Transmitter Release Differs at Snake Twitch and Tonic Endplates During Potassium-Induced Nerve Terminal Depolarization

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Connor, Elizabeth A., Anna Dunaevsky, David J. G. Griffiths, Jean C. Hardwick, and Rodney L. Parsons. Transmitter release differs at snake twitch and tonic endplates during potassium-induced nerve terminal depolarization. J. Neurophysiol. 77: 749–760, 1997. Twitch and tonic muscle fibers of snake skeletal muscle differ in their synaptic as well as mechanical properties. These experiments were aimed at determining the basis of the difference in vesicular release properties of nerve terminals at twitch and tonic endplates. Miniature endplate currents (MEPCs) were recorded from voltage-clamped garter snake muscle fibers depolarized by high K+ in either a control Ca2+ or high-Ca2+ solution. MEPC frequency increased at twitch and tonic endplates and remained elevated for 8 h during depolarization in control Ca2+. At twitch endplates depolarized in the presence of high Ca2+, an increase in MEPC frequency was followed by a progressive decline. In contrast, MEPC frequency remained elevated in high Ca2+ at tonic endplates. The observed decrease in MEPC frequency at depolarized twitch endplates in high Ca2+ was not a function of the level of depolarization or initial MEPC frequency, nor was it due to a reduction in MEPC amplitude and loss of MEPCs in baseline noise. An optical assay of presynaptic function in which the activity-dependent dye FM1-43 was used confirmed that quantal release differs at twitch and tonic endplates. Most twitch nerve terminals were labeled by FM1-43 during prolonged depolarization with control Ca2+ or after brief depolarization with high Ca2+. In contrast, the number of twitch nerve terminals and the degree to which they were stained was greatly reduced after prolonged exposure to high K+ and high Ca2+, whereas depolarized tonic endplates were well stained by FM1-43 during brief and prolonged exposure to high Ca2+. FM1-43 staining also revealed variable levels of quantal release between individual boutons at twitch endplates after prolonged depolarization in high-Ca2+ solution. The observed reduction in presynaptic function at twitch nerve terminals after prolonged depolarization in high-Ca2+ solution was reversible and therefore not due to irreversible damage to terminal boutons. MEPC frequency increased at both twitch and tonic endplates when either Sr2+ or Ba2+ was substituted for high Ca2+ during K+-induced depolarization. Over time, in Sr2+ or Ba2+ solutions, MEPC frequency remained elevated at tonic endplates but declined at twitch endplates with a time course similar to that observed in high Ca2+. MEPC amplitudes at both endplates remained constant. We conclude that the regulation of quantal release differs in nerve terminals innervating twitch and tonic endplates and postulate that differential intraterminal accumulation of Ca2+ may underlie the observed difference in presynaptic function.

INTRODUCTION

Skeletal muscles of the snake contain two classes of fibers, twitch and tonic muscle fibers (Morgan and Proske 1984). These two muscle fiber types exhibit different mechanical, biochemical, morphological, and electrical properties (Lichtman and Wilkinson 1987; Morgan and Proske 1984; Wilkinson and Lichtman 1985; Wilkinson and Nemeth 1989; Wilkinson et al. 1991). Each twitch fiber has a single endplate that receives innervation from one motoneuron and upon neural stimulation produces an action potential accompanied by a muscle fiber twitch (Lichtman and Wilkinson 1987; Wilkinson and Lichtman 1985). In contrast, tonic fibers are multiply innervated along their length, with each endplate often receiving inputs from multiple motoneurons (Lichtman and Wilkinson 1987; Lichtman et al. 1985). Tonic fibers do not conduct action potentials but rather produce graded contractures upon neural stimulation (Lichtman and Wilkinson 1987; Morgan and Proske 1984). The kinetics and voltage dependence of acetylcholine receptor-channel complexes also differ at twitch and tonic endplates (Connor et al. 1984; Dionne 1989; Dionne and Parsons 1981; Ruff and Spiegel 1990). Thus the synaptic as well as mechanical properties of twitch and tonic muscle fibers contribute to their functionally different responses to neural stimulation.

Presynaptic function also is fundamentally different for motoneuron terminals innervating twitch and tonic muscle fibers. The quantal content of endplate potentials is higher at twitch endplates than at tonic endplates (Morgan and Proske 1984). Furthermore, at tonic endplates, the endplate potential facilitates with repetitive stimulation whereas at twitch endplates the endplate potential exhibits depression. Previously, Coniglio et al. (1993) demonstrated a striking difference in miniature endplate current (MEPC) frequency at snake twitch and tonic endplates during prolonged depolarization. Specifically, nerve terminals innervating tonic muscle fibers sustained quantal release more effectively than terminals innervating twitch fibers when depolarized in an isotonic K+-induced solution containing high Ca2+. MEPC frequency was sustained at both twitch and tonic endplates for the duration of depolarization in control Ca2+. These results suggested that the intrinsic release properties of nerve terminals innervating twitch and tonic endplates differ.

The experiments presented here were aimed at determining the basis for the difference in vesicular release properties of nerve terminals innervating twitch and tonic endplates. This study combines the novel technique of optical imaging of presynaptic function using an activity-dependent dye with electrophysiological recording. We determined that neither
the level of depolarization, a reduced MEPC amplitude, nor the initial MEPC frequency account for the difference in presynaptic function at twitch and tonic neuromuscular junctions. Further, we used a noninvasive optical assay of presynaptic function to demonstrate that nerve terminals innervating tonic endplates remain functional after prolonged depolarization in high calcium. Snake tonic muscle fibers are much smaller than twitch fibers (Dionne and Parsons 1981), making them more susceptible to injury during impalement. Mechanical disturbance of motoneuron terminals can stimulate bursts of MEPCs (Van der Kloot and Molgo 1994). We eliminated the possibility that the sustained MEPC frequency at tonic endplates may be artifactual and may result from mechanical injury rather than a difference in transmitter release properties. The optical assay of presynaptic function also revealed differential levels of release between subsets of boutons at individual twitch nerve terminals, an observation not evident with the use of electrophysiological methods. Finally, we tested the effect of other divergent cations on depolarization-induced MEPC frequency at twitch and tonic endplates to gain insight into the role and specificity of Ca\(^{2+}\) in regulation of MEPC frequency.

