Synaptic vesicle release at the neuromuscular junction (NMJ) is essential to sustain synaptic transmission. A subset of synaptic vesicles (defined as the “readily releasable” pool, RRP) is docked at active zones and available for release (Richards et al. 2003; Rizzoli and Betz 2004). However, only a fraction of these vesicles are released with each neural impulse. Synaptic vesicles located in close proximity to active zones do not contribute to neurotransmitter release until recruited to the RRP and thus constitute the “cycling” pool (Rosenmund and Stevens 1996; Zucker and Regehr 2002). Recycled vesicles can return to a “cycling” pool of vesicles, the RRP, or to a larger “reserve” pool located further away from the active zones (Richards et al. 2003; Sudhof 2004). Vesicles in the reserve pool are not released unless recruited to the cycling pool or RRP, usually to sustain long-term transmission.

Although much is known about synaptic vesicle pools at mammalian NMJs (Ellisman et al. 1976; Mantilla et al. 2004; Pieribone et al. 1995; Reid et al. 1999; Wilson 1979), their composition. At terminals innervating type IIX and/or IIB fibers, the initial decline in QC during repetitive stimulation matched the predicted depletion of docked synaptic vesicles. In contrast, at terminals innervating type I or IIA fibers, a faster than predicted decline in QC with repetitive stimulation suggests that a decrease in the probability of release at these terminals plays a role in addition to depletion of docked vesicles. Differences in QC decline likely reflect fiber-type specific differences in activation history and correspond with well-described differences in neuromuscular transmission across muscle fiber types.

METHODS

Experiments were conducted on male, Sprague-Dawley rats (n = 24, ~300 g body wt). All experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee and were conducted in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ducted in strict accordance with the American Physiological Society Animal Care guidelines. Animals were killed prior to the terminal experiment by exsanguination under deep ketamine (80 mg/kg) and xylazine (20 mg/kg) intramuscular anesthesia.

**Morphology of the presynaptic terminal**

**CONFOCAL IMAGING OF PRESYNAPTIC TERMINALS.** Methods for imaging presynaptic terminals at type-identified diaphragm muscle fibers have been described previously (Mantilla et al. 2004). Briefly, the diaphragm muscle was excised together with the phrenic nerve and placed in oxygenated Rees-Simpson solution [containing (in mM) 135 Na+, 5 K+, 2 Ca2+, 1 Mg2+, 120 Cl−, and 25 HCO3−] bubbled with 95% O2-5% CO2 at room temperature. The muscle was pinned in a silicon rubber (Sylgard; DowCorning, Midland, MI)-coated dish at −1.5 times resting length (approximate optimal length for force generation). The styryl dye FM4-64 (5 μM; Molecular Probes, Eugene, OR) and Alexa 488-labeled α-bungarotoxin (1 μg/ml; Molecular Probes) were added to the bath to label pre- and postsynaptic elements of the NMJ, respectively. The phrenic nerve was stimulated at 10 Hz (0.5-ms supramaximal pulses with a 67% duty cycle) for a 20-min period using a suction electrode with stimulation driven by an A-M Systems 2100 isolated pulse generator (A-M Systems, Carlsborg, WA). Visible contractions of the diaphragm muscle served to verify successful nerve stimulation in all cases. During stimulation, presynaptic terminals were labeled with FM4-64 as synaptic vesicle release and recycling occurred. After stimulation, excess FM4-64 (not taken up by the synaptic vesicles) was removed by continuous washing of the nerve–muscle preparation with Rees-Simpson solution for 30 min. Thereafter, FM4-64-labeled presynaptic terminals were imaged using an Olympus Fluoview 200 laser-scanning confocal system (Olympus America, Melville, NY) mounted on an upright Olympus BX50WI microscope using a ×40 0.8 NA water-immersion lens (Olympus). The step size of the confocal system was set at 3.0 μm, and a series of optical slices was obtained through the depth of each NMJ. During confocal imaging, both Alexa 488 and FM4-64 fluorescence were excited by illumination at 488 nm using an Argon laser, and fluorescence emission was detected at 510–540 nm (Alexa 488) and 570 nm (FM4-64) using a dichroic filter. Only superficially (≤ 50-μm depth from surface) presynaptic terminals were imaged. In general, >60 presynaptic terminals were examined per diaphragm (both right and left sides). Each image stack consisted of approximately seven image planes (3 μm optical slices) at 12-bit resolution (0.25 μm²/pixel). Image processing and analysis was performed using Metamorph 6.3.1 (Universal Imaging, Downingtown, PA). Each image stack was manually aligned, and maximum intensity projections were obtained. Presynaptic terminals with overlapping axons and/or those not en face with the plane of the optical slice were excluded from further analyses. From the projection image, presynaptic terminals were manually circumscribed making sure that the entire terminal was included. Presynaptic terminals innervating type I or Ia fibers (expressing MHC isoforms MHCslow or MHC2A, respectively) were distinguished from those innervating type IIx and/or IIb fibers (expressing MHC2X or MHC2B, respectively) based on NMJ morphology as previously described (Mantilla et al. 2004; Prakash et al. 1996). The planar area of individual presynaptic terminals was determined from the circumscribed region of interest.

