New insights into short-term synaptic facilitation at the frog neuromuscular junction

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Submitted 12 March 2014; accepted in final form 5 September 2014

Ma J, Kelly L, Ingram J, Price TJ, Meriney SD, Dittrich M. New insights into short-term synaptic facilitation at the frog neuromuscular junction. J Neurophysiol 113: 71–87, 2015. First published September 10, 2014; doi:10.1152/jn.00198.2014.—Short-term synaptic facilitation occurs during high-frequency stimulation, is known to be dependent on presynaptic calcium ions, and persists for tens of milliseconds after a presynaptic action potential. We have used the frog neuromuscular junction as a model synapse for both experimental and computer simulation studies aimed at testing various mechanistic hypotheses proposed to underlie short-term synaptic facilitation. Building on our recently reported excess-calcium-binding-site model of synaptic vesicle release at the frog neuromuscular junction (Dittrich M, Pattillo JM, King JD, Cho S, Stiles JR, Meriney SD. Biophys J 104: 2751–2763, 2013), we have investigated several mechanisms of short-term facilitation at the frog neuromuscular junction. Our studies place constraints on previously proposed facilitation mechanisms and conclude that the presence of a second class of calcium sensor proteins distinct from synaptotagmin can explain known properties of facilitation observed at the frog neuromuscular junction. We were further able to identify a novel facilitation mechanism, which relied on the persistent binding of calcium-bound synaptotagmin molecules to lipids of the presynaptic membrane. In a real physiological context, both mechanisms identified in our study (and perhaps others) may act simultaneously to cause the experimentally observed facilitation. In summary, using a combination of computer simulations and physiological recordings, we have developed a stochastic computer model of synaptic transmission at the frog neuromuscular junction, which sheds light on the facilitation mechanisms in this model synapse.

short-term synaptic plasticity; frog neuromuscular junction; synaptic vesicle release; MCell; stochastic simulation
in the frog NMJ hypothesized that residual Ca$^{2+}$ was not free but rather remained bound to Ca$^{2+}$ sensors and thereby enhanced vesicle release during future excitation events. The idea that residual bound Ca$^{2+}$ could enhance subsequent stimuli evolved to include the possibility of multiple Ca$^{2+}$ binding sites, some mediating vesicle fusion and release and others responsible for facilitation during subsequent stimuli (Atluri and Regehr 1996; Kamlya and Zucker 1994; Tang et al. 2000). Other studies suggested that facilitation resulted from saturation of local Ca$^{2+}$ buffers such as calbindin-D28k and parvalbumin, giving rise to increased availability of free Ca$^{2+}$ during subsequent stimuli (Blatow et al. 2003; Matveev et al. 2004; Müller et al. 2007).

Additional insight into the nature of the Ca$^{2+}$ binding sites responsible for transmitter release and facilitation came from studies of the effect of exogenous Ca$^{2+}$ buffers. These experiments showed that the addition of a fast exogenous Ca$^{2+}$ buffer [e.g., fura-2, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)] significantly reduced both facilitation and initial transmitter release (Mukhamedyarov et al. 2009; Tanabe and Kijima 1992; Yamada and Zucker 1992). In contrast, the addition of a slow Ca$^{2+}$ buffer such as ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) reduced facilitation without substantially affecting single-action potential-triggered transmitter release (Mukhamedyarov et al. 2009; Suzuki et al. 2000; Tanabe and Kijima 1992). The differential effect of EGTA on high-frequency stimulus-triggered facilitation versus single-action potential-evoked vesicle fusion suggests that two distinct processes acting on different time scales are involved. While there is clear evidence that Ca$^{2+}$ binding to synaptotagmin is involved in the action potential-triggered fast fusion step (Chapman 2008; Lynch et al. 2007; Rizo and Rosenmund 2009; Südhof and Malenka 2008), the NMJ no distinct molecular player mediating facilitation has so far been identified. In contrast, studies at the calyx of Held have shown that facilitation may be mediated by Ca$^{2+}$ binding to neuronal Ca$^{2+}$-sensor proteins (NCSs) that directly enhance Ca$^{2+}$ flux through P/Q-type voltage-gated Ca$^{2+}$ channels (VGCCs) during repeated stimuli (Catterall et al. 2013; Catterall and Few 2008; Mochida et al. 2008). Since the NMJ is thought to only contain N-type VGCCs (Kerr and Yoshihiski 1984) for which no Ca$^{2+}$ binding-induced facilitation has been reported, it is presently not known whether this mechanism might also act at the NMJ.

