Ca\textsuperscript{2+} Dependence of the Binomial Parameters \(p\) and \(n\) at the Mouse Neuromuscular Junction

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Wang X, Pinter MJ, Rich MM. Ca\textsuperscript{2+} dependence of the binomial parameters \(p\) and \(n\) at the mouse neuromuscular junction. J Neurophysiol 103: 659–666, 2010. First published November 25, 2009; doi:10.1152/jn.00708.2009. The Ca\textsuperscript{2+} dependence of synaptic quantal release is generally thought to be restricted to probability of vesicular release. However, some studies have suggested that the number of release sites (\(n\)) at the neuromuscular junction (NMJ) is also Ca\textsuperscript{2+} dependent. In this study, we recorded endplate currents over a wide range of extracellular Ca\textsuperscript{2+} concentrations and found the expected Ca\textsuperscript{2+} dependency of release. A graphical technique was used to estimate \(p\) (probability of release) and \(n\) using standard binomial assumptions. The results suggested \(n\) was Ca\textsuperscript{2+} dependent. The data were simulated using compound binomial statistics with variable \(n\) (Ca\textsuperscript{2+} dependent) or fixed \(n\) (Ca\textsuperscript{2+} independent). With fixed \(n\), successful simulation of increasing Ca\textsuperscript{2+} required that \(p\) increase abruptly at some sites from very low to high values. Successful simulation with variable \(n\) required the introduction of previously silent release sites (\(p = 0\)) with high values of \(p\). Thus the success of both simulations required abrupt, large increases of \(p\) at a subset of release sites with initially low or zero \(p\). Estimates of the time course of release obtained by deconvolving evoked endplate currents with average miniature endplate currents decreased slightly as Ca\textsuperscript{2+} increased, thus arguing against sequential release of multiple quanta at higher Ca\textsuperscript{2+} levels. Our results suggest that the apparent Ca\textsuperscript{2+} dependence of \(n\) at the NMJ can be explained by an underlying Ca\textsuperscript{2+} dependence of a spatially variable \(p\) such that \(p\) increases abruptly at a subset of sites as Ca\textsuperscript{2+} is increased.

INTRODUCTION

The Ca\textsuperscript{2+} dependence of vesicular release is a widely recognized property of synapses. However, despite years of work, uncertainty remains about the mechanism(s) that govern this dependence and which properties of the release process Ca\textsuperscript{2+} controls. Early work established that the probability of quantal release (\(p\)) from a given release site is controlled by the concentration of external Ca\textsuperscript{2+} (Del Castillo and Katz 1954; Dodge and Rahamimoff 1967; Katz and Miledi 1965). Based largely on statistical modeling of the release process, the number of release sites (\(n\)) was thought to be fixed such that only probability of release (\(p\)) was altered with changes in external Ca\textsuperscript{2+} (for review, see Korn and Faber 1987).

Subsequent studies at the neuromuscular junction (NMJ) reported, however, that \(n\) is also controlled by extracellular Ca\textsuperscript{2+} (Bennett et al. 1975, 1977; Di Gregorio et al. 1996; Miyamoto 1975; Searl and Silinsky 2002, 2003). One study reported that \(n\) had a much steeper Ca\textsuperscript{2+} dependence than \(p\) (Bennett et al. 1975). Others have argued that the Ca\textsuperscript{2+} dependence of \(n\) is only apparent and arises because of technical limitations or incorrect assumptions made during calculations used to determine \(p\) and \(n\) (Barton and Cohen 1977; Brown et al. 1976; Clements and Silver 2000; Lustig et al. 1986; Redman 1990; Zucker and Regehr 2002). Specific concerns involve assumptions that \(p\) is constant over time and uniform among all release sites at a synapse (Korn and Faber 1987; Miyamoto 1986; Provan and Miyamoto 1993; Redman 1990; Regehr and Stevens 2001; Silver 2003). Another concern is the assumption that single sites only release contents of single vesicles (Auger and Marty 2000). Because of these concerns, there is no agreement at present about the role of Ca\textsuperscript{2+} in regulating the number of functional synaptic release sites.