**Methods**

Experiments were performed on visually identified twitch and tonic muscle fiber endplates in the costocutaneous muscle of garter snakes (*Thamnophis*) at room temperature (20–23°C). Snakes were killed by rapid decapitation, and muscle preparations were dissected and pinned in Sylgard-coated plastic dishes. Preparations were initially kept in a normal-Na\(^+\) solution containing (in mM) 159 NaCl, 2.5 KCl, 1.0 CaCl\(_2\), 4.2 MgCl\(_2\), and 1.0 N-2-hydroxyethylpiperazine-\(N'\)-2-ethanesulfonic acid, pH 7.3 (Coniglio et al. 1993; Connor et al. 1984). Quantal transmitter release was induced by exposure to depolarizing solutions in which all or a portion of NaCl was replaced by an equimolar amount of potassium propionate (K\(^+\); 35, 60, and 160 mM) (Coniglio et al. 1993). Unless otherwise noted, depolarization was produced with a 35 mM K\(^+\) solution. All high-K\(^+\) solutions contained CsCl (5 mM) to facilitate the voltage clamping of depolarized muscle fibers (Coniglio et al. 1993; Connor et al. 1984). In most experiments, the concentration of Ca\(^{2+}\) in high-K\(^+\) solutions was either 1.0 mM Ca\(^{2+}\) with 4.2 mM Mg\(^{2+}\) added (control Ca\(^{2+}\)) or 3.6 mM Ca\(^{2+}\) with no added Mg\(^{2+}\) (high Ca\(^{2+}\)). In some experiments, 3.6 mM Ca\(^{2+}\) was replaced with either 3.6 mM Sr\(^{2+}\) or 3.6 mM Ba\(^{2+}\) in a 35 mM K\(^+\) solution.

**Electrophysiological methods**

Twitch and tonic muscle fibers were identified with the use of criteria described in detail in previous reports (Coniglio et al. 1993; Connor et al. 1984; Dionne and Parsons 1981). There are two subtypes of twitch fibers in snake muscle: slower-twitch and faster-twitch fibers (Wilkinson and Lichtman 1985). In these experiments we did not distinguish between twitch fiber type. MEPCs were recorded from twitch or tonic endplates after various lengths of time in high-K\(^+\) solution with the use of a two-microelectrode voltage-clamp system (Coniglio et al. 1993; Connor et al. 1984). We chose to voltage clamp the muscle fibers to negative membrane potentials to increase the driving force for MEPCs and thereby increase the signal-to-noise ratio. MEPCs were recorded from twitch fibers voltage clamped to −150 mV, whereas MEPCs from tonic endplates were not maintained at −100 mV because tonic fibers could not be maintained at −150 mV without muscle fiber deterioration. Records of MEPCs were stored on a PCM recorder (Vetter).

The frequency of MEPCs as a function of recording time was estimated from visual inspection of computer traces and this estimate approximated quantal release. Determination of MEPC frequencies by visual inspection is limited at high rates of release because of overlap of individual events. To increase the efficiency of estimating MEPC frequency, muscle fibers were voltage clamped to negative membrane potentials (see above) and MEPCs were displayed with an extended time base to facilitate the identification of individual event peaks. Under these conditions, MEPC frequencies of <300 MEPCs per second were readily discerned and it is likely that frequency estimates were accurate reflections of presynaptic activity. At MEPC frequencies of >300 MEPCs per second, however, the estimated value serves only as an approximation of quantal release. These estimates of high MEPC frequency are likely to underestimate the actual frequency, with the error being progressively greater at increasing rates of release.

Amplitude measurements were made on individual digitized MEPCs with clear rise transitions from baseline to peak with the use of the SCAN program (generously provided by Dr. John Dempster, University of Strathclyde, Glasgow, Scotland). For recordings from tonic endplates, MEPCs with rapid rise times to peak were selected for amplitude analysis, thus excluding any MEPCs that may have originated from distant endplates (Dionne and Parsons 1981). The amplitude distribution of MEPCs recorded from a given endplate was used to estimate the variation in size of quanta released at that endplate.

**Optical assay of vesicle release and recycling**

Twitch and tonic neuromuscular junctions were visualized by staining with rhodamine-conjugated peanut agglutinin (PNA; Sigma, St. Louis MO), which marks synaptic and terminal Schwann cell basal laminae (Ko 1987). Muscle preparations were exposed to PNA (33 mg/ml; dissolved in either normal-Na\(^+\) solution or high-K\(^+\) solution) for 15 min and then rinsed. Optical estimates of vesicle release and recycling were made with the use of potassium-stimulated transmitter release coupled with uptake of the fluorophore FM1-43 (2 μM; Molecular Probes, Eugene, Oregon) into recycling synaptic vesicles (Betz and Bewick 1993; Betz et al. 1992). Muscle preparations were exposed for 5 min to FM1-43 in 35 mM K\(^+\) solution containing either control Ca\(^{2+}\), 3.6 mM Ca\(^{2+}\), 3.6 mM Sr\(^{2+}\), or 3.6 mM Ba\(^{2+}\), then washed for 15 min in normal-Na\(^+\) solution before viewing. Preparations were exposed to FM1-43 either immediately upon depolarization or after 1–8 h in high-K\(^+\) solution. Nerve terminals were visualized and images were obtained with a Dage VE-1000 SIT camera, digitized, averaged (×16), and stored with the use of the NIH Image software package version 1.54 (Dunaevsky and Connor 1995). Twitch and tonic neuromuscular junctions, identified by PNA stain (red emission filter, >590 nm), were scored as either uniformly stained, partially stained, or unstained by FM1-43 (green emission filter, 520–560 nm). A bouton exhibiting fluorescence above the background level was considered stained. Staining was scored as uniform if all boutons of a nerve terminal were stained, whereas a terminal with at least one but not all boutons stained was scored partially stained. For each muscle a percentage of the total number of identified twitch or tonic nerve terminals stained by FM1-43 was determined. Some figures were created as montages of neuromuscular junctions imaged at different focal planes.

In control experiments we determined that nerve terminals exposed to FM1-43 in normal-Na\(^+\) solution were not stained. Further, we found that FM1-43-labeled twitch and tonic terminals destained during subsequent depolarization in the absence of dye.