**ELECTRON MICROSCOPY OF PRESYNAPTIC TERMINALS.** The methods for electron microscopy of presynaptic terminals at type-identified diaphragm muscle fibers were described previously (Mantilla et al. 2004). Briefly, animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.6). The diaphragm muscle was excised, stretched to ~1.5 times resting length and postfixed overnight in 2% paraformaldehyde and 2% glutaraldehyde in PBS (both right and left sides of the diaphragm separately). After a wash in PBS, the tissue was incubated with 2% osmium tetroxide, partially dehydrated in a series of graded ethyl alcohols while progressively lowering the temperature to ~20°C, and subsequently infiltrated in 1:1 ethanol LR White resin (Energy Beam Sciences, Agawam, MA) overnight. After this, each specimen was transferred into fresh LR White for 60 min while being brought to room temperature. Specimens were then embedded in fresh LR White while allowing for polymerization at 55°C for 2–3 days. Thin sections of diaphragm muscle (100 nm) were obtained by ultramicrotomy and mounted on nickel grids to dry. Specimen grids were then visualized using a Jeol 1200 transmission electron microscope (JEOL-USA, Peabody, MA) at 60 kV.

**ACTIVE ZONE SIZE AND DISTRIBUTION.** All sections were screened for NMJs using low-magnification (×5,000–7,000). For each NMJ, digital images were obtained at ×25,000 and imported into a comprehensive image analysis program (AnalyzeAVW, Mayo Foundation) for 3-D reconstruction (Robb et al. 1989). Individual NMJs were classified as innervating type I or Ia fibers versus type IIx and/or IIb fibers based on morphology as previously described (Fahim et al. 1984; Mantilla et al. 2004). Consecutive digital images for each NMJ were manually aligned, oriented, and placed into a stack after visual registration. On average, a given presynaptic terminal was contained in 35–45 serial sections. Pixel dimensions were 5.26 nm in the x-y axis. Voxel dimensions were considered to be isometric. Specimen thickness was used to set image spacing so that individual images were then 5.5 × 5.5 μm (x and y axes) by 0.1 μm (z axis). Object maps were then created by manually segmenting pre- and postsynaptic elements, active zones and Schwann cells. Each NMJ was rendered in 3-D using linear interpolation between slices. Object maps for the Schwann cells and presynaptic terminals were digitally removed to facilitate visualization of active zones (Fig. 1). The length of each active zone was digitally traced and measured within individual slices. Using a nearest neighbor approximation, the distance between individual active zones was measured in 3-D, and the average distance between active zones was then calculated for each terminal.

**NUMBER OF SYNAPTIC VESICLES IN THE RRP AND THE CYCLING POOL.** At identified active zones, the number of docked synaptic vesicles were manually counted (i.e., the RRP). The total number of synaptic vesicles in the RRP was estimated using a method previously developed by Schikorski and Stevens (2001), but because active zones were usually only found in two adjacent serial sections, a factor of 2 was used to account for the 3-D distribution of synaptic vesicles at each active zone. Synaptic vesicles in close proximity to the active zone (within 200 nm) were also counted after creating a region of interest in individual digital images using Metmorph. These counts were used to determine the number of vesicles in the cycling pool by multiplying the number of active zones per terminal by the mean number of vesicles surrounding each active zone (assuming a correction factor of 3 to account for the depth of the active zone and surrounding vesicle pool in 3-D).