Disadvantages of previous studies of \(p\) and \(n\) at the NMJ include use of low external Ca\textsuperscript{2+} (Bennett et al. 1975; Searl and Silinsky 2003) or use of higher Ca\textsuperscript{2+} levels and voltage recording compromised by nonlinear summation of endplate potentials (Di Gregorio et al. 1996; Miyamoto 1975). In this study, endplate currents were recorded from crushed muscle fibers (Argentieri et al. 1992; Barstad and Lillehei 1968; Glavinovic 1979) in normal and high Ca\textsuperscript{2+} solutions, thus avoiding complications of voltage recording and the need for pharmacologic treatments to inhibit release. Release was characterized using binomial assumptions and a graphical technique to estimate \(p\) and \(n\) (Clements 2003; Clements and Silver 2000; Silver 2003). The main concern of previous studies involved the assumption that \(p\) is uniform at all \(n\). To address this concern, statistical modeling was used to assess how \(p\) and \(n\) could be varied to match data obtained as Ca\textsuperscript{2+} concentrations were raised. The results of this study suggest that the number of high probability release sites increases at the mouse NMJ as Ca\textsuperscript{2+} is increased and that the process involves a sudden shift from a low or zero \(p\) to high \(p\) at a subset of release sites.

METHODS

Ethical approval

Two to 4-mo-old mice were killed using CO\textsubscript{2} inhalation, and the tibialis anterior muscle was removed. All procedures involving animals were approved by the Wright State LACUC committee.

NMJ recording

For all experiments, the recording chamber was continuously perfused with Ringer solution containing (in mM) 118 NaCl, 0.7 MgCl\textsubscript{2}, 3.5 KCl, 26.2 NaHCO\textsubscript{3}, 1.7 NaH\textsubscript{2}PO\textsubscript{4}, and 5.5 glucose (pH 7.3–7.4,
20–22°C, equilibrated with 95% O₂-5% CO₂. Ca²⁺ concentration was changed as indicated to vary quantal content (m) between 30 and 100. To determine whether effects on m were caused by changes in divalent cation concentration, data from four muscles were obtained while total divalent cation concentration was maintained at 2.7 mM. Recordings from two muscles were obtained in solution with 2.6 mM Ca²⁺ and 0.1 mM Mg²⁺. In these muscles, m was close to 71, and the variance of m [Var(m)] was close to 3.5. Records from two other muscles were obtained in solution with 1.2 mM Ca²⁺ and 1.5 mM Mg²⁺. In these muscles, m was close to 42, and Var(m) was close to 12. These values agreed well with data plotted in Fig. 2, which was obtained by varying Ca²⁺ without keeping the concentration of divalent cations fixed.

Endplate recordings were performed as previously described (Wang et al. 2004). After dissection, the tibialis anterior muscle was partially bisected and folded apart to flatten the muscle. After pinning in a sylastic dish, muscle strips were stained with 10 µM 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-Di-2ASP, Invitrogen, Carlsbad, CA) and imaged with an upright epifluorescence microscope. Muscle fibers were crushed away from the endplate band to eliminate contractions after nerve stimulation, and two-electrode voltage clamp was used to set the holding potential to ~−45 mV. The nerve to the tibialis anterior was stimulated with 0.2-ms current pulses at 0.5 Hz using a bipolar electrode (FHC, Bowdoin, ME). Stimulus intensity was maintained at 2× threshold for the appearance of EPCs. Endplates were imaged and muscle fibers were imaged within 100 µm of the endplate to ensure good space clamp of the endplate region.

MEPCs were recorded for 1 min without stimulation. Because there is no spontaneous firing of axon action potentials in our preparation, TTX was not applied during recording of MEPCs. Fibers with <30 MEPCs in 1 min of recording were discarded. The average MEPC sample size was 50. MEPC CV averaged 26%. Calculations of MEPCs in 1 min of recording were discarded. The average MEPC TTX was not applied during recording of MEPCs. Fibers with average MEPC for that endplate.