Given the lack of direct experimental data on the presynaptic Ca$^{2+}$-dependent facilitation mechanism, computational modeling can provide crucial insight and also aid in consolidating physiological and biochemical evidence into a functional hypothesis at a microscopic level. As an initial step toward this goal we recently developed an excess-calcium-binding-site model of action potential-triggered vesicle release at the frog NMJ (Dittrich et al. 2013) (Fig. 1A). Using stochastic reaction-diffusion simulation via MCell (Kerr et al. 2008; Stiles and Bartol 2001), we were able to show that a model of the frog NMJ with eight synaptotagmin molecules on each vesicle (corresponding to 40 Ca$^{2+}$ binding sites) without any ad hoc Ca$^{2+}$ binding site cooperativity could predict experimentally known properties of single-action potential-triggered vesicle fusion (Dittrich et al. 2013). In the current study we found that our previous model was not able to predict the experimentally observed facilitation during multiple stimuli at high frequency. Thus, via a step-by-step evolution of our excess-calcium-binding-site model, and constrained by experimental observations, we have tested the viability of several facilitation mechanisms at the microscopic level. We found that a model with a second class of Ca$^{2+}$ binding sites, distinct from synaptotagmin, provided good agreement with our experimental constraints. In addition, we identified a novel fusion mechanism based on the association of Ca$^{2+}$-bound synaptotagmin with the presynaptic membrane. Our results yield new insight into the facilitation mechanism at the frog NMJ and also provide constraints on the number and kinetic properties of a potential second sensor site whose precise molecular identity is presently unknown.

METHODS

Excess-calcium-binding-site model. We started our investigation of short-term synaptic facilitation at the frog NMJ by using our previously developed excess-calcium-binding-site model (Dittrich et al. 2013) and simulated it using MCell (www.mcell.org). Our model contained a realistic three-dimensional representation of a frog NMJ active zone (AZ) created via CellBlender (www.mcell.org) based on published averages (Fig. 1A). The mesh geometry was created in CellBlender and then exported directly into MCell’s model description language (MDL). Twenty-six synaptic vesicles of 50-nm diameter were arranged in two rows lateral to a trough containing VGCCs at a vesicle-channel stoichiometry of 1:1 (Luo et al. 2011) and at locations suggested by published estimates (Heuser et al. 1979; Pawson et al. 1998; Stanley et al. 2003). During each single or repeated action potential stimulation event, VGCCs opened stochastically (see below), giving rise to Ca$^{2+}$ influx into the terminal, Ca$^{2+}$ diffusion within the terminal, Ca$^{2+}$ binding to sensor sites on synaptic vesicles, and vesicle fusion once a sufficient number of Ca$^{2+}$ ions had bound in a prescribed binding pattern we called a vesicle fusion mechanism. Key model parameters are listed in Table 1.

Vesicular Ca$^{2+}$ sensor sites. The arrangement and number of synaptotagmin Ca$^{2+}$ binding sites on synaptic vesicles were identical to those employed in our previous study (Dittrich et al. 2013). Forty Ca$^{2+}$ sensor sites corresponding to eight synaptotagmin molecules with five Ca$^{2+}$ binding sites each were located at the bottom of individual synaptic vesicles on mesh tiles opposite to the presynaptic membrane (Fig. 1B). This arrangement of Ca$^{2+}$ sensor sites was used in both our control and persistent-binding model. Our second sensor site facilitation model included additional sensor sites [number of second sensor sites (ntv) = 8, 12, 16, 28, 68, 144] on vesicle mesh tiles located in an annular region directly above the synaptotagmin molecules (see Fig. 4A). These second sensor sites represented the Ca$^{2+}$ binding sites (of unknown stoichiometry) on the as yet unknown proteins that might be involved in facilitation.

VGCC and Ca$^{2+}$ binding kinetics. The kinetics of the VGCCs was identical to that in our excess-calcium-binding-site model (Dittrich et al. 2013) and consisted of three closed states ($C_0$, $C_1$, $C_2$) and one open state ($O$) that interconverted according to the following scheme

\[
\begin{align*}
3\alpha & \quad 2\alpha & \quad \alpha & = k \\
C_0 & \Leftrightarrow C_1 & \Leftrightarrow C_2 & \Leftrightarrow O \Rightarrow Ca^{2+} \\
\beta & \quad 2\beta & \quad 3\beta
\end{align*}
\]