**Electrophysiological measurements of synaptic events**

The methods for recording miniature and evoked end-plate potentials (mEPP and EPP, respectively) at diaphragm muscle fibers have been previously described (Fournier et al. 1991). Briefly, the diaphragm muscle together with the phrenic nerve were dissected, overstretched beyond optimal sarcomeric length (~2× resting length) and pinned to the bottom of a silicon rubber-coated dish containing Rees-Simpson solution bubbled with 95% O2-5% CO2 kept at room temperature. Presynaptic terminals were visualized by incubating the diaphragm muscle-phrenic nerve preparation in 5 μM FM4-64 while stimulating at 10 Hz for 20 min using a suction electrode as described in the preceding text. The preparation was then washed in Rees-Simpson solution containing 6 μM μ-conotoxin GiIIb (Bachem California, Torrance, CA) to prevent muscle contraction and allowed...
to rest for 30 min before electrophysiological recordings were obtained. Superficial en face presynaptic terminals labeled with FM4-64 were visualized at 16-bit resolution using a Photometrics Cascade: 512F cooled charge-couple device camera (Photometrics-Roper Scientific, Tucson, AZ) mounted on an Olympus BX51WI microscope equipped with a high-speed DramV illuminator (PTI). Preparations were illuminated at 488 ± 5 nm using a neutral density filter to decrease intensity by ~95%. Emitted fluorescence was detected at >570 nm. Individual presynaptic terminals innervating type I or IIa versus type IIx and/or IIb fibers were identified based on morphological differences as previously described (Mantilla et al. 2004; Prakash et al. 1996), and recordings were obtained at selected terminals to obtain a sample of both groups within a single diaphragm preparation. A borosilicate glass micropipette filled with 3 M KCl (15–20 MΩ) was inserted into the muscle fiber at the very edge of the identified presynaptic terminal (usually within 2 μm). Direct visualization of the end-plate with a ×60 lens allowed consistent placement of the micropipette tip. Therefore any differences in membrane input resistance that may interfere with the individual recordings were considered too small to affect mEPP amplitude. Recordings were obtained using a Duo 773 electrometer (World Precision Instruments, Sarasota, FL), digitally stored on a Pentium 5 computer and processed using AxoScope 9.2 software (Axon Instruments-Molecular Devices, Union City, CA). Initial resting membrane potentials were more negative than ~60 mV and remained stable throughout the experiment. Amplitudes and frequency of spontaneous mEPPs were determined over a 10-min period. Thereafter, evoked EPPs were measured during repetitive supramaximal phrenic nerve stimulation at 20 or 50 Hz (0.5-ms pulse duration).

Statistical analysis

All comparisons across groups of type-identified presynaptic terminals (innervating type I or IIa vs. type IIx and/or IIb muscle fibers) were performed using one-way ANOVA. Differences were analyzed post hoc using the Tukey-Kramer honestly significant difference test using JMP 6.0 (SAS Institute, Cary, NC) when appropriate. Statistical significance was established at the 0.05 level. All experimental data are presented as means ± SE unless otherwise specified.

RESULTS

Fiber type-specific differences in surface area of presynaptic terminals and FM4-64 uptake during repetitive stimulation

Presynaptic terminals were labeled with FM4-64 throughout the muscle, and excellent visualization of presynaptic structures was evident. As previously reported, some axon staining remained after washing (likely due to dye seeping into the thick myelin sheath), yet minimal staining of the muscle fiber membrane occurred. Diaphragm presynaptic terminals present at type I or IIa fibers (fiber type based on MHC composition) and at type IIx and/or IIb fibers were also readily identified according to reported differences in terminal morphology (Mantilla et al. 2004; Prakash et al. 1996). NMJs at type I or IIa fibers are more compact and less complex than those at type IIx and/or IIb fibers. On average, ~15 type-identified NMJs were sampled per hemidiaphragm (n = 12 hemidiaphragms for these measurements). Presynaptic terminals showed expected fiber type differences in size (planar surface area) with those NMJs at type I or IIa fibers (n = 78) having significantly smaller surface area compared with those at type IIx and/or IIb (n = 60) fibers (Table 1; P < 0.05). FM4-64 uptake was heterogeneous within individual terminals with punctate areas of greater brightness (likely active zones). Average FM4-64 fluorescence intensity was consistently higher at terminals innervating type I or IIa fibers compared with those innervating type IIx and/or IIb fibers (Mantilla et al. 2004).

Active zone distribution at presynaptic terminals

Using transmission electron microscopy, individual synaptic terminals were clearly identified and classified as either present at type I or IIa fibers versus type IIx and/or IIb fibers according to reported differences in terminal morphology (Mantilla et al. 2004; Prakash et al. 1996). NMJs at type I or IIa fibers are more compact and less complex than those at type IIx and/or IIb fibers. On average, ~15 type-identified NMJs were sampled per hemidiaphragm (n = 12 hemidiaphragms for these measurements). Presynaptic terminals showed expected fiber type differences in size (planar surface area) with those NMJs at type I or IIa fibers (n = 78) having significantly smaller surface area compared with those at type IIx and/or IIb (n = 60) fibers (Table 1; P < 0.05). FM4-64 uptake was heterogeneous within individual terminals with punctate areas of greater brightness (likely active zones). Average FM4-64 fluorescence intensity was consistently higher at terminals innervating type I or IIa fibers compared with those innervating type IIx and/or IIb fibers (Mantilla et al. 2004).