For measurement of EPC variance, ≥20 stimulations of the nerve were performed at 0.5 Hz. However, even at this low rate of stimulation, there was slight depression of the EPC during the first three pulses when Ca²⁺ was 5.0 mM. This depression led to overestimation of EPC variance so the first three EPCs were discarded from measurement of EPC variance, leaving ≥17 values for analysis. The average number of EPCs measured to determine EPC variance was 22. Endplate recordings in which there was movement artifact or a change in EPC amplitude with time were discarded because such artifacts led to large increases in variance. To determine the Ca²⁺ dependence of p and n, m and Var(m) were measured for at least five endplates in each muscle studied. For each muscle, the average m and Var(m) were calculated. The average of m and Var(m) for each muscle were averaged to generate plots.

Deconvolution analysis of EPCs was performed as previously described (Rich et al. 2002). Deconvolution analysis provides an estimate of the rate at which quanta (represented by the average MEPC) are released to account for the features of the average EPC (Borges et al. 1995; Diamond and Jahr 1995; Van der Kloot 1988a,b). Measurement obtained from the time course of synaptic release included maximum amplitude, time-to-peak, and half-width. To obtain an estimate of total release expressed in quantal units, the time course of synaptic release was integrated from its onset to a point where the function returned to baseline.

Statistical modeling

The binomial equation has been commonly used to describe the statistical properties of synaptic release (Korn and Faber 1987; Redman 1990). In this relationship, the probability of synaptic release (p) determines the number of release sites (N) that are activated per trial out of a maximum number of available release sites (m). To obtain estimates of the parameters n and p from experimental data under the assumption of binomial release statistics, we used a graphical approach described previously (Clements and Silver 2000). This approach is based on the relationships defining the expected number E(N) and variance of release sites activated per trial under binomial constraints. Thus

\[
E(N) = np
\]

\[
Var(N) = np(1 - p)
\]

The assumptions are made that the measured average m is equivalent to the expected number of activated release sites and that Var(m) is equivalent to the variance in the number of release sites activated per trial. Thus

\[
m = E(N) = np
\]

\[
Var(m) = Var(N) = m(1 - m/n)
\]

Estimates of n and p are obtained by plotting experimental values of m and Var(m) on a grid composed of parabolas and straight lines generated by determining m and Var(m) while varying p and holding n constant (to generate the parabolas) and by determining m and Var(m) while varying n and holding p constant (to generate the straight lines).

One of the major assumptions made in applying binomial statistics to describe the release properties of a synapse that involves many release sites (such as the NMJ) is the equivalence of p at all n (Redman 1990; Zucker and Regehr 2002). To assess how violations of this and other assumptions may have contributed to the graphical determination of n and p from experimental data, we performed a series of simulations in which various binomial constraints were eliminated. The simulations were based on a binomial random number generator supplied by values of n and p. Values of p were determined in a number of ways. In the default setting, p was assumed to be constant across all n. To simulate the case of nonuniform p, the selected value of p was assumed to represent the average p of all n, and each n was assigned a value determined by a β distribution random number generator supplied by the average p value and an assumed CV. In most cases of uniform and nonuniform p, values of p remained constant during simulation runs. In other instances, p was assumed to vary with time at individual n. Initially, values of p were assigned to sites as in the nonuniform case described above. During each trial, values were reassigned using the initially assigned value of p as the mean of a β distribution and an assumed CV to describe time variability. Computer code was written in C, and random number generators were obtained from the RANDLIB library (M.D. Anderson Cancer Center, Houston, TX).

RESULTS

At the mouse NMJ, mean quantal amplitude (amplitude of the MEPC) can easily be determined and used to directly calculate quantal content (m). We measured EPC amplitude, variance of EPC amplitude, and average MEPC amplitude to directly calculate Var(m) (Fig. 1). This allowed us to directly estimate changes in p and n for individual NMJs (Fig. 1). This analysis assumes that variance of MEPC amplitude does not contribute significantly to variance of EPC amplitude. To verify this assumption, we measured MEPC variance at 59 NMJs from four muscles and found that, although CV of MEPCs (26%) was greater than CV of EPCs (maximum 10% in 1 mM Ca²⁺; Table 1), MEPC variance was low relative to EPC variance. Whereas MEPC variance was 0.11 ± 0.01 nA², in 1 mM Ca²⁺, EPC variance was close to 13 nA², and in 5 mM external Ca²⁺, EPC variance was still >3 nA². Thus for
all concentrations of extracellular Ca$^{2+}$ used, MEPC variance was ~4% of EPC variance. Because variances are additive, these data show that MEPC variance constitutes a very small fraction of EPC variance such that we could ignore MEPC variance in all subsequent analysis of Var($m$).