The time-dependent rate constants $\alpha$, $\beta$, and $k$ were parameterized based on experimentally measured action potentials and whole cell Ca$^{2+}$
The time dependence of $\alpha$, $\beta$, and $k$ for repeated stimuli (paired-pulse and 5-pulse experiments) were generated by stitching together the appropriate number of single action potential waveforms interspersed by the appropriate interstimulus intervals (ISIs) at a resting potential of $-60$ mV. Once in the open state, VGCCs released Ca$^{2+}$ ions into the presynaptic terminal with a time-dependent rate constant $k$. Ca$^{2+}$ ions then diffused within the terminal and could either bind to Ca$^{2+}$ sensor sites on vesicles (synaptotagmin and/or second sensor binding sites) or 2 mM of static Ca$^{2+}$ buffer molecules distributed randomly and uniformly throughout the terminal according to

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**Fig. 1.** Overview of excess-calcium-binding-site model in the frog neuromuscular junction (NMJ). A: snapshot of the frog NMJ model, rendered via Cell-Blender. Top: front view of frog NMJ model. Large spheres represent 26 docked synaptic vesicles arranged in 2 parallel rows. Tiles on the bottom of each vesicle with small and large dots represent unbound and bound synaptotagmin Ca$^{2+}$ binding sites, respectively. Voltage-gated Ca$^{2+}$ channels (VGCCs) are located in the trough between docked synaptic vesicles (arrow). Small spheres within the terminal represent free Ca$^{2+}$ ions and bound endogenous Ca$^{2+}$ buffer (arrows). Not shown are a large number of unbound buffer molecules for visual clarity. Bottom: top view of frog NMJ model, showing the 1-to-1 stoichiometry of 26 synaptic vesicles to VGCCs. B: arrangement of synaptotagmin binding sites on the bottom of the synaptic vesicle. The 8 synaptotagmin molecules present on each vesicle are highlighted in different shades of gray, and each contains 5 Ca$^{2+}$ binding sites on individual mesh triangles. White spheres indicate bound Ca$^{2+}$ ions. The particular vesicle shown is considered released according to the excess-calcium-binding-site model since 3 synaptotagmin molecules (cassette of 5 adjacent binding sites shaded the same) each have at least 2 Ca$^{2+}$ ions bound. C: experimentally, a stimulus train at 100 Hz provides facilitation, while the MCell model shows depression. D: average number and concentration of free Ca$^{2+}$ ions in a sampling box underneath vesicles (average over $n = 260,000$ vesicles). Top axis indicates the time of action potential peaks for different stimulus numbers. E: location and dimension of sampling box underneath vesicles used to measure the local Ca$^{2+}$ concentration.
Synaptotagmin-like calcium binding site (synaptotagmin) k on

Diffusion coefficient for free Ca^{2+} ions D = 6 \times 10^{-6} \text{ cm}^2 \text{s}^{-1} Winslow et al. 1994

VGCC kinetic properties See METHODS, conductance of open VGCC: 2.4 pS Dittrich et al. 2013

Synaptotagmin-like calcium binding site (synaptotagmin) k on = 1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} Davis et al. 1999

k off = 6,000 \text{ s}^{-1} Xu et al. 1997

Endogenous calcium buffer Concentration = 2 mM Yazejian et al. 2000

k on = 1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}
k off = 1,000 \text{ s}^{-1}

Synaptotagmin/lipid interaction rates in persistent-binding model k_{lipid, on} = 2 \times 10^3 \text{ M}^{-1} \text{s}^{-1} Based on

k_{lipid, off} = 10 \text{ s}^{-1}

Second calcium sensor binding site (Y sensor) k on = 6 \times 10^6 \text{ M}^{-1} \text{s}^{-1} Hui et al. 2005

k off = 88 \text{ s}^{-1}

BAPTA Ca^{2+} binding kinetics k on = 4 \times 10^6 \text{ M}^{-1} \text{s}^{-1} Matveev et al. 2006

k off = 88 \text{ s}^{-1}

EGTA Ca^{2+} binding kinetics k on = 1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} Eggermann et al. 2012

k off = 0.77 \text{ s}^{-1}

with the appropriate rate constants taken from the literature (see RESULTS and Table 1). In our model, we chose 2 mM of static Ca^{2+} buffer for computational efficiency because it is intended to capture the average effect of the true underlying buffer conditions. To establish the validity of this assumption we examined several models with low concentrations of buffer and mixtures of different concentrations of fixed and mobile buffer. To model the effect of mobile buffer we used an approximation in which only Ca^{2+}-bound buffer molecules could diffuse while unbound buffer remained fixed (this is a good approximation unless the buffer concentration becomes very small). Under all buffer conditions tested, we obtained quantitatively similar models with only minor parameter adjustments (data not shown).