**TABLE 1.** Measurements and calculated values resulting from the 2- and 3- dimensional electron microscopy images at type-identified rat diaphragm neuromuscular junctions

<table>
<thead>
<tr>
<th></th>
<th>Type I and IIa</th>
<th>Type IIx and/or IIb</th>
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<tbody>
<tr>
<td>Presynaptic terminal area, μm²</td>
<td>398 ± 11</td>
<td>712 ± 22*</td>
</tr>
<tr>
<td>Number of docked vesicles per active zone</td>
<td>3.01 ± 0.16</td>
<td>3.14 ± 0.20</td>
</tr>
<tr>
<td>Distance between active zones, μm</td>
<td>0.99 ± 0.06</td>
<td>1.16 ± 0.11</td>
</tr>
<tr>
<td>Number of active zones per terminal</td>
<td>231</td>
<td>455*</td>
</tr>
<tr>
<td>Size of readily releasable pool</td>
<td>1385</td>
<td>2728*</td>
</tr>
<tr>
<td>Presynaptic terminal area occupied by active zones, %</td>
<td>8.5</td>
<td>9.4</td>
</tr>
</tbody>
</table>

All values are means ± SE. *, statistically significant difference from terminals innervating type I or IIa diaphragm muscle fibers (P < 0.05).
to previously described morphological characteristics (Fahim et al. 1984; Mantilla et al. 2004). Nine presynaptic terminals were completely reconstructed in 3-D from serial electron microscopy images (5 terminals at type I or IIa fibers and 4 at type IIx and/or IIb fibers; Fig. 1). Synaptic structures could be easily identified and active zones were clearly visualized as dense areas surrounded by synaptic vesicles (Fig. 2). Each presynaptic terminal contained multiple active zones. The average distance between active zones was \( \sim 1 \) \( \mu \)m and was not significantly different across fiber types \( (P > 0.05) \); Table 1).

To determine the number of active zones per terminal, we assumed an even (triangle-based) distribution across the entire presynaptic terminal surface area (Fig. 3). Each side of the equilateral triangles was set to be equal to the average distance between active zones as measured in the 3-D reconstructions. We assumed equal distribution of active zones throughout the terminal by placing the active zones (as indicated by the red dots) at each vertex of each triangle but did not allow for any active zones on the surface borders because no active zones were found at this morphological interface. Although active zones are not found equally spaced throughout the terminal, the number of active zones per terminal is dependent on the surface area of the terminal. Therefore the calculated average number of active zones per terminal was lower at terminals innervating type I or IIa fibers (range: 171–324; 95% confidence interval, CI) than at those innervating type IIx and/or IIb fibers (range: 343–627; 95% CI; Table 1).

**FIG. 2.** Two-dimensional electron microscopy image of a rat diaphragm NMJ showing specific areas of interest including active zones and docked synaptic vesicles. Dense areas aligned with junctional folds across the synaptic cleft identify active zones. The size of the cycling pool was determined by counting the number of synaptic vesicles in the vicinity of an active zone (within 200 \( \mu \)m), whereas the readily releasable pool (RRP) was estimated from the number of docked vesicles at active zones (those vesicles in physical contact with the dense bar; see METHODS). Bar represents 200 \( \mu \)m.

**FIG. 3.** Representative depiction of the method used to determine the number of active zones per terminal assuming homogenous distribution. The average distance \( (d) \) between active zones was determined from the 3-dimensional (3-D) reconstruction of an NMJ. Equilateral triangles of side \( d \) were rendered over the presynaptic terminal surface area assuming a triangular distribution of active zones (see METHODS). The number of active zones per terminal was thus calculated assuming equal distribution of active zones throughout the terminal (as indicated by the red dots) while not allowing for any active zones on the surface borders.

**Synaptic vesicle pools at type-identified presynaptic terminals**

Within presynaptic terminals, clear-content vesicles were easily identifiable in the electron microscopy images. Synaptic vesicles were found primarily clustered in close proximity to active zones, yet they were also present throughout the presynaptic terminal.

Vesicles abutting the dense active zone area at the presynaptic terminal membrane were considered to be “docked” (Fig. 2) in agreement with previous studies (Murthy et al. 2001; Schikorski and Stevens 1997). Occasional vesicle fusion events at the terminal membrane were evident, indicating endo- or exocytosis events. When examined in the individual electron microscopy images, the number of docked vesicles per active zone was not significantly different across fiber types (Table 1); approximately three docked vesicles per active zone for terminals at type I or IIa fibers \( (n = 86) \) and type IIx and/or IIb fibers \( (n = 42) \). In agreement with previous observations (Ellisman et al. 1976), the average area of an active zone did not differ across presynaptic terminals \( (0.147 \pm 0.001 \mu m^2) \) for terminals at both type I or IIa fibers and type IIx and/or IIb fibers. Extrapolating from these measurements, we estimated the number of synaptic vesicles in the RRP (i.e., docked vesicles) using a method previously developed by Schikorski and Stevens (2001). The total number of vesicles docked at all active zones within a single terminal (i.e., the RRP) is in the range of 1,029–1,943 (95% CI) in NMJs at type I or IIa fibers and in the range of 2,060–3,765 (95% CI) in those at type IIx and/or IIb diaphragm fibers (Table 1).

