Synaptic Vesicle Distribution and Release at Rat Diaphragm Neuromuscular Junctions

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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 muscle fibers were used to perform electrophysiological recordings show that the number of synaptic vesicles released with each stimulus (i.e., the quantal content, QC) decreases over time. It is controversial whether synaptic depression occurs solely from depletion of the RRP (Elmqvist and Quastel 1965; Glavinovic 1979; Reid et al. 1999; Wilson 1979) and/or from a decrease in the probability of release (Betz and Christensen and Martin 1970). Whether these mechanisms differ across NMJs with different activation histories is unknown.

Activity is important in determining the structure and function of a synapse (Hebb 1949). The diaphragm muscle is a particularly active muscle of mixed fiber type composition. Based on myosin heavy chain (MHC) composition, diaphragm motor units differ in activation history and susceptibility to fatigue (Johnson and Sieck 1993; Sieck and Fournier 1989). Presynaptic terminals at type I or IIA diaphragm fibers have smaller cycling pool size despite greater average synaptic vesicle density at active zones compared with those at type IIX and/or IIB fibers (Mantilla et al. 2004). We hypothesize that the more frequently recruited terminals (at type I or IIA fibers) have smaller QC compared with terminals at type IIX and/or IIB fibers and that the mechanisms involved in QC decline vary across fiber types in accordance with known differences in activation history. In the present study, we examined the distribution of synaptic vesicle pools at presynaptic terminals at type-identified diaphragm muscle fibers using novel three-dimensional (3-D) reconstructions of serial electron microscopy images, measured the rate of decline in QC during repetitive stimulation at individual NMJs and compared fiber-type differences in the release of synaptic vesicles.

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.
duced 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 (~50–500 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 IIa 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 Zeiss 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 (Analyz3AVW, Mayo Foundation) for 3-D reconstruction (Robb et al. 1989). Individual NMJs were classified as innervating type I or IIa 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 Metamorph. 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 Ila 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).

**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 Ila 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 Ila 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 Ila 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 homogeneous 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 Ila 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 Ila fibers versus type IIx and/or IIb fibers according

<table>
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<tr>
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<th>Type I and Ila</th>
<th>Type IIx and/or IIb</th>
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<tbody>
<tr>
<td>Presynaptic terminal area</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, cm²</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>
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All values are means ± SE, *statistically significant difference from terminals innervating type I or Ila diaphragm muscle fibers (P < 0.05).
fibers (range: 343–627; 95% CI; Table 1). Dendrites were
innervated by terminals that provided significantly less
number of active zones per terminal was lower at terminals
innervating type I or IIa fibers (range: 171–324; 95% con-
fidence interval, CI) than at those innervating type IIx and/or IIb
fibers (range: 1,029–1,943; 95% CI). 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 ~1 μm 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 is dependent on the
surface area of the terminal. Therefore the calculated average
terminal was thus calculated assuming equal distribution of active zones
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 pre-
synaptic 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 ± 0.001 μm² 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 dif-
fered between presynaptic terminals at the different muscle
fiber types, in agreement with a previous report (Mantilla
et al. 2004). Within 200 nm 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 <

**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 nm), 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 nm.**
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.

**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 IIa 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 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
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 replenishment 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 I 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 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 recycled per pulse by type IIx and IIb fibers (~1011 298 vesicles/s) is higher than terminals at type I or IIa fibers (~1011 207 vesicles/s).

**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). Insert: 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 RRP 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.
These results also indicate that there are two components to particularly within the first 0.5 to 1.0 s of repetitive stimulation. Varying across diaphragm NMJs at different muscle fiber types, with a higher rate of replenishment in terminals at type Ix and/or Ilb fibers (≈300 vesicles/s) than at type I or Ila 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 Ix and/or Ilb fibers and only after ≈1.0 s for terminals at type I or Ila fibers.

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 Ix and/or Ilb fibers (expressing MHC2X and/or MHC2B). At type I or Ila fibers, vesicles available to be recycled was established as the average difference between the actual QC and the predicted QC decline after ≈200–600 s of continuous stimulation (a period during which relatively stable release was observed and the RRP would have been depleted if not replenished). This maximum rate of vesicle replenishment (through recycled vesicles alone) was then considered in estimating RRP depletion, again assuming no change in the probability of release. Although there is still a large discrepancy with the actual data within the first 2 min of stimulation (Fig. 8), it is evident that recruitment of vesicles into the RRP and/or a decrease in the probability of release must also be involved in maintaining the size of the RRP.

To elucidate the time dependence of QC release across fiber types, differences in QC decline were compared across stimulation rates (Fig. 9). Given that the initial phase of QC decline can be predicted with greater fidelity (Aristizabal and Glavinovic 2003), only the first 17 pulses were considered (i.e., 330 ms at 50 Hz or 850 ms at 20 Hz). The probability of release over time was calculated assuming no replenishment of the RRP. Fiber-type differences in probability of release become more apparent at the different stimulation frequencies (Fig. 10). Whereas the probability of release did not necessarily change with varying stimulation frequency at presynaptic terminals innervating type I or Ila fibers, increasing stimulation frequency resulted in a greater decline in QC (and thus in probability of release) at terminals innervating type Ix and/or Ilb 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 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 Ix and/or Ilb fibers (expressing MHC2X and/or MHC2B). At type I or Ila fibers, after difference between the actual QC and the predicted QC decline was established as the average 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 I or IIa fibers (≈200 vesicles/s) than at type Ix and/or Ilb fibers (≈300 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 Ix and/or Ilb fibers and only after ≈1.0 s for terminals at type I or Ila fibers.

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**DISCUSSION**

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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 1999).
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 (Mantilla 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 underestimated 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 Lavdis 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 (Dobrunz 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.

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REFERENCES


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