Assuming uniform and stationary $p$ for all $n$, we plotted changes in $p$ and $n$ as $m$ by changing external Ca$^{2+}$. Both the average value of $p$ and $n$ changed with alterations in external Ca$^{2+}$ when $p$ was $>0.5$ (Fig. 2; Table 1). Although this could be interpreted as showing Ca$^{2+}$ dependence of both $p$ and $n$, it is possible that the increase in $n$ with higher concentrations of extracellular Ca$^{2+}$ is an artifact that is because of assumptions of uniform and stationary $p$ (Korn and Faber 1987; Miyamoto 1986; Provan and Miyamoto 1993; Redman 1990; Regehr and Stevens 2001; Silver 2003).

We examined the assumptions of uniform and stationary $p$ to determine whether they could account for the findings presented in Fig. 2 and Table 1. The binomial model of release assumes that the value of $p$ at each release site is uniform over time (stationary). One way in which $p$ might not be stationary is that the mean value of $p$ for the entire synapse might drift during data collection. This would have profound effects on ANOVA. We thus only analyzed data from endplates in which recordings of both EPCs and MEPCs were stable over the recording intervals (see example in Fig. 1). Another way that $p$ might be nonstationary is for $p$ at individual release sites to vary with time while the overall mean $p$ is stationary. To quantify the effect of nonstationary $p$ at individual release sites on ANOVA, we developed a model of binomial release with nonstationary $p$. We allowed $p$ at each site to vary over time around a mean value of $p$ for that site that was assigned from a distribution of mean release probabilities. When nonstationary $p$ was included in the model, it had no effect on estimates of $p$ and $n$ (data not shown). Thus the effects of nonstationary $p$ at individual release sites were ignored in ANOVA.

The binomial model of release also assumes that $p$ is uniform between synaptic sites. Focal extracellular recordings and impalement at different sites along the length of the terminal in the frog NMJ suggests that release probabilities differ between synaptic sites of a single terminal (Bennett and Lavidis 1982; Bennett et al. 1986; D’Alonzo and Grinnell 1988; Faber 1987; Miyamoto 1986; Provan and Miyamoto 1993; Redman 1990; Regehr and Stevens 2001; Silver 2003).

![Figure 1](http://jn.physiology.org/)

**TABLE 1.** Effect of changes in extracellular Ca$^{2+}$ on $m$, $p$, and $n$

<table>
<thead>
<tr>
<th>Ca$^{2+}$</th>
<th>$m$</th>
<th>Var($m$)</th>
<th>$p$</th>
<th>$n$</th>
<th>CV</th>
<th>Number of Muscles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>33.6 ± 1.3</td>
<td>11.0 ± 1.1</td>
<td>.67 ± .04</td>
<td>50.4 ± 2.7</td>
<td>9.8 ± 0.6%</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>40.9 ± 1.0</td>
<td>7.6 ± 1.0</td>
<td>.81 ± .02</td>
<td>50.3 ± 5.4</td>
<td>6.9 ± 0.8%</td>
<td>5</td>
</tr>
<tr>
<td>1.6</td>
<td>58.3 ± 4.1</td>
<td>7.9 ± 0.8</td>
<td>.86 ± .01</td>
<td>67.6 ± 4.5</td>
<td>4.9 ± 0.3%</td>
<td>4</td>
</tr>
<tr>
<td>2.0</td>
<td>69.8 ± 6.7</td>
<td>4.3 ± 0.3</td>
<td>.93 ± .01</td>
<td>71.7 ± 6.8</td>
<td>3.1 ± 0.3%</td>
<td>5</td>
</tr>
<tr>
<td>5.0</td>
<td>90.9 ± 2.1</td>
<td>3.5 ± 0.7</td>
<td>.96 ± .01</td>
<td>94.6 ± 2.3</td>
<td>2.0 ± 0.2%</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are mean ± SE. $m$ was calculated by dividing the amplitude of the endplate current by the amplitude of the average miniature endplate current. For each muscle, the mean values of each parameter were calculated. The average of each parameter was calculated by taking the mean of the muscle means.