The significance of the effect of exogenous Ca^{2+} buffer BAPTA and EGTA on vesicle release and paired-pulse facilitation (PPF) were tested with a one-way ANOVA (P < 0.01) followed by Tukey’s post hoc test (P < 0.01). All statistical analyses were conducted with MATLAB (v. 7.8.0, MathWorks). We used a short simulation time step (\(dt = 10 \text{ ns}\)) to ensure accurate spatial sampling of the confined regions between VGCCs and the Ca^{2+} binding sites on vesicles. During each simulation run, we kept track of Ca^{2+} ions emitted from individual open Ca^{2+} channels and recorded which sensor sites on synaptic vesicles bound Ca^{2+} ions from which VGCC and pulse (during multistimulus experiments). This allowed us to analyze whether and to what degree Ca^{2+} ions from different stimuli contributed to release of synaptic vesicles. Analysis programs written in C++ and Python were then used to analyze the number and timing of vesicle release events after simulation. With the exception of the runs to compute the Ca^{2+} release relationship (CRR), all simulations were conducted at a physiological (in the frog) external Ca^{2+} concentration of 1.8 mM. Simulations were set up and analyzed on a local workstation (2.4 GHz Core2-Duo iMac) and simulated on several computer clusters at the Pittsburgh Supercomputing Center (Salk, an SGI Altix 4700 shared-memory NUMA system with 144 Itanium 2 processors; Axon, a 256 core cluster with 64 quad-core 2.5 GHz Intel Xeon E5420 CPUs). A typical duration for a single paired-pulse simulation with 10-ms ISI was \(\sim 2 \text{ h}\).

Table 2. Average number of released vesicles and PPF for a range of persistent-binding models with different values for \(k_{lipid, on}\) and \(k_{lipid, off}\)

<table>
<thead>
<tr>
<th>(k_{lipid, on} \text{ s}^{-1})</th>
<th>0.2</th>
<th>2</th>
<th>10</th>
<th>20</th>
<th>100</th>
<th>200</th>
<th>2,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n_r)</td>
<td>PPF</td>
<td>(n_r)</td>
<td>PPF</td>
<td>(n_r)</td>
<td>PPF</td>
<td>(n_r)</td>
<td>PPF</td>
</tr>
<tr>
<td>(2 \times 10^4)</td>
<td>1.95</td>
<td>1.36</td>
<td>1.95</td>
<td>1.36</td>
<td>1.95</td>
<td>1.33</td>
<td>1.92</td>
</tr>
<tr>
<td>(2 \times 10^3)</td>
<td>1.78</td>
<td>1.38</td>
<td>1.83</td>
<td>1.32</td>
<td>1.78</td>
<td>1.32</td>
<td>1.79</td>
</tr>
<tr>
<td>(1 \times 10^4)</td>
<td>1.52</td>
<td>1.38</td>
<td>1.5</td>
<td>1.4</td>
<td>1.49</td>
<td>1.43</td>
<td>1.48</td>
</tr>
<tr>
<td>(2 \times 10^3)</td>
<td>0.68</td>
<td>1.68</td>
<td>0.6</td>
<td>1.58</td>
<td>0.47</td>
<td>1.45</td>
<td>0.4</td>
</tr>
</tbody>
</table>

PPF, paired-pulse facilitation; \(n_r\), no. of released vesicles. Values shown in bold match our experimental constraints (\(n_r = 0.41–0.59, \text{ PPF} = 1.45–1.75\)).
Table 3. Average $n_y$, and PPF for a range of second sensor models for different choices of $n_y$, $k_{on}$ for synaptotagmin, and $N$

<table>
<thead>
<tr>
<th>$n_y$</th>
<th>$k_{on}$ M$^{-1}$s$^{-1}$</th>
<th>$n_y$</th>
<th>PPF</th>
<th>$n_y$</th>
<th>PPF</th>
<th>$n_y$</th>
<th>PPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1 $\times$ 10$^8$</td>
<td>1.0</td>
<td>1.26</td>
<td>0.2</td>
<td>1.61</td>
<td>0.1</td>
<td>2.09</td>
</tr>
<tr>
<td>28</td>
<td>1 $\times$ 10$^8$</td>
<td>1.4</td>
<td>1.50</td>
<td>0.5</td>
<td>1.74</td>
<td>0.1</td>
<td>2.13</td>
</tr>
<tr>
<td>68</td>
<td>1 $\times$ 10$^8$</td>
<td>3.1</td>
<td>1.23</td>
<td>1.5</td>
<td>1.94</td>
<td>0.7</td>
<td>2.34</td>
</tr>
<tr>
<td>144</td>
<td>1 $\times$ 10$^8$</td>
<td>6.2</td>
<td>1.32</td>
<td>3.8</td>
<td>1.75</td>
<td>2.3</td>
<td>2.13</td>
</tr>
<tr>
<td>16</td>
<td>4 $\times$ 10$^8$</td>
<td>1.9</td>
<td>1.18</td>
<td>0.6</td>
<td>1.49</td>
<td>0.2</td>
<td>1.66</td>
</tr>
<tr>
<td>28</td>
<td>4 $\times$ 10$^8$</td>
<td>2.5</td>
<td>1.21</td>
<td>1.0</td>
<td>1.48</td>
<td>0.3</td>
<td>1.68</td>
</tr>
<tr>
<td>68</td>
<td>4 $\times$ 10$^8$</td>
<td>4.0</td>
<td>1.22</td>
<td>2.1</td>
<td>1.54</td>
<td>1.0</td>
<td>1.86</td>
</tr>
</tbody>
</table>