**RESULTS**

**MEPC frequency at twitch fiber endplates exposed to high calcium and different external potassium concentrations**

We first tested the role of nerve terminal depolarization in the decline in MEPC frequency at depolarized twitch endplates. Previously, exposure to high Ca\(^{2+}\) during prolonged depolarization with a 160 mM K\(^+\) solution resulted in an increase in MEPC frequency at twitch endplates, followed by a progressive decline until many endplates were silent (Coniglio et al. 1993). To determine whether a similar decline in MEPC frequency occurred at twitch endplates at other levels of depolarization, we measured MEPC frequency at twitch endplates depolarized by varying the external K\(^+\). As an indicator of nerve terminal resting membrane potential, we measured the membrane potential of twitch muscle fibers exposed to varying levels of external K\(^+\) and assumed that the extent of nerve terminal depolarization varies with elevated K\(^+\) in a similar fashion. As expected, twitch fiber membrane potential was progressively more depolarized when the extracellular K\(^+\) concentration was raised from 35 to 160 mM (35 mM: \(-37.2 \pm 0.7\) mV, mean \(\pm SE, n = 50\) muscle fibers; 60 mM: \(-26.5 \pm 0.5\) mV, \(n = 32\) fibers; 160 mM: \(-3.4 \pm 0.7\) mV, \(n = 14\) fibers).

We found a similar pattern of MEPC frequency, an increase followed by a progressive decrease, at twitch endplates when muscle preparations were exposed to high-Ca\(^{2+}\) solutions with K\(^+\) concentrations ranging from 35 to 160 mM (Fig. 1). The MEPC frequency decreased along a similar time course at different levels of external K\(^+\). Thus the progressive decline in quantal release at twitch endplates occurs over a range of levels of depolarization. The remainder of the experiments were performed with the use of a 35 mM K\(^+\) solution because in this solution muscle fibers were less depolarized and easier to voltage clamp to negative membrane potentials.

**Calcium dependence of MEPC frequency at twitch and tonic endplates in response to depolarization**

We next determined whether the progressive decline in MEPC frequency in response to depolarization by 35 mM K\(^+\) was dependent on Ca\(^{2+}\) concentration and muscle fiber type. Exposure to 35 mM K\(^+\) solution containing control Ca\(^{2+}\) resulted in an increase in MEPC frequency at twitch fiber endplates that was sustained for prolonged periods (Figs. 2A and 4A). Depolarization of twitch nerve terminals in the presence of high Ca\(^{2+}\) resulted initially in a greater MEPC frequency that then declined to ~50 MEPCs per second (Figs. 2B and 4A). Few or no MEPCs were recorded at many individual twitch endplates after 2 h of depolarization, whereas at an occasional endplate the MEPC frequency was as high as 100 per second. During high-K\(^+\) depolarization, MEPC frequency at tonic endplates was also enhanced in the presence of high Ca\(^{2+}\) compared with control Ca\(^{2+}\) (Figs. 3 and 4B). Unlike the response at twitch endplates, however, MEPC frequency was sustained at tonic endplates during prolonged exposure to high-K\(^+\) solutions with either control or high Ca\(^{2+}\).

**MEPC amplitude in high-potassium solutions containing control or high calcium**

We determined mean MEPC amplitudes at various times during exposure to 35 mM K\(^+\) to test whether the decline in MEPC frequency at twitch endplates in high Ca\(^{2+}\) was due to a decrease in MEPC amplitude. Analysis of mean MEPC amplitude at twitch and tonic endplates during exposure to high-K\(^+\) solutions containing control or high Ca\(^{2+}\) demonstrated that MEPC amplitude is unchanged for the duration of depolarization (Fig. 5, A and B). The values of mean MEPC amplitude obtained from individual twitch muscle fibers were more variable than those of tonic fibers. Because snake muscle MEPC amplitudes vary as a function of fiber size (Wilkinson et al. 1992), we attribute this difference in variability to our selection of larger tonic muscle fibers for impalement, whereas MEPC recordings were obtained from twitch fibers over a wider range of fiber diameters.

We also considered that the MEPC amplitude distribution in depolarized preparations was no longer unimodal even though the mean MEPC amplitude was unchanged. For example, two populations of MEPCs, small and large amplitude, could yield a mean MEPC amplitude that is an inaccurate indicator of quantal size. This we tested by constructing MEPC amplitude histograms for twitch and tonic fiber endplates after a 6- to 8-h exposure to a high-K\(^+\) solution containing high Ca\(^{2+}\). MEPC amplitude histograms remained essentially unimodal for both twitch (Fig. 5C) and tonic (data not shown) endplates, although some large-amplitude MEPCs were observed at twitch endplates. Thus, during potassium-induced depolarization in the presence of high Ca\(^{2+}\), the mean MEPC amplitude serves as a good estimate of quantal size at both twitch and tonic endplates.

**Optical analysis of vesicle release and recycling at twitch and tonic nerve terminals**

We postulate that the ability of tonic nerve terminals to sustain quantal release more effectively than twitch nerve
The sustained quantal release at tonic terminals represents an intrinsic difference in presynaptic function between these nerve terminal types (Coniglio et al. 1993). However, the sustained quantal release at tonic terminals might be an artifact produced by microelectrode impalement. Endplates of smaller tonic muscle fibers are harder to visualize and thus successful impalement with two microelectrodes is more difficult than in twitch fibers. To ensure that the high MEPC frequency observed at tonic endplates was not due to mechanical stimulation, the dye FM1-43, which provides an assay of synaptic vesicle release and recycling, was used to obtain an optical measurement of presynaptic release (Betz et al. 1992).