The number of vesicles surrounding each active zone differed between presynaptic terminals at the different muscle fiber types, in agreement with a previous report (Mantilla et al. 2004). Within 200 \( \mu \)m of an active zone, there were 27 ± 1 vesicles at terminals innervating type I or IIa fibers versus 23 ± 1 vesicles at those innervating type IIx and/or IIb fibers \( (P < \)
0.05). Based on these values, the size of the cycling pool was estimated to comprise 18,711 vesicles (13,851–26,244; 95% CI) in terminals at type I or IIa fibers and 31,395 vesicles (23,667–43,332; 95% CI) in those at type IIx and/or IIb fibers.

Despite the greater synaptic vesicle density per active zone in terminals at type I or IIa fibers compared with those innervating type IIx and/or IIb fibers, the size of the RRP and cycling pool was dictated primarily by the number of active zones in each terminal, which in itself is a function of terminal surface area (i.e., size).

**Quantal content differs across diaphragm presynaptic terminals**

In the electrophysiology experiments, individual NMJs were visualized after labeling with FM4-64 and classified as present at type I or IIa versus type IIx and/or IIb fibers (see METHODS). Intracellular recordings of mEPP amplitude and frequency as well as EPP amplitude were then obtained at type-identified diaphragm NMJs (n = 5 terminals at type I or IIa fibers; n = 8 terminals at type IIx and/or IIb fibers). mEPP amplitude was larger for terminals present at type I or IIa fibers than for those at type IIx and/or IIb fibers (Table 1; Fig. 4). The rise time was comparable across terminals, suggesting that the differences in mEPP amplitude were not related to differences in postsynaptic receptor kinetics. Importantly, mEPP amplitude or rise time did not differ when measured before or after repeated phrenic nerve stimulation (data not shown); thus no evidence of postsynaptic desensitization was found after repeated activation. mEPP frequency also did not differ across terminals (~200 events/min; Fig. 4).

To determine the QC released per stimulus, EPP amplitudes were divided by mean terminal mEPP amplitude (Fig. 5), after correcting for nonlinear summation (McLachlan and Martin 1981). Assuming that all vesicles released contribute equally to EPP amplitude, the average initial QC for terminals at type I or IIa fibers and at type IIx and/or IIb fibers were 40.0 (±2.2) and 67.0 (±5.0), respectively, (P < 0.05).

**Repetitive stimulation results in a decline in quantal content**

After 10 min of stimulation (assuming no postsynaptic desensitization), the total number of vesicles released was calculated to be 114,067 ± 24,094 and 214,468 ± 22,768 vesicles for terminals at type I or IIa diaphragm fibers and type IIx and/or IIb fibers, respectively. Overall, the decline in QC did not appear to differ between terminals during the entire course of the 10-min period of continuous 20-Hz stimulation (Fig. 5). However, after normalizing to the initial QC for each terminal, a significant difference across fiber types became evident within the first 2.5 s of continuous stimulation (Fig. 5B; inset). The relative change in QC was greater at terminals innervating type I or Ia fibers than at those innervating type IIx and/or IIb fibers during the initial 2.5 s of stimulation.

Only a small fraction of active zones and synaptic vesicles are needed to account for the QC released with each stimulus. For example, even if one vesicle were released from each active zone, only ~15% of all active zones would release during the initial stimulus. After 10 s of stimulation, vesicles would be released from only 8% of all active zones. The maximum number of active zones required to account for the number of vesicles released during repetitive nerve stimulation decreases even further as QC declines. Indeed if the percentage of vesicles released is determined by dividing the number of docked vesicles (i.e., the morphologically defined RRP) by the QC determined electrophysiologically, the initial fraction of vesicles released from the RRP is ~2.9% for terminals at type I or IIa fibers and ~2.5% for terminals at type IIx and/or IIb fibers. Assuming no change in RRP size after 10 s of continuous stimulation (i.e., complete replenishment of the RRP), the percent of docked vesicles released would decrease to ~1.7% for terminals at all fiber types.

**Estimation of quantal decline at type-identified presynaptic terminals**

A stepwise approach to the study of QC decline was used to identify mechanistic differences in the release of synaptic vesicles across fiber types, comparing the results of electrophysiological recordings obtained at type-identified diaphragm NMJs to the decline in QC based on simple assumptions of pool depletion and estimates of synaptic vesicle pool size (Figs. 6–8).

First the depletion of vesicles from the RRP was plotted with pulse number assuming no replenishment of this pool and no

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**FIG. 4.** Comparison of miniature endplate potential (mEPP) amplitude (A) and mEPP frequency (B) at type-identified diaphragm NMJs. Mean mEPP amplitudes are significantly greater for NMJs at type I or IIa fibers (*P < 0.05) than at type IIx and/or IIb fibers (Δ; P < 0.05). However, no significant difference is evident in mEPP frequency across NMJs at the different muscle fiber types. C: representative recording from a NMJ at a type I or IIa rat diaphragm fiber.
change in the probability of release. Presynaptic terminals innervating type IIx and/or IIb fibers displayed an initial decline in QC (over the 1st 0.5 s or 10 pulses) that could be completely accounted for by depletion of the RRP (Fig. 6B).