$n_y$, no. of second sensor sites; $N$, total no. of active synaptotagmin and second sensor sites required to trigger fusion. Values shown in bold best match our experimental constraints ($n_y = 0.41$–0.59, PPF = 1.45–1.75).

**Vesicle fusion mechanisms.** The vesicle fusion mechanism utilized as part of our control and persistent-binding model was as described previously (Dittrich et al. 2013). Synaptotagmin molecules simultaneously occupied by at least two Ca$^{2+}$-ions were considered active, and vesicles were released as soon as three out of a total of eight synaptotagmin molecules became active.

The fusion mechanism for our second sensor model was a straightforward extension of this release mechanism. More specifically, a vesicle was released once a given number of synaptotagmin and/or second sensor sites were active simultaneously. Since we did not know how many second sensor sites were present on the unknown protein mediating facilitation, second sensor sites were considered active once bound by a single Ca$^{2+}$-ion. In addition, we used a Metropolis-Hastings (Hastings 1970; Metropolis et al. 1953) sampling protocol to decide which vesicles should be released using

$$P = \min(\exp(-\frac{E_0 - n_y \cdot \Delta E_y - n_Y \cdot \Delta E_Y}{k_B T}), N)$$

for calculating the fusion probability. Here, $E_0 = 40 \cdot k_B T$ is the free energy barrier for vesicle fusion (Li et al. 2007; Martens et al. 2007), $n_y$ and $n_Y$ are the number of active synaptotagmin and Y sites, and $\Delta E_y$ and $\Delta E_Y$ are the respective reductions in free energy barrier toward vesicle fusion.

For our persistent-binding model shown in Fig. 2, since the number of active synaptotagmin molecules could only be determined after simulation (Dittrich et al. 2013), the lipid binding step into and out of the persistent state also had to be computed after simulation. To this end, we converted the reaction rate constants for the persistent step into reaction probabilities and then used a Monte Carlo scheme to sample transitions of activated synaptotagmin molecules into a persistent state and back.

Since the transition from the active to the lipid bound state and back was modeled as a unimolecular process, we used the exponential relationship

$$P = 1 - \exp(-k \cdot t)$$

to convert reaction rate constants to reaction probabilities. For example, using $k_{lipid-off} = 10$ s$^{-1}$ (Hui et al. 2005) and $dr = 0.5$ μs yielded $P_{lipid-off} = 5 \times 10^{-6}$. Similarly, a $k_{lipid-on} = 2 \times 10^8$ s$^{-1}$ and $dr = 0.5$ μs resulted in $P_{lipid-on} = 1 \times 10^{-4}$. Here, $dr = 0.5$ μs corresponded to our chosen data output interval.

The postsimulation analysis of the transition kinetics into and out of the persistent state was an approximation since Ca$^{2+}$-ions, which were sequestered within a persistent synaptotagmin state during analysis, could still bind and unbind Ca$^{2+}$-ions in the underlying MCell simulation. However, since at most three of eight synaptotagmin sites would be persistent simultaneously this effect had limited impact on overall system dynamics. For all multipulse simulations, the small number of spontaneous release events between pulses were not counted unless vesicles remained in a fusion-competent state until the beginning of the next pulse.