Presynaptic function at neuromuscular junctions was assessed with FM1-43 during both brief (5 min) and prolonged (8 h) exposure to high-K\textsuperscript+ solution containing either control Ca\textsuperscript2+ or high Ca\textsuperscript2+. These preparations were also stained with PNA to identify twitch and tonic neuromuscular junctions. Brief depolarization (5 min) in the presence of control Ca\textsuperscript2+ resulted in uniform staining of most nerve terminals at twitch endplates, whereas no nerve terminals innervating tonic fibers were stained (Table 1; Fig. 6, A and B). These data suggest that the level of release elicited at tonic nerve terminals by high-K\textsuperscript+ solution containing control Ca\textsuperscript2+ is insufficient to produce visible clusters of FM1-43-labeled synaptic vesicles at release sites. Brief stimulation with a
A

Twitch

- CONTROL CALCIUM
- HIGH CALCIUM

MEPC AMPLITUDE (nA)

0 3 6 9 12 15

TIME (MINUTES)

B

Tonic

- CONTROL CALCIUM
- HIGH CALCIUM

MEPC AMPLITUDE (nA)

0 3 6 9 12 15

TIME (MINUTES)

C

NUMBER OF EVENTS

-25 -20 -15 -10 -5 0

MEPC AMPLITUDE (nA)

FIG. 5. Size and distribution of MEPC amplitudes recorded from individual twitch and tonic muscle fiber endplates is unchanged by prolonged depolarization. A and B: muscle fibers were exposed to a 35 mM K+ solution containing either control Ca2+ (●) or high Ca2+ (△). A: mean MEPC amplitudes were recorded from individual twitch fiber endplates voltage clamped to −150 mV from ≥3 muscles. B: mean MEPC amplitudes were recorded from individual tonic fiber endplates voltage clamped to −100 mV from ≥3 muscles. C: MEPC distribution is plotted for a twitch fiber endplate voltage clamped to −150 mV after a 450-min exposure to 35 mM K+ solution containing high Ca2+. The mean and mode values for the MEPC amplitude are −7.2 and −6.8 nA, respectively.

high-K+ solution containing high Ca2+ produces a higher rate of release and resulted in uniform staining of both twitch and tonic nerve terminals (Fig. 6, C and D). The relative intensity of staining at twitch nerve terminals was greater when terminals were depolarized in the presence of high Ca2+ compared with control Ca2+. Thus this optical assay effectively distinguished between levels of vesicle release and recycling at snake twitch and tonic neuromuscular junctions, an observation consistent with the estimates of MEPC frequency.

FM1-43 incorporation was next tested at twitch and tonic nerve terminals after prolonged depolarization. An 8-h depolarization in high-K+ solution containing control Ca2+ resulted in uniform staining of most twitch nerve terminals by FM1-43, whereas the majority of tonic nerve terminals was not stained (Table 1; Fig. 6, E and F). After prolonged exposure to high-K+ solution containing high Ca2+, nearly all tonic nerve terminals were stained, indicating that vesicular release was maintained at tonic endplates during prolonged depolarization (Fig. 6, G and H). In contrast, approximately a third of twitch nerve terminals remained unstained after prolonged high-K+ depolarization in the presence of high Ca2+ (Table 1; Fig. 6, G and H). Interestingly, at a majority of the twitch nerve terminals labeled by FM1-43, the staining was nonuniform in that only a subset of boutons was stained, suggesting that the level of quantal release and vesicle recycling varied among individual boutons (Fig. 6, I and J). The intensity of FM1-43 stain in these labeled boutons was often quite strong.

The FM1-43 staining of only a subset of boutons at twitch nerve terminals depolarized for long periods in the presence of high-Ca2+ solution may indicate that the rate of presynaptic release between boutons normally differs. Differential release rates between boutons would be detectable under conditions in which presynaptic release is suppressed; boutons releasing at higher rates would be stained by FM1-43, whereas boutons releasing at rates below the minimum for observing FM1-43 stain would be unstained. To test whether presynaptic release rates of boutons at normal twitch nerve terminals are detectably different, we determined the pattern...
TABLE 1.  

<table>
<thead>
<tr>
<th>[Ca(^{2+})](_{\text{solution}}), mM</th>
<th>Time in High-K(^{+}), Solution, h</th>
<th>Twitch</th>
<th>Tonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0</td>
<td>97 ± 5 (6)</td>
<td>1 ± 1 (6)</td>
</tr>
<tr>
<td>1.0</td>
<td>8</td>
<td>93 ± 8 (3)</td>
<td>0 ± 0 (3)</td>
</tr>
<tr>
<td>3.6</td>
<td>0</td>
<td>97 ± 3 (9)</td>
<td>1 ± 1 (9)</td>
</tr>
<tr>
<td>3.6</td>
<td>8</td>
<td>8 ± 6 (9)</td>
<td>54 ± 10 (9)</td>
</tr>
</tbody>
</table>

Values in Average Percentage of Neuromuscular Junctions Stained by FM1–43 are means ± SE, with numbers of muscle preparations in parentheses. Each muscle was exposed to high-K\(^{+}\) solution for an additional 5 min during staining with FM1–43. Uniform stain: all nerve terminal boutons were stained. Partial stain: only a subset of nerve terminal boutons was stained. Values in these columns are average percentages of neuromuscular junctions stained by FM1–43. For each data point, muscle preparations from ≥3 different snakes were analyzed and 14–53 twitch or tonic neuromuscular junctions were examined per muscle preparation.

of FM1–43 incorporation at twitch nerve terminals exposed to 35 mM K\(^{+}\) solution containing different concentrations of Ca\(^{2+}\) (0–1.0 mM) and 4.2 mM Mg\(^{2+}\) (Dodge and Rahamimoff 1967). Muscles were dissected in a 0 mM Ca\(^{2+}\) Na\(^{+}\) solution and then incubated in Na\(^{+}\) solution containing a chosen level of Ca\(^{2+}\) and 4.2 mM Mg\(^{2+}\) before staining with FM1–43. In 1.0 mM Ca\(^{2+}\) and 4.2 mM Mg\(^{2+}\) (control Ca\(^{2+}\)) solution, nearly all twitch nerve terminals were uniformly stained by FM1–43 (Table 2). As the Ca\(^{2+}\) concentration decreased, the number of twitch nerve terminals stained by FM1–43 decreased, as did the relative intensity of stain. At all concentrations of Ca\(^{2+}\), nearly all of the twitch nerve terminals stained with FM1–43 were uniformly stained; only rarely was a partially stained nerve terminal observed. These results suggest that the rate of vesicular release between boutons at normal twitch nerve terminals is not detectably different as measured by FM1–43 incorporation.