However, at presynaptic terminals innervating type I or IIa fibers, the observed decline in QC occurred much faster than can be attributed to depletion of vesicles in the RRP, specifically over the initial 0.5 s of continuous 20-Hz stimulation (Fig. 6A). Thus a decrease in the probability of release of synaptic vesicles in the RRP may account for the difference in QC decline across fiber types in the first second of repetitive stimulation. As expected, after 0.5–1.0 s of repetitive stimulation, continued depletion of the RRP would result in a significantly greater decline in QC than was actually observed. Even if depletion of the “cycling pool” (the RRP plus those vesicles located in close proximity to the active zone) was considered (Fig. 6), replenishment from the cycling pool alone cannot support continuous vesicular release. The initial slower rate of QC decline predicted by an unchanging probability of release from the RRP with no repletion of this pool suggests that the cycling pool of synaptic vesicles contributes vesicles to the RRP through recruitment during the first 5 s of repetitive stimulation (especially at terminals innervating type IIx and/or IIb fibers).

Therefore the replenishment and the rate of replenishment of the RRP during repetitive 20-Hz stimulation were estimated from the difference between the predicted depletion of the RRP and the observed QC decline (Fig. 7). A significant difference in the need for vesicle replenishment exists across fiber types: at terminals innervating type IIx and IIb fibers, replenishment must occur within 0.42 s from the onset of stimulation, whereas for terminals at type I or IIa fibers, replenishment is not necessary until 0.96 s from the onset of stimulation. The replenishment rates also differ across fiber type with a higher rate for terminals at type IIx and/or IIb fibers (~298 vesicles/s) than terminals at type I or IIa fibers (~207 vesicles/s).

Finally, the rate of vesicle recycling was estimated based on the difference between the observed rate of QC decline and the rate of QC decline shown in Fig. 7. The maximum number of vesicles released per terminal type over 200 pulses (Fig. 5B) divided by the duration of the pulse train (100 s) gives the rate of vesicle recycling in vesicles/s. The rate of vesicle recycling was significantly higher at terminals innervating type IIx and/or IIb fibers than at terminals at type I or IIa fibers.

FIG. 5. A: average decline in quantal content (QC) over a 10-min period of continuous 20-Hz stimulation at type I or IIa (○) and type IIx and/or IIb terminals (●). A rapid early decline in QC (1st 100 s) at both types of NMJ terminals is followed by a slower (late) decline in released QC (beyond 300 s). Inset: immediate decline in QC occurring within the first 0.5 s. B: relative change in released QC (normalized to the initial for each terminal type) is similar across type I or IIa and type IIx and/or IIb terminals in both the early and late phases of the decline curve. However, fiber type differences are apparent in the immediate decline in QC (<2.5 s; inset), with a greater relative change at terminals innervating type I or IIa fibers compared with those at type IIx and/or IIb fibers (P < 0.05; MANOVA).

FIG. 6. The decline in QC during repetitive 20-Hz stimulation was estimated based on a fixed probability of release from the RRP (●) or from the cycling pool of synaptic vesicles (○) as described in METHODS. A, measured QC data. These estimates of vesicle pool depletion assumed no replenishment of vesicles either by recycling released vesicles or recruitment from a reserve pool. Note that continued vesicle release would result in complete depletion of the RPP in ~10 s and the cycling pool in ~130 s at both types of diaphragm terminals. Interestingly, the change in QC between 0.5 and 5 s parallels the predicted rate of decline based on depletion of the cycling pool at both fiber types, suggesting an important role for vesicle recruitment from this pool in this period. However, beyond this time point, sustained quantal release will depend on additional forms of vesicle replenishment, either from a reserve pool of vesicles, additional vesicle synthesis and/or a variable rate of vesicle recycling.

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describe the decline in QC resulting from repetitive stimulation: a rapid phase (<0.5 s) and a delayed phase (<2.5 s). These novel findings may underlie important fiber-type differences in adaptation to exercise, inactivity and disease. Additionally, the 3-D electron microscopy reconstructions of NMJs provide novel insight into the distribution of active zones and the synaptic vesicles associated with them, and the structural differences are likely contributing to the functional differences across fiber-type. The results further indicate that synaptic depression results from a depletion of synaptic vesicles in the RRP, a change in the probability of vesicle release, and insufficient vesicle pool replenishment.

The estimated QC decline based on depletion of the docked vesicles (RRP) correlates well with the rapid phase of decline only at terminals innervating type IIx and/or IIb fibers (expressing MHC2X and/or MHC2B). At type I or IIa fibers

**DISCUSSION**

The results of the present study indicate that the underlying mechanisms of synaptic depression with repetitive stimulation vary across diaphragm NMJs at different muscle fiber types, particularly within the first 0.5 to 1 s of repetitive stimulation. These results also indicate that there are two components to

FIG. 7. The rate of replenishment of the RRP during repetitive stimulation was estimated from the difference between the predicted depletion of the RRP and the actual QC measurements. The RRP replenishment rate differs across NMJs at different fiber types, with a higher rate of replenishment in terminals at type Ix and/or IIb fibers (~300 vesicles/s) than at type I or IIa fibers (~200 vesicles/s). In addition, a significant difference exists in the timing for initial replenishment after the onset of stimulation: replenishment occurs after ~0.4 s for terminals at type IIx and/or IIb fibers and only after ~1.0 s for terminals at type I or IIa fibers.