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and the CV of mean \( p \) were varied. We were again able to closely approximate the data shown in Fig. 2 (Fig. 3A, ■). However, to replicate the data in Fig. 2, it was necessary to include a high spatial variability of mean \( p \). When mean \( p \) was 0.35 and 0.45, it was necessary to use a CV for \( p \) of 0.9. As mean \( p \) was increased, there was never an increase in the fraction of sites with \( p \) ranging from 0.3 to 0.7. Instead, there was a reduction in the percentage of sites with \( p < 0.1 \) and an increase in sites with \( p > 0.9 \) (Fig. 3B, bottom row). Thus the data in Fig. 2 can be simulated by introducing spatially non-uniform \( p \) and varying either \( p \) alone or by varying both \( p \) and \( n \). In both modeling situations, however, it is necessary to postulate that as extracellular \( \text{Ca}^{2+} \) is increased, some synaptic sites abruptly change from being functionally silent (0 or low \( p \)) to operational with high \( p \). There are no indications from this modeling that, after \( p \) is increased abruptly, these sites behave any differently than other sites.

**Time course of release**

Our modeling suggests that the apparent increase in \( n \) as \( \text{Ca}^{2+} \) is increased can be explained by abrupt increases in \( p \) at a subset of release sites. An alternate explanation is that the apparent increase in \( n \) occurs because of release of multiple vesicles in rapid succession (prolonged release) when extracellular \( \text{Ca}^{2+} \) is high (Auger and Marty 2000; Regehr and Stevens 2001). To address this possibility, the time course of release was measured using deconvolution of the EPC waveform as previously described (Rich et al. 2002). Sequential release of vesicles would lead to widening of the release waveform that results from deconvolution of average EPCs with average MEPCs from the same endplate. Shown in Fig. 4 are the average EPC, MEPC, and deconvolution waveforms from an endplate in solution containing 1 mM \( \text{Ca}^{2+} \) and an endplate in 5 mM \( \text{Ca}^{2+} \). The initial synchronous wave of vesicular release is greater in 5 mM \( \text{Ca}^{2+} \), but there was no statistically significant difference in the time to peak of the release waveform (Fig. 4; Table 2). A statistically significant narrowing of the half-width of the peak release waveform occurred in 5 mM \( \text{Ca}^{2+} \) rather than a widening of half-width as

**Data simulations**

We performed two simulations of the data obtained at various levels of external \( \text{Ca}^{2+} \). In the first simulation, \( p, n, \) and the CV of mean \( p \) were allowed to vary. Release was simulated with five different sets of parameters designed to match the data points in Fig. 2 and Table 1. The values of mean \( p, n, \) and the distributions of \( p, n \) used for simulating release for each of the data points are shown in the top row of Fig. 3B. The values resulting from the simulation are plotted in Fig. 3A and closely match the data shown in Fig. 2. In the second simulation, \( n \) was \( \text{Ca}^{2+} \) independent and fixed at 100. Only mean \( p \)
would be expected if there were sequential release of vesicles (Table 2).