To determine the onset and time course of the loss of uniform staining at individual twitch nerve terminals, we assayed presynaptic activity at twitch nerve terminals depolarized in the presence of high Ca\(^{2+}\) for different lengths of time. The percentage of twitch nerve terminals uniformly stained by FM1–43 gradually decreased with longer periods of exposure to high-K\(^{+}\) and high-Ca\(^{2+}\) solution (Table 3). Conversely, the percentage of twitch nerve terminals partially stained by FM1–43 increased with length of depolarization (Table 3), as did the proportion of unstained terminals. Interestingly, after 1 and 2 h in high-Ca\(^{2+}\) depolarizing solution, partially stained twitch nerve terminals had a majority of boutons stained by FM1–43, whereas at longer times of depolarization (4–8 h), fewer than half of the boutons were stained by FM1–43 at partially stained nerve terminals. At all times of depolarization, a majority of tonic nerve terminals was uniformly stained by FM1–43 (Table 3).

Long-term potassium-induced depolarization has been shown previously to produce changes in the ultrastructure of nerve terminals that are reversed over several hours in Na\(^{+}\) solution (Coniglio et al. 1993). We tested whether functional recovery of vesicular release would occur at twitch nerve terminals after prolonged depolarization in the presence of high Ca\(^{2+}\). Muscles were depolarized for 6 h in the presence of high Ca\(^{2+}\), and some preparations were then washed for 2 h in normal-Na\(^{+}\) solution and then tested for the ability to incorporate FM1–43. After 6 h of depolarization, 15% of twitch nerve terminals from four muscles (96 nerve terminals) were uniformly stained by FM1–43. Forty-four percent of nerve terminals were partially stained and 42% were unstained. The level of presynaptic release in muscles that were washed for 2 h in normal-Na\(^{+}\) solution after depolarization was strikingly different. In three muscles assayed with FM1–43 after a 2-h wash, 50% of 73 twitch nerve terminals were uniformly stained by FM1–43, whereas an additional 46% were partially stained. These data demonstrate that the observed reduction in presynaptic function of twitch nerve terminals after prolonged depolarization in the presence of high Ca\(^{2+}\) appears to be reversible.

Results from other experiments further demonstrated that boutons that failed to stain with FM1–43 after prolonged depolarization were still functional and not disrupted. After 6 h of depolarization in the presence of high Ca\(^{2+}\), the presynaptic function of a nerve terminal was assayed with FM1–43 and a few of its boutons were faintly stained (Fig. 7, A and B). After a 2-h wash and restaining, we reidentified the nerve terminal. We observed that many more boutons of this nerve terminal were labeled with FM1–43 and the staining was more intense (Fig. 7C).

**Initial MEPC frequency induced by high-K\(^{+}\) depolarization**

The initial MEPC frequencies elicited at twitch nerve terminals by depolarization in the presence of high Ca\(^{2+}\) exceeded those at tonic nerve terminals (Fig. 4). To test the possibility that the progressive decline in MEPC frequency at twitch nerve terminals is a result of a high initial MEPC frequency, we depolarized muscles in the presence of high calcium for 30–60 min to elicit high MEPC frequencies. Muscles were then depolarized for an additional 6–7 h in high-K\(^{+}\) and control Ca\(^{2+}\) solution and presynaptic function was optically assessed with FM1–43. In two such muscles, 91% of twitch nerve terminals (30 of 33) were uniformly stained by FM1–43 after 7–8 h of total depolarization. These data are similar to those from a muscle depolarized for 7 h in the presence of control Ca\(^{2+}\); all twitch nerve terminals (33 of 33, 1 muscle) were uniformly stained by FM1–43. Muscles depolarized in the presence of control calcium for 8 h yielded similar results (Table 1). These data indicate that an initial high-frequency burst of MEPCs alone is not sufficient to bring about the progressive decline in MEPC frequency observed at twitch nerve terminals after prolonged depolarization in the presence of Ca\(^{2+}\).

**MEPC frequency at twitch fiber endplates depolarized with strontium or barium substituted for high calcium**

The observation that MEPC frequency declined at twitch endplates after prolonged depolarization in the presence of high Ca\(^{2+}\) but not control Ca\(^{2+}\) suggests that Ca\(^{2+}\) might be involved in the time-dependent decrease in quantal release. One consequence of nerve terminal depolarization is that
intraterminal Ca^{2+} concentration increases as voltage-dependent Ca^{2+} channels open and is likely to be greater in preparations depolarized in the presence of high Ca^{2+}. To determine the role of Ca^{2+} and its specificity in the decline of MEPC frequency at depolarized twitch endplates, MEPC frequency was determined in preparations depolarized by high-K^{+} solution in which Sr^{2+} or Ba^{2+} were substituted for high Ca^{2+}. Strontium and barium can permeate voltage-activated Ca^{2+} channels and therefore accumulate in nerve terminals (Hagiwara and Byerly 1981). At twitch fiber endplates treated with either Sr^{2+} or Ba^{2+} in the high-K^{+} solution, MEPC frequency increased and then declined with a time course similar to that observed when preparations were exposed to high-K^{+} solutions containing high Ca^{2+} (Fig. 8A). This decline in MEPC frequency was unique to twitch fibers in that MEPC frequency at tonic endplates remained elevated during prolonged exposure to high-K^{+} solution containing either Sr^{2+} or Ba^{2+} (Fig. 8B). This MEPC frequency decline at twitch endplates in the presence of Sr^{2+} or Ba^{2+} was not accompanied by a reduction in mean MEPC amplitude (Fig. 9A) or an altered MEPC amplitude distribution (Fig. 9, B and C), although some large-amplitude MEPCs

FIG. 6. Twitch and tonic nerve terminals exhibit different presynaptic function during depolarization in a 35 mM K^{+} solution containing control or high Ca^{2+}. Twitch (†) and tonic (arrowhead) neuromuscular junctions were stained with both rhodamine-labeled peanut agglutinin (PNA) (A, C, E, G, and I) and FM1-43 (B, D, F, H, and J) after brief or prolonged exposure to 35 mM K^{+} solution containing either control or high levels of Ca^{2+}. A and B: 5 min in high K^{+} containing control Ca^{2+}. C and D: 5 min in high K^{+} containing high Ca^{2+}. E and F: 8 h in high K^{+} containing control Ca^{2+}. G–J: 8 h in high K^{+} containing high Ca^{2+}. Nerve terminals innervating tonic endplates were not stained by FM1-43 in high-K^{+} solution containing control Ca^{2+} (A, B, E, and F). Although both twitch and tonic nerve terminals were stained by FM1-43 after a brief exposure to high-K^{+} solution containing high Ca^{2+} (C and D), only tonic fibers were consistently stained after prolonged depolarization in the presence of high Ca^{2+} (G and H). Only a subset of boutons is stained by FM1-43 at a twitch neuromuscular junction depolarized for 8 h in the presence of high Ca^{2+} (I and J), suggesting that there are variable levels of quantal release and vesicle recycling between individual boutons. Bar: 50 μm.
during staining with FM1-43. Uniform stain: all nerve terminal boutons could not be attributed to a progressive reduction in MEPC

exposed to differing concentrations of calcium

TABLE 2. FM1-43 staining at twitch neuromuscular junctions exposed to differing concentrations of calcium