FIG. 8. The decline in QC during repetitive 20-Hz stimulation was estimated based on a fixed probability of release from the RRP (black circles) as indicated in METHODS. During the initial 0.5 s of continuous stimulation, the measured change in QC release (A) occurs much faster than could be accounted for by depletion of the RRP at terminals innervating type I or IIa fibers (A). Thus a decrease in the probability of release must also take place in this period. After the 1st 1.0 s, continued depletion of the RRP would result in a significantly greater decline in QC than observed. Thus some type of replenishment of the RRP must occur beyond this time point. In contrast, at type IIx and/or IIb terminals (B), the immediate decline in QC over the initial 0.5 s could be completely accounted for by a depletion of the RRP. After 0.5 s of continuous stimulation, continued depletion of the RRP would have resulted in a significantly greater decline in QC than is actually observed, again suggesting repletion of this pool beyond this time point. Interestingly, when a fixed-rate of recycling of released vesicles into the RRP is considered in the depletion of the RRP (C; see METHODS), the QC was predicted to continue to decline below observed values beyond the initial 0.5- to 1.0-s period. Thus additional recruitment of vesicles into the RRP, possibly from the cycling pool is suggested.

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(expressing MHC\textsubscript{Slow} or MHC\textsubscript{2A}), depletion of the RRP cannot solely account for the rapid decline in QC; thus a decrease in the probability of release is necessary. The delayed phase of QC decline is likely influenced by RRP replenishment from recycling and/or recruitment of vesicles from a cycling pool. Differences in replenishment across muscle fiber types are also consistent with the known differences in presynaptic terminal surface area and RRP size (Mantilla et al. 2004). To support sustained transmission, recycling likely predominates at terminals innervating type I or IIa fibers, whereas recruitment from the reserve pool predominates at terminals innervating type IIx and/or IIb fibers. It is possible that differences in initial QC and QC decline reflect fiber type-specific differences in activation history.

**Differences in QC decline are likely related to differences in activity**

It is well known that the recruitment order of motor units imposes different activation histories across fiber types within a mixed muscle such as the diaphragm (Johnson and Sieck 1993; Sieck and Fournier 1989). Inspiratory pressures required for normal breathing can be generated by recruitment of motor units composed of type I and IIa fibers. Motor units composed of type IIx or IIb fibers are only recruited during the infrequent motor behaviors associated with increased demand (e.g., coughing and sneezing). These differences in activation history across motor units generally correspond to their susceptibility to fatigue (Clamann and Robinson 1985; Sieck and Fournier 1989). NMJs at slow- or fast-twitch fatigue-resistant motor units (comprising type I or IIa fibers, respectively) are less susceptible to failure (defined electromyographically) than those at fast-twitch, fatigable motor units (comprising type IIx or IIb fibers). In addition, glycogen depletion with varying levels of activation differs across diaphragm muscle fiber types, reflecting differences in overall muscle use during repeated nerve stimulation (Johnson and Sieck 1993). However, fiber-type differences in quantal release were previously unknown.

In the present study, the size of the RRP (defined morphologically) was found to vary across diaphragm fiber types and RRP size is proportional to the number of active zones within a terminal, which in turn depends on terminal dimension (as determined from 3-D reconstructions). The larger RRP at terminals innervating type IIx and/or IIb diaphragm fibers is consistent with their higher initial QC compared with terminals innervating type I or IIa fibers. Thus the number of vesicles released may depend on the number of vesicles available in general agreement with previous studies (Reid et al. 1999; Wilson 1979).

Differences in RRP size across fiber types may relate to differences in activation history. Using FM dyes to label recycled vesicles, Reid et al. (1999) found that nerve terminals at soleus and extensor digitorum longus (EDL) muscles (composed of type I or IIa fibers and type IIx and IIb fibers, respectively) differ in quantal release characteristics, which generally correspond to their different in vivo activity patterns. Other studies using electrophysiological recordings also demonstrate that EDL terminals have a higher initial QC (15–30%) than soleus terminals (Gertler and Robbins 1978; Reid et al. 1999; Wood and Slater 1997). The activation history of specific muscle fibers depends on fiber type, even within a single, active muscle such as the diaphragm. In this sense, the overall number of vesicles recycled would need to be higher at terminals innervating type I or IIa fibers (compared with type IIx and/or IIb) due to their more frequent recruitment (Johnson...
and Sieck 1993; Sieck and Fournier 1989). Significantly greater vesicle recycling (measured using FM4-64 uptake) was evident during repetitive phrenic stimulation at terminals innervating type I or IIA fibers compared with those innervating type IIx and/or IIb fibers (Manilla et al. 2004). These results agree with previous studies examining hind limb muscles of a predominantly homogenous fiber type composition. For instance, QC decreases to a greater extent (relative to the initial QC values) in the rat EDL than in the soleus muscle (~90 vs. ~65%, respectively) during repetitive activation (Reid et al. 1999). Thus it is likely that specific NMJ adaptations in vesicle release and recycling directly relate to the in vivo activity patterns to which they are exposed (Betz et al. 1993; Millar et al. 2002; Reid et al. 1999; Stevens and Wesseling 1998).