The increase in peak release rate agreed with the increase predicted by the increase in p and the apparent increase in n. As extracellular Ca\(^{2+}\) is increased from 1 to 5 mM, p increases from 0.67 to 0.96 (close to a 50% increase; Table 1), and n increases from 50 to 95 (close to a 100% increase; Table 1). Thus if the apparent increase in n is a result of simultaneous release of vesicles, the peak of the deconvolution waveform should increase by close to 200% (p × n, 1.5 × 2 = 3, a 200% increase). As predicted, the deconvolution waveform had a peak rate of vesicular release that was increased by close to 200% in 5 mM Ca\(^{2+}\) (Fig. 4; Table 2). Thus if a single release site releases more than one vesicle in solution containing high Ca, the release occurs synchronously. There is precedence for synchronous multiquantal release from what may be single synaptic sites (Llano et al. 2000; Oertner et al. 2002; Wadiche and Jahr 2001). The primary difficulty posed for our analysis by the possibility of synchronous multivesicular release is that it might partially saturate postsynaptic receptors and thus cause underestimation of both m and n (Silver 2003). Otherwise, this release mode is functionally similar to activating release sites and has little effect on conclusions (Clements 2003).

### Table 2. Ca\(^{2+}\) dependence of temporal characteristics of vesicular release

<table>
<thead>
<tr>
<th></th>
<th>1 mM Ca(^{2+})</th>
<th>5 mM Ca(^{2+})</th>
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<tbody>
<tr>
<td>Peak rate, vesicles/ms</td>
<td>92.6 ± 6.4*</td>
<td>285.0 ± 16.4</td>
</tr>
<tr>
<td>Time to peak of release, ms</td>
<td>0.326 ± 0.009</td>
<td>0.353 ± 0.019</td>
</tr>
<tr>
<td>Half-width of peak release, ms</td>
<td>0.324 ± 0.006*</td>
<td>0.256 ± 0.011</td>
</tr>
<tr>
<td>m by deconvolution</td>
<td>38.5 ± 2.6*</td>
<td>145.3 ± 8.9</td>
</tr>
<tr>
<td>n by EPC/MEPC</td>
<td>33.6 ± 1.3*</td>
<td>90.9 ± 2.1</td>
</tr>
<tr>
<td>Difference between MEPC and EPC half-width (ms)</td>
<td>0.193 ± 0.017*</td>
<td>0.901 ± 0.051</td>
</tr>
</tbody>
</table>

Values are means ± SE. The first 4 rows represent values obtained by deconvolution of EPCs by the average MEPC for the endplate being analyzed. m by deconvolution is measured by integration of the deconvolution waveform and includes both early and prolonged release of vesicles. n by EPC/MEPC is measured by dividing the peak amplitude of the EPC by the peak amplitude of the MEPC and only includes early release. See Table for abbreviations. *P < 0.01 between 1 mM Ca\(^{2+}\) and 5 mM Ca\(^{2+}\).

**DISCUSSION**

The Ca\(^{2+}\) dependence of the binomial parameter n used to describe synaptic release has remained unclear. In this study, we recorded endplate currents over a physiological range of Ca\(^{2+}\) concentrations (Fig. 5; Table 2). Although prolonged release did not alter estimates of n, it caused a significant increase in m in solution containing 5 mM Ca\(^{2+}\) when m was measured using deconvolution (Table 2). Prolonged release may be related to delayed release of vesicles described previously as the period of elevated spontaneous quantal release that occurs after nerve stimulation (Rahamimoff and Yaari 1973; Van der Kloot and Molgo 1994). It seems likely that both processes are caused by residual Ca\(^{2+}\) (Rahamimoff and Yaari 1973; Van der Kloot and Molgo 1994; Zucker and Regehr 2002).

**FIG. 4.** Ca\(^{2+}\) dependence of the time course of vesicular release. A: top: the EPC and MEPC used for deconvolution from an endplate recorded from in 1 mM Ca\(^{2+}\). Bottom: the EPC and MEPC used for deconvolution from an endplate recorded from in 5 mM Ca\(^{2+}\). The vertical scales for EPCs and MEPCs are 20 and 1 nA, respectively. B: the deconvolution waveforms from the endplate current traces shown in A. The peak rate of vesicular release is significantly higher in 5 mM Ca\(^{2+}\). The vertical scale for the deconvolution waveforms is in quanta per millisecond, and the time base is the same as in A. C: the deconvolution waveforms from B have been normalized and superimposed. Although the peak of the deconvolution waveform is similar in time to peak and width, the presence of prolonged release of vesicles after the peak is more prominent in the deconvolution waveform from the endplate in 5 mM Ca\(^{2+}\).