<table>
<thead>
<tr>
<th>[Ca(^{2+})](,) mM</th>
<th>Uniform stain</th>
<th>Partial stain</th>
<th>Unstained</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 ± 0 (3)</td>
<td>0 ± 0 (3)</td>
<td>100 ± 0 (3)</td>
</tr>
<tr>
<td>0.2</td>
<td>2 ± 2 (3)</td>
<td>4 ± 2 (3)</td>
<td>94 ± 1 (3)</td>
</tr>
<tr>
<td>0.4</td>
<td>46 ± 15 (3)</td>
<td>2 ± 2 (3)</td>
<td>51 ± 12 (3)</td>
</tr>
<tr>
<td>0.8</td>
<td>75 ± 11 (3)</td>
<td>1 ± 1 (3)</td>
<td>24 ± 11 (3)</td>
</tr>
<tr>
<td>1.0</td>
<td>97 ± 5 (6)</td>
<td>1 ± 1 (6)</td>
<td>3 ± 2 (6)</td>
</tr>
</tbody>
</table>

Values in Average Percentage of Twitch Neuromuscular Junctions Stained by FM1-43 are means ± SE, with numbers of muscle preparations in parentheses. Uniform stain: all nerve terminal boutons were stained. Partial stain: only a subset of nerve terminal boutons was stained. Unstained: no nerve terminal boutons were stained. Values in these columns are average percentages of neuromuscular junctions stained by FM1-43. For each data point, muscle preparations from ≥3 different snakes were analyzed and 15–31 twitch neuromuscular junctions were examined per muscle preparation.

were recorded that resembled those seen during depolarization in the presence of high Ca\(^{2+}\). MEPC amplitude also remained constant and amplitude histograms appeared unimodal at tonic endplates exposed for up to 8 h to a high-K\(^+\) solution containing Sr\(^{2+}\) or Ba\(^{2+}\) (data not shown).

Optical analysis of presynaptic function of twitch and tonic nerve terminals after an 8-h depolarization in the presence of 3.6 mM Sr\(^{2+}\) or Ba\(^{2+}\) confirmed that twitch nerve terminals selectively fail to maintain presynaptic release levels with prolonged depolarization in the presence of elevated divalent cations (Table 4). When muscle preparations were exposed to Sr\(^{2+}\) or Ba\(^{2+}\) for 5 min during staining with FM1-43, most twitch and tonic nerve terminals were uniformly stained by FM1-43. After an 8-h preincubation with depolarizing solution and 3.6 mM Sr\(^{2+}\) or Ba\(^{2+}\), a majority of tonic nerve terminals was uniformly stained by FM1-43. Twitch nerve terminals, however, were mainly unstained by FM1-43, although partial staining was sometimes observed. Al-

TABLE 3. Time course of FM1-43 staining at twitch and tonic neuromuscular junctions during high-K\(^+\) depolarization in the presence of 3.6 mM Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Time in 3.6 mM Ca(^{2+}) and High-K(^+) Solution, h</th>
<th>Average Percentage of Neuromuscular Junctions Stained by FM1-43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Twitch</td>
</tr>
<tr>
<td></td>
<td>Uniform stain</td>
</tr>
<tr>
<td>0</td>
<td>97 ± 3 (9)</td>
</tr>
<tr>
<td>1</td>
<td>78 ± 7 (4)</td>
</tr>
<tr>
<td>2</td>
<td>60 ± 8 (4)</td>
</tr>
<tr>
<td>4</td>
<td>42 ± 18 (4)</td>
</tr>
<tr>
<td>8</td>
<td>8 ± 6 (9)</td>
</tr>
</tbody>
</table>

Values in Average Percentage of Neuromuscular Junctions Stained by FM1-43 are means ± SE, with numbers of muscle preparations in parentheses. Each muscle was exposed to high-K\(^+\) solution for an additional 5 min during staining with FM1-43. Uniform stain: all nerve terminal boutons were stained. Partial stain: only a subset of nerve terminal boutons was stained. Values in these columns are average percentages of neuromuscular junctions stained by FM1-43. For each data point, muscle preparations from ≥3 different snakes were analyzed and 17–53 twitch or tonic neuromuscular junctions were examined per muscle preparation.

FIG. 7. Twitch nerve terminal, stained with PNA (A), displayed weak partial FM1-43 stain (B) after a 6-h depolarization in a high-K\(^+\), high-Ca\(^{2+}\) solution. C: same nerve terminal was restained with FM1-43 after an additional 2 h of washing in normal-Na\(^+\) solution. Boutons that were unstained by FM1-43 after prolonged depolarization were intact and able to incorporate detectable levels of FM1-43 after the 2-h wash. Bar: 30 \(\mu\)m. Though the pattern of FM1-43 staining in Sr\(^{2+}\) and Ba\(^{2+}\) solutions was similar to that observed in high-Ca\(^{2+}\) solution, a larger percentage of nerve terminals was stained at least partially by FM1-43 when exposed to Ca\(^{2+}\) as compared with Sr\(^{2+}\) or Ba\(^{2+}\).

D I S C U S S I O N

The results of the present study demonstrate that the presynaptic performance of nerve terminals innervating twitch and tonic muscle fibers differs in response to potassium-induced depolarization. With the use of the styryl dye FM1-43 as an optical assay, we demonstrated that nerve terminals innervating tonic fibers maintain high rates of quantal release whereas nerve terminals at twitch endplates do not. We also show that the inability of twitch nerve terminals to sustain high levels of quantal release occurs over a range of external K\(^+\) concentrations, and is due to neither a time-dependent decrease in MEPC amplitude nor the initial frequency of MEPC release. These results support the hypothesis that there are intrinsic differences in the presynaptic mechanisms regulating quantal transmitter release in nerve terminals innervating snake twitch or tonic endplates.