What accounts for the decline in QC?

Several studies highlight a predominant effect of RRP depletion in the QC decline with repetitive stimulation at both rat (Bennett and Florin 1974; Glavinovic 1979; Wilson 1979) and human NMJs (Elmqvist and Quastel 1965; Liley and North 1953). Using intracellular recordings at rat diaphragm NMJs, Wilson (1979) reported that QC decline results from a decrease in the releasable store, whereas the probability of release remains constant. However, in these experiments, the size of the releasable store (RRP) was estimated based on the QC, the probability of release was assumed to be constant (because it was not possible to determine whether the proportion of vesicles released from the RRP changed over time), and greater impairment of the larger type IIx or IIb fibers could not be excluded. Thus any change in probability of release and its possible contribution to the decline in QC during repetitive nerve stimulation were likely unrecognized.

Synaptic depression at rat hind limb muscles results from inadequate recycling and/or recruitment of vesicles into the RRP and its gradual depletion (Reid et al. 1999). It is highly unlikely that the RRP would ever completely deplete with normal or even elevated activity levels. Inspiratory duration in the rat is <200 ms, diaphragm duty cycle is ~25–35% (Kong and Berger 1986). Complete depletion of the RRP would require continuous stimulation for ~10s (Fig. 6) given that the role of RRP depletion would, if anything, be overestimated assuming no change in the probability of release and if any replenishment of the RRP is ignored. Although the RRP comprised a very small fraction of the total number of synaptic vesicles (Table 1), the number of synaptic vesicles in the RRP far exceeds the number of vesicles released with each stimulus (~35-fold larger for terminals at type I or IIA fibers and ~41-fold larger for terminals at type IIx and/or IIb fibers). Even if only one vesicle is released per active zone, only ~15% of active zones are utilized initially (assuming no postsynaptic desensitization). This surprisingly large surplus of active zones and synaptic vesicles remaining available for release further suggests that vesicle depletion is not the sole cause for QC decline (certainly the rapid initial phase of QC decline) and suggests that a decrease in the probability of release plays an important role in QC decline with repetitive stimulation. In agreement with this view, synaptic depression occurred from both a decrease in the probability of release as well as RRP depletion at amphibian (Betz 1970) and mammalian NMJs (Christensen and Martin 1970) and at hippocampal synapses (Stevens and Wesseling 1999).

Fiber-type differences in the probability of release may only reflect the average probability of release of all active zones within each terminal. However, significant heterogeneity in release probability may exist across active zones. Using electrophysiological recordings at toad NMJs, some active zones have very high probability of release (those that release first), whereas others in the same terminal have release probabilities at or near zero (Bennett and Lavidis 1989). The notion of nonuniform release probabilities across individual release sites was also noted at central synapses (Walmsley et al. 1988). Assuming that similar differences exist in the rat diaphragm muscle, it is exciting to speculate that terminals at type I or IIA fibers have a greater proportion of active zones with a low probability of release compared with terminals at type IIx and IIb fibers. Differential expression of exocytotic or Ca2+ regulating proteins may exist across NMJs at the different fiber types; these possibilities remain to be explored.

Using different stimulation frequencies, fiber type differences became more apparent during the initial phase of QC decline (Figs. 9 and 10). These results indicate that stimulation frequency may influence the probability of synaptic vesicle release at individual active zones and that a maximal rate of RRP replenishment or “priming” may exist. Although it has not been demonstrated that the probability of synaptic vesicle release may be frequency dependent, both the rate of vesicle release and the fraction of vesicles released per pulse vary with stimulation frequencies in frog NMJs (Betz and Bewick 1993). In addition, the rate of QC decline may vary across active zones within the same terminal (Glavinovic 1995). Indeed, the probability of release may depend on the number of primed synaptic vesicles in the RRP (Dobranz 2002). It is not known if these mechanisms also exist at mammalian NMJs. Regardless of the mechanism, the results of the present study indicate that the differences in synaptic vesicle release observed across fiber types are entirely consistent with their activation history and susceptibility to fatigue, revealing both structural and functional adaptations at diaphragm NMJs in accordance with their physiological demands.

G R A N T S

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R E F E R E N C E S


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