**Recording transmitter release from frog neuromuscular junction.** Adult northern leopard frogs (Rana pipiens) were anesthetized with 0.4% tricaine and double pithed in compliance with protocols reviewed and approved by the Institutional Animal Use and Care Committee at the University of Pittsburgh. The cutaneous pectoris muscle was dissected from the frogs and placed in normal frog Ringer (NFR, in mM: 5 glucose, 116 NaCl, 10 HEPES buffer, 2 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, pH 7.4). For recordings, the nerve-muscle preparation was placed in a Sylgard-coated recording chamber. Evoked transmitter release was elicited by drawing the nerve into a suction electrode and stimulating (in pairs, or in trains at 1–100 Hz) with a current that was 10 times the threshold required to elicit a muscle contraction. Microelectrodes were pulled from borosilicate glass, filled with 3 M potassium acetate (resistance 40–60 MΩ), and impaled into muscle cells to allow recording of nerve stimulation-evoked end-plate potentials (EPPs) from postsynaptic muscle cells close to visually identified NMJs as described previously (Cho and Meriney 2006). All data were collected and analyzed with Clampex10 software (Axon Instruments).

**RESULTS**

**Experimental model constraints.** We used a range of experimental studies to help constrain our computational model. First, we measured the magnitude of transmitter release during pairs of stimuli as estimated by the size of postsynaptic EPPs. We then plotted the ratio of the second EPP to the first (paired-pulse ratio, PPR) at different ISIs (Fig. 2E). At a short ISI of 10 ms, we measured a significant PPR of 1.57 ± 0.34 (n = 66), which decayed to baseline with increasing ISI. Next, we measured EPP amplitudes during short trains of five stimuli at 100 Hz (Fig. 1C), which showed that tetanic facilitation at the frog NMJ increased substantially by a factor of 2.5 between the first and fifth stimulation events. These data provided insight into the timescales underlying facilitation and furnished important constraints for our modeling studies described below. In particular, we used J) the time dependence of the PPR as a function of ISI (10–100 ms) and 2) the growth of tetanic facilitation during a five-pulse stimulus train at 100 Hz as constraints for our model building.

Next, we considered the effect of exogenous Ca$^{2+}$-buffer on facilitation in the frog NMJ AZ. Experimentally, it is well known that addition of the fast Ca$^{2+}$-buffer BAPTA reduces both vesicle release and facilitation in the frog NMJ (Mukhamedyarov et al. 2009; Naraghi and Neher 1997; Suzuki et al. 2000; Tanabe and Kijima 1992). In contrast, addition of the slow Ca$^{2+}$-buffer EGTA to the nerve terminal cytoplasm reduces facilitation but has a much smaller impact on vesicle release than BAPTA at comparable buffer concentrations (De-
Table 4. Average \( n_r \), PPF, and CRR for a range of second sensor energy models for different choices of \( n_Y, k_w \) of synaptotagmin, \( \Delta E_s \), and \( \Delta E_v \)

<table>
<thead>
<tr>
<th>( \Delta E_s )</th>
<th>( \Delta E_v )</th>
<th>( n_r )</th>
<th>PPF</th>
<th>CRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>14.00</td>
<td>0.18</td>
<td>1.65</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
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<td>0.20</td>
<td>1.57</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
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<td>0.30</td>
<td>1.46</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>16.00</td>
<td>0.35</td>
<td>1.59</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
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<td>0.36</td>
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</tr>
<tr>
<td>11</td>
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<td>12</td>
<td>16.00</td>
<td>0.56</td>
<td>1.29</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\( n_r = 8, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \)

| \( n_r = 12, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \) | \( n_r = 16, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \) | \( n_r = 28, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \) |
|-----------------|-----------------|----------|------|-----|
| 9               | 14.00           | 0.28     | 1.71 | N/A |
| 9               | 15.00           | 0.30     | 1.76 | N/A |
| 10              | 14.00           | 0.35     | 1.63 | N/A |
| 10              | 15.00           | 0.43     | 1.61 | N/A |
| 10              | 16.00           | 0.54     | 1.68 | N/A |
| 11              | 14.00           | 0.50     | 1.45 | N/A |
| 11              | 15.00           | 0.55     | 1.47 | N/A |
| 12              | 14.00           | 0.51     | 1.64 | N/A |

\( n_r = 28, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \)

| \( n_r = 68, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \) | \( n_r = 144, k_w = 1 \times 10^9 \, M^{-1} \, s^{-1} \) | \( n_r = 16, k_w = 4 \times 10^7 \, M^{-1} \, s^{-1} \) |
|-----------------|-----------------|----------|------|-----|
| 10              | 0.50            | 1.82     | 4.68 |     |
| 10              | 0.47            | 1.77     | 4.76 |     |
| 10              | 0.40            | 1.79     | 5.37 |     |
| 9               | 0.38            | 1.92     | 4.72 |     |
| 8               | 0.24            | 1.94     | 5.27 |     |