The decrease in MEPC frequency at twitch endplates could not be attributed to a progressive reduction in MEPC amplitude. MEPC amplitudes were the same in preparations depolarized with a 35 mM K\(^+\) solution containing either control or high Ca\(^{2+}\) and remained constant for up to 8 h of depolarization. This result differs from a previous report in
which mean MEPC amplitudes were progressively reduced when nerve terminals were depolarized in a 160 mM K+ solution containing high Ca2+ (Coniglio et al. 1993). We propose that the differing results reflect differences in experimental conditions. In Coniglio et al. (1993), muscle preparations were maintained in isotonic potassium so that nerve terminals were depolarized to ~0 mV. In the present study, muscles were maintained in 35 mM potassium, so the extent of nerve terminal depolarization was likely to be significantly less. We suggest that the progressive decrease in MEPC amplitude observed in preparations in isotonic potassium resulted from a reduction in vesicle filling, and further that processes regulating vesicle filling may be affected by both the extent and duration of membrane depolarization.

FM1-43 staining confirmed that vesicle fusion and recycling continued at high rates in nerve terminals innervating tonic muscle fibers under conditions in which MEPC frequencies had declined greatly at twitch endplates. The combination of electrophysiological and optical measurements of presynaptic function allows an estimation of the sensitivity with which FM1-43 can detect vesicle release and recycling in this system. In general, tonic endplates depolarized with a high-K+ solution containing control Ca2+ had a MEPC frequency of <0.5 per second and were unstained by FM1-43. Because many tonic endplates are multiply innervated, the rate of MEPC frequency from an individual terminal is likely to be <0.5 per second (Lichtman et al. 1985). In contrast, under conditions in which twitch and tonic endplates had MEPC frequencies of ~200 per second, nerve terminals were well stained by FM1-43. The intensity of FM1-43 staining also was an index of the level of MEPC frequency. For instance, nerve terminals innervating twitch endplates were more strongly stained by FM1-43 during a brief depolarization in the presence of high Ca2+ than during depolarization in the presence of control Ca2+.

Throughout this paper, the rates of quantal release were expressed as MEPC frequency per endplate. It is also interesting to consider the extent of presynaptic release per bouton given that the number of boutons differs at twitch and tonic neuromuscular junctions. There are roughly 3 times as many boutons at snake twitch neuromuscular junctions than at tonic endplates (Wilkinson and Lichtman 1985). The resting levels of release at individual twitch and tonic boutons are significantly different with MEPC frequency at twitch nerve terminals (~0.4 MEPCs per s) in control calcium solution, ~10-fold greater than that at tonic nerve terminals (~0.03 MEPCs per s) (Coniglio et al. 1993). Depolarization, in the presence of control calcium, the MEPC frequency at both twitch and tonic nerve terminals increases ~1,000-fold. Taking bouton estimates per endplate of 60 for twitch nerve terminals and 20 for tonic nerve terminals, the release rate per bouton at rest and during potassium stimulation is less at tonic boutons than at twitch boutons. This is consistent with our observations that tonic boutons were unstained by FM1-43 in control calcium whereas twitch boutons were stained. A difference in active zone number per bouton, as reported by Walrond and Reese (1985), could account for the difference in release rates at twitch and tonic boutons.

FM1-43 staining provided information about the release properties of nerve terminals at twitch endplates not attainable with electrophysiological recording. During brief depolarization of twitch nerve terminals with high-K+ solution containing either control or high Ca2+, all boutons were similarly stained by FM1-43, suggesting that roughly equivalent numbers of quanta were being released from each bouton. This was confirmed in preparations in which quantal release was reduced by lowering the external Ca2+ concentration. After prolonged exposure to a high-K+ solution containing high Ca2+, the partial pattern of FM1-43 staining of twitch endplates indicated that quantal release was no longer uniform among individual boutons at these twitch terminals, suggesting that the signal for the decline in quantal release may be local, thus allowing individual boutons within a given terminal to respond independently.

Experiments in which Sr2+ or Ba2+ was substituted for 3.6 mM Ca2+ in a high-K+ depolarizing solution demon-
FIG. 9. Size and distribution of MEPC amplitudes are plotted for twitch endplates, voltage clamped to 0 mV, during prolonged depolarization by 35 mM K+ solution containing either Sr²⁺ or Ba²⁺. A: mean MEPC amplitude does not decrease at twitch fiber endplates during prolonged exposure to high-K⁺ solution containing Sr²⁺ (○) or Ba²⁺ (●) substituted for Ca²⁺. For each solution, data were obtained from 3 muscles. B: MEPC distribution is plotted for a twitch fiber exposed for ~400 min to a 35 mM K⁺ solution containing 3.6 mM Sr²⁺. C: MEPC distribution is plotted for a twitch fiber exposed for ~400 min to a 35 mM K⁺ solution containing 3.6 mM Ba²⁺. The mean and mode values for the MEPC amplitudes are ~9.5 and ~9.1 nA (B) and ~10.4 and ~9.8 nA (C), respectively.

Demonstrated that both divalent cations can stimulate quantal release at potassium-depolarized twitch and tonic endplates. Both Sr²⁺ and Ba²⁺ are permeable through voltage-gated Ca²⁺ channels (Hagiwara and Byerly 1981). Although not as effective as Ca²⁺, Sr²⁺ supports neurally evoked transmitter release at neuromuscular junctions (Miledi 1966; Silinsky et al. 1995). Barium, under some conditions, supports transmitter release from motor nerve terminals; it activates the posttetanic rise in MEPC frequency but fails to support evoked release (Quastel and Saint 1988; Silinsky 1977, 1978; Van der Kloot and Molgo 1994). These experiments also demonstrated that during prolonged depolarization in the presence of Sr²⁺ or Ba²⁺, presynaptic release at twitch endplates was not sustained, whereas an elevated MEPC frequency and FM1-43 staining persisted at tonic endplates. These results suggest that the process governing the progressive decline in MEPC frequency at twitch but not tonic endplates is not Ca²⁺ specific and can be supported by other divalent cations that have Ca²⁺-like properties.