**FIG. 5.** Prolonged release of vesicles widens the EPC but does not contribute to EPC amplitude. A: the EPC traces from Fig. 4 and the superimposed inverted deconvolution waveforms for the same endplates (gray). Prolonged release of vesicles occurs after the peak of the EPC (dotted line). B: the normalized fitted MEPCs (gray) and average EPCs (black) from the same endplates in A. The EPC recorded in 1 mM Ca\(^{2+}\) has a half-width that is only slightly wider than the fitted MEPC from the same endplate. However, the EPC recorded in 5 mM Ca\(^{2+}\) has a significantly wider half-width than the MEPC from the same endplate because of prolonged release of vesicles.
extracellular Ca\(^{2+}\) concentrations and used a graphical technique (Clements 2003; Clements and Silver 2000; Silver 2003) to estimate probability of release (\(p\)) and number of release sites (\(n\)), assuming simple binomial statistics. The results suggested that both \(p\) and \(n\) were Ca\(^{2+}\) dependent. Modeling of the data was performed using compound binomial statistics with fixed \(n\) (not Ca\(^{2+}\) dependent) or variable \(n\) (Ca\(^{2+}\) dependent). The results were similar in a fundamental way; in both cases, sites with low or zero \(p\) changed abruptly to sites with high \(p\) as Ca\(^{2+}\) increased. An alternate explanation of our data are that sites begin to release multiple quanta in quick succession as Ca\(^{2+}\) is raised. Rapid sequential release of vesicles would increase the duration of peak release rates, but deconvolution of endplate currents showed no prolongation and thus argued against this mechanism. Our data and modeling suggest that the apparent Ca\(^{2+}\) dependence of \(n\) can be explained by abrupt increases in \(p\) at a subset of release sites.

**Physical correlates of \(p\) and \(n\)**

The physical meanings of the statistical parameters \(p\) and \(n\) play a crucial role in the interpretation of our data. Although it is generally agreed that \(p\) represents the probability of quantal release, there is uncertainty as to how many vesicles are specified by this parameter. In the simplest interpretation, each release site (active zone) has only one releasable vesicle that has a probability of release \(p\) (Korn and Faber 1991; Redman 1990). For the purpose of studying the Ca\(^{2+}\) dependence of \(n\), it does not matter whether \(p\) represents the probability of release for a single vesicle or group of vesicles at a release site.

Evidence from the frog NMJ indicates that \(n\) represents the number of active zones (Kelly and Robbins 1987; Wernig 1975). Alternatively, \(n\) may represent the number of releasable (docked) vesicles (Searl and Silinsky 2002; Stevens 2003). If \(n\) represents the number of active zones, potential mechanisms that could account for Ca\(^{2+}\) dependence of \(n\) would include the presence of vesicles at active zones or the number of functional active zones. If \(n\) represents the number of releasable vesicles, changes could represent either priming of vesicles to make them release ready or movement of vesicles nearer the membrane. For simplicity, \(n\) will be discussed in the following text as if it represents the number of synaptic release sites, but conclusions could just as easily be applied if \(n\) represents release sites with docked vesicles or release-ready vesicles; in that case, the issue becomes the Ca\(^{2+}\) dependence of vesicle docking or recruitment of vesicles into the releasable pool. Despite the uncertainty of the physical correlates of \(p\) and \(n\), evidence indicates that these are independently regulated parameters controlling release because treatment with phorbol esters or adenosine at the NMJ alter \(n\) without changing \(p\) (Searl and Silinsky 2003).