\( n_r = 28, k_w = 4 \times 10^7 \, M^{-1} \, s^{-1} \)

| \( n_r = 28, k_w = 4 \times 10^7 \, M^{-1} \, s^{-1} \) | \( n_r = 16, k_w = 4 \times 10^7 \, M^{-1} \, s^{-1} \) | \( n_r = 68, k_w = 4 \times 10^7 \, M^{-1} \, s^{-1} \) |
|-----------------|-----------------|----------|------|-----|
| 9               | 0.48            | 1.56     | 5.33 |     |
| 9               | 0.63            | 1.64     | 3.94 |     |
| 8               | 0.81            | 1.40     | 4.49 |     |
| 8               | 0.64            | 1.78     | 3.19 |     |
| 8               | 0.48            | 1.59     | 5.32 |     |
| 8               | 0.50            | 1.86     | 5.03 |     |
| 5               | 0.56            | 1.71     | 5.23 |     |
| 5               | 0.83            | 1.65     | 4.97 |     |

CRR, \( \text{Ca}^{2+} \) release relationship; \( \Delta E_s \), energy contribution toward fusion for active synaptotagmin; \( \Delta E_v \), energy contribution toward fusion for second sensor sites; N/A, CRR was not computed. Values shown in bold best match our experimental constraints (\( n_r = 0.41–0.59, \) PPF = 1.45–1.75).

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to and enhance subsequent stimuli (Bertram et al. 1996; Katz and Miledi 1968; Matveev et al. 2006). Since our previous excess-calcium-binding-site model demonstrated that synaptotagmin’s binding kinetics for Ca$^{2+}$ did not meet this requirement, we considered a different mechanism based on the association of Ca$^{2+}$-bound synaptotagmin with the lipid bilayer. Recent biochemical evidence has suggested that Ca$^{2+}$-bound C2 domains of synaptotagmin penetrate and associate with the lipid membrane and that this process is vital to exocytosis (Bai et al. 2002; Hui et al. 2006; Paddock et al.

Fig. 2. Summary of the persistent-binding-site model. A: schematic view of the state diagram of the persistent-binding-site model. The active state corresponds to synaptotagmin (Syt) with at least 2 Ca$^{2+}$ ions bound but not yet interacting with the lipid membrane. The persistent state describes an active synaptotagmin molecule interacting with the lipid membrane. B: Ca$^{2+}$ release relationship (CRR) of the persistent-binding-site model. Shown is a log-log plot of average vesicle release vs. external [Ca$^{2+}$] and a linear regression with a slope of 5.12 for the CRR. AZ, active zone. C: histogram of computed vesicle release latencies in the persistent-binding-site model together with the experimentally measured values (Katz and Miledi 1965a). D: experimental and modeled facilitation growth during a 5-pulse stimulus train at 100 Hz. E: experimental and modeled decay of paired-pulse facilitation (PPF) as a function of interstimulus interval. Solid line is a double-exponential fit of the experimental data. F: PPF and average vesicle release ($n_r$) under varying BAPTA concentrations. G: PPF and average $n_r$ under varying EGTA concentrations. *Statistically significant values (1-way ANOVA followed by Tukey’s post-hoc test, $P < 0.01$).
Interestingly, the reported kinetics of the lipid-synaptotagmin interaction in solution features high \( k_{\text{on}} \) (10\(^10\) M\(^{-1}\)s\(^{-1}\)) and low \( k_{\text{off}} \) (12 s\(^{-1}\)) values (Hui et al. 2005). This suggests that a high-affinity lipid-bound synaptotagmin state might persist long enough to yield facilitation during high-frequency stimulation.

To investigate whether such a protein-lipid association process could underlie facilitation at the frog NMJ, we incorporated this hypothesis into our model. This so-called persistent-binding model consists of a simple two-state model and is illustrated schematically in Fig. 2A. The model comprised an initial \( Ca^{2+} \) binding step to synaptotagmin to yield an active state, followed by lipid association of synaptotagmin to form a longer-lasting persistent state. Since we did not know the effective lipid concentration at the presynaptic membrane close to synaptic vesicles, we modeled the lipid-synaptotagmin binding process as a simple unimolecular process. Similar to our previous excess-calcium-binding-site model, synaptotagmin became active once two of its five binding sites bound \( Ca^{2+} \) ions (see Table 1) (Dittrich et al. 2013). Once active, synaptotagmin molecules reversibly entered the persistent state with lipid association rates \( k_{\text{lipid\_on}} \) and \( k_{\text{lipid\_off}} \). Vesicle release occurred once at least three synaptotagmin molecules were simultaneously in this persistent state.