Because it is likely that calcium plays a role in the differential release observed between tonic and twitch nerve terminals, one would like to know the mechanism by which calcium might act to selectively depress presynaptic release in twitch nerve terminals. During nerve terminal depolarization, Ca²⁺ influx through voltage-gated Ca²⁺ channels raises intraterminal Ca²⁺ levels. The observed difference in presynaptic function between twitch and tonic nerve terminals could result from differing levels of Ca²⁺ influx during depolarization. Intraterminal Ca²⁺ levels during depolarization might be markedly less in tonic than in twitch nerve terminals because there are fewer Ca²⁺ channels in active zones of tonic nerve terminals compared with twitch terminals (Walrond and Reese 1985).

One possible effect of prolonged exposure to high-K⁺ and high-Ca²⁺ solutions is that twitch nerve terminals are irreversibly damaged as a consequence of prolonged calcium entry. Indeed, Coniglio et al. (1993) found that long-term depolarization of nerve terminals by exposure to 160 mM K⁺ in the presence of both control and high Ca²⁺ levels resulted in ultrastructural changes including loss of synaptic
TABLE 4. FM1-43 staining at twitch and tonic neuromuscular junctions during brief or prolonged depolarization in the presence of Ca$^{2+}$, Sr$^{2+}$, or Ba$^{2+}$

<table>
<thead>
<tr>
<th>Divalent Cation</th>
<th>Time in High-K$^+$ Solution, h</th>
<th>Average Percentage of Neuromuscular Junctions Stained by FM1-43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uniform stain</td>
<td>Partial stain</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0</td>
<td>97 ± 3 (9)</td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>0</td>
<td>98 ± 2 (4)</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0</td>
<td>96 ± 5 (4)</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>8</td>
<td>8 ± 6 (9)</td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>8</td>
<td>0 ± 0 (4)</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>8</td>
<td>8 ± 5 (4)</td>
</tr>
</tbody>
</table>

Values in Average Percentage of Neuromuscular Junctions Stained by FM1-43 are means ± SE, with numbers of muscle preparations in parentheses. Each muscle was exposed to high-K$^+$ solution for an additional 5 min during staining with FM1-43. Uniform stain: all nerve terminal boutons were stained. Partial stain: only a subset of nerve terminal boutons was stained. Values in these columns are average percentages of neuromuscular junctions stained by FM1-43. For each data point, muscle preparations from 2 different snakes were analyzed and 17–53 twitch or tonic neuromuscular junctions were examined per muscle preparation.

vesicles, mitochondrial swelling, and appearance of intramembranous inclusions. The results reported here indicate that twitch nerve terminals recovered presynaptic function after muscles were returned to normal-Na$^+$ solution after prolonged depolarization. Thus the failure of twitch nerve terminals to release neurotransmitter during prolonged depolarization is not due to irreversible damage to nerve terminal boutons.

More likely is the possibility that the elevated intraterminal calcium level in twitch nerve terminals suppresses the release process itself. Two sites of calcium action are the vesicular release mechanism and the vesicle membrane recycling mechanism. The primary effect of an increase in intraterminal Ca$^{2+}$ concentration is stimulation of quantal release. As intraterminal Ca$^{2+}$ continues to rise, vesicle membrane endocytosis can become inhibited (von Gersdorff and Matthews 1994). Thus an elevated intraterminal Ca$^{2+}$ concentration would result in suppression of presynaptic function as a consequence of the combined stimulation of vesicle release and inhibition of vesicle recycling. This mechanism of calcium action would result in silent twitch nerve terminals that are depleted of synaptic vesicles. Alternatively, elevated intraterminal Ca$^{2+}$ may directly inhibit mechanisms regulating vesicular release at twitch endplates during prolonged depolarization in the presence of high Ca$^{2+}$. Under these conditions, nerve terminals would not necessarily be depleted of synaptic vesicles. Preliminary evidence suggesting that vesicle depletion is not the sole cause of the decreased MEPC frequency comes from twitch endplates that were silent after prolonged depolarization with high-K$^+$ solution containing high Ca$^{2+}$. In some preparations, transient bursts of MEPCs were recorded at otherwise silent twitch endplates. Also, high-frequency bursts of MEPCs could sometimes be mechanically elicited from silent twitch endplates either by stretching the nerve or impaling the nerve terminal (Parsons and Griffiths, unpublished observations). We suggest that both mechanisms, inactivation of the release process and vesicle depletion, may be involved in the decrease in MEPC frequency at twitch nerve terminals during prolonged depolarization in the presence of high Ca$^{2+}$. An ultrastructural analysis of the changes in nerve terminal morphology is currently underway. The results of these studies should demonstrate whether vesicle depletion plays a primary role in determining the progressive decrease in MEPC frequency with partial depolarization and elevated calcium concentrations.

In summary, results of the present study demonstrate that presynaptic function is different at snake twitch and tonic endplates after prolonged depolarization with high K$^+$; nerve terminals at twitch endplates do not sustain quantal release as effectively as nerve terminals at tonic endplates. The inability of frog twitch terminals to sustain quantal release during prolonged depolarization has also been reported by Molenaar and Oen (1988). It would be interesting to test whether a similar difference in presynaptic function occurs at snake twitch and tonic endplates with neural stimulation because the dynamics and perhaps mechanisms regulating spontaneous and neurally evoked release may differ (Zefirov et al. 1995). It is also interesting to speculate about the physiological implications of the differences in twitch and tonic nerve terminal properties. Twitch nerve terminals control phasic motor units and therefore a large amount of neurotransmitter is released to ensure the generation of a suprathreshold endplate potential. In contrast, tonic fibers are designed to maintain neurally evoked contractures for prolonged periods. In this case, neurotransmitter release may not need to be massive but must be sustained for prolonged periods. We postulate that a critical level of intraterminal Ca$^{2+}$ in twitch nerve terminals may be a factor promoting the progressive decline in quantal release. Future studies comparing the type and distribution of Ca$^{2+}$ channels and the dynamics of intraterminal Ca$^{2+}$ accumulation during stimulation of nerve terminals innervating twitch and tonic muscle fibers may determine the mechanism underlying the observed difference in presynaptic function reported here. Finally, we have demonstrated that the use of FM1-43 as an optical assay of presynaptic release provides additional insight into the dynamics of presynaptic function between boutons at individual neuromuscular junctions.

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