**Potential mechanisms underlying increased \(n\) or abrupt increases in \(p\) at some release sites**

We were able to model the apparent Ca\(^{2+}\) dependence of \(n\) by manipulations of \(p\). When \(n\) was Ca\(^{2+}\) dependent (not fixed), the apparent increase in \(n\) could be modeled by introducing new (previously silent or 0 \(p\)) release sites as Ca\(^{2+}\) was raised. When \(n\) was Ca\(^{2+}\) independent (fixed), the experimental data were fit by abruptly increasing \(p\) among a subset of initially low \(p\) sites as Ca\(^{2+}\) was raised. Despite including spatially variable \(p\), the data could not be modeled in either case by only increasing \(p\) in a graded manner among previously active sites. Thus in both cases, simulation of the data required abrupt increases of \(p\) at some release sites as Ca\(^{2+}\) was raised.

These results suggest that some release sites at the NMJ possess low or zero \(p\) in normal Ca\(^{2+}\) and are thus functionally silent. The recruitment of such sites likely plays a role in activity-induced potentiation of release and other forms of plasticity at the motor terminal that may help to increase the reliability of motor unit activation. Because acetylcholine receptors are present at such high density in the postsynaptic membrane (Salpeter 1987), it seems probable that mechanisms underlying silent release sites are located presynaptically. Presynaptic mechanisms underlying silent synapses have been described at the goldfish Mauthner cell (Lin and Faber 1988a,b) and the crayfish NMJ (Wojtowicz et al. 1991, 1994).

Increases in extracellular Ca\(^{2+}\) could be acting via several mechanisms to abruptly increase \(p\). Because increased extracellular Ca\(^{2+}\) increases Ca\(^{2+}\) entry, Ca\(^{2+}\) may diffuse farther during presynaptic action potentials. Because intracellular Ca\(^{2+}\) is carefully buffered, the Ca\(^{2+}\) gradient within the nerve terminal may be very sharp (Gilmanov et al. 2008; Nowycky and Pinter 1993; Oheim et al. 2006; Stanley 1997). It has been suggested in several systems including the NMJ that Ca\(^{2+}\) sensors for release are located at different distances from the sites of Ca\(^{2+}\) entry (Alvarez et al. 2008; Kennedy et al. 1999; Qian and Saggau 1999). The combination of a steep Ca\(^{2+}\) gradient that extends a variable distance from Ca\(^{2+}\) channels coupled with sensors at variable distances from Ca\(^{2+}\) channels could result in some vesicles with initially low \(p\) being exposed to sudden increases in Ca\(^{2+}\) concentration and thus sudden increases in \(p\). It is conceivable that such location variability of Ca\(^{2+}\) sensors for release could also contribute to spatial variability of \(p\) among release sites that do not differ otherwise in function. A second way that increased extracellular Ca\(^{2+}\) could abruptly increase \(p\) is by increasing resting Ca\(^{2+}\). The frequency of MEPCs was increased when extracellular Ca\(^{2+}\) was elevated (data not shown), consistent with elevation of intracellular Ca\(^{2+}\). Increased resting Ca\(^{2+}\) could act directly on release by summing with entering Ca\(^{2+}\) to increase \(p\) (Zucker and Regehr 2002). Although this would seem more likely to increase \(p\) at all release sites in a graded manner, it could couple with increased Ca\(^{2+}\) entry as described above to contribute to abrupt increases in \(p\). Alternatively, increased resting Ca\(^{2+}\) could trigger second messenger cascades that abruptly increase \(p\) by mechanisms such as increasing the number of release-ready (docked) vesicles. A third possibility is that Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular stores suddenly increases the Ca\(^{2+}\) concentration at a subset of release sites. At the frog NMJ, ryanodine receptor-mediated Ca\(^{2+}\)-induced Ca\(^{2+}\) release causes enhancement of evoked release (Narita et al. 2000). In that study, Ca\(^{2+}\) release from intracellular stores during presynaptic action potentials was accompanied by prolonged time to peak of the endplate potential. As we found no prolongation in the time course of peak release, it seems unlikely that this mechanism occurs that the mouse NMJ.

In summary, the Ca\(^{2+}\) dependence of release at the mouse NMJ can be fit without postulating that \(n\) is Ca\(^{2+}\) dependent. The apparent Ca\(^{2+}\) dependence of \(n\) can be accounted for by an...
abrupt increase in p at a subset of release sites. Once recruited, these “reluctant” release sites behave normally.

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