To determine values for \( k_{\text{lipid\_on}} \) and \( k_{\text{lipid\_off}} \) that would satisfy constraints 1–7, we conducted a parameter sweep. As shown in Table 2, increasing \( k_{\text{lipid\_on}} \) while keeping \( k_{\text{lipid\_off}} \) fixed increased vesicle release and reduced PPF. On the other hand, increasing \( k_{\text{lipid\_off}} \) led to a strong reduction in PPF. Using values for \( k_{\text{lipid\_on}} \) and \( k_{\text{lipid\_off}} \) of 2,000 s\(^{-1}\) and 10 s\(^{-1}\), respectively, our persistent-binding model agreed well with most of our constraints. The average number of released vesicles per action potential and AZ during a single stimulus was 0.47, and the histogram of release latencies was narrow and in good agreement with experimental data (Fig. 2C). The computed CRR was 5.12 (Fig. 2B) and thus close to—albeit slightly larger than—the experimentally observed value of 4.2 (Dittrich et al. 2013). Importantly, our persistent-binding model showed a significant increase in facilitation in response to a series of stimuli (Fig. 2D), albeit somewhat slower than observed experimentally. Furthermore, this model also captured the experimentally observed decay in PPF as a function of the ISI (Fig. 2E). However, as the ISI was increased, the PPF of our model decreased less steeply than measured experimentally (Fig. 2E). Nevertheless, given the simplicity of our persistent-binding model, the observed agreement was remarkably good.

Our determined value for \( k_{\text{lipid\_off}} \) (10 s\(^{-1}\)) is in good agreement with the experimental data for lipid unbinding by
synaptotagmin (12 s$^{-1}$) (Hui et al. 2005). Since our value for $k_{\text{lipid, on}}$ is an effective rate constant that implicitly contains the (unknown) concentration of lipids at the presynaptic membrane, it was not straightforward to compare its value with experimental binding data obtained in the presence of a well-defined lipid concentration.

To investigate the effects of exogenous Ca$^{2+}$ buffer on synaptic transmission in our persistent-binding model, we added increasing concentrations of BAPTA and EGTA to our model (in mM: 0.1, 0.5, 1.0, 2.0) in addition to the 2 mM of endogenous buffer. As shown in Fig. 2F, vesicle release decreased quickly as the BAPTA concentration was increased. In contrast, the PPF remained constant up to 0.5 mM of BAPTA and decreased slowly at higher concentrations. In particular, at a BAPTA concentration of 0.5 mM at which release was reduced by $\sim$70%, we did not observe any significant reduction in PPF in our model, in contrast to experimental observations (see above). Furthermore, with increasing concentrations of EGTA (Fig. 2G), our simulations showed a slow decrease in vesicle release and basically unchanged PPF, with the exception of the data point at 1 mM EGTA, which exhibited a small, but statistically significant drop in PPF. Thus, while our persistent-binding-site model showed significant facilitation and also agreed well with most of our previous single-pulse constraints, it exhibited only a limited ability to reproduce the experimentally measured effect of exogenous Ca$^{2+}$ buffers BAPTA and EGTA on synapse function. We therefore wondered if we could find another extension of our basic excess-calcium-binding-site model that would yield facilitation in agreement with all our experimental constraints.

A model with additional second sensors sites shows facilitation and agrees well with all our constraints. The involvement of spatially and kinetically distinct Ca$^{2+}$ binding sites in vesicle fusion and facilitation, respectively, has been proposed previously (Bennett et al. 2004; Matveev et al. 2006; Tang et al. 2000; Yamada and Zucker 1992). However, very little is known about the detailed molecular nature and number of potential second sensor sites involved in facilitation. Thus we wondered if, based on our excess-calcium-binding-site model, we could both confirm the viability of this mechanism and also shed light on its molecular nature. To this end, we added to each vesicle a second set of Ca$^{2+}$ binding sites (Y sites) (Tang et al. 2000) in addition to the 40 existing sites on synaptotagmin. Given that we currently do not know the localization of these Y binding sites, we assumed them to be close to but spatially distinct from the synaptotagmin molecules. Thus the newly introduced Y binding sites were placed in an annular region on the bottom of synaptic vesicles directly above the synaptotagmin molecules (Fig. 4A). However, the precise location of Y binding sites on the bottom of synaptic vesicles has little impact on the model. For example, a configuration in which Y binding sites were placed in the same area on the bottom of synaptic vesicles where synaptotagmin molecules were located provided virtually identical results to the current model with only minor parameter adjustments (data not shown). The Ca$^{2+}$ binding kinetics of the second sensor sites were modeled based on literature values for the crayfish NMJ, $k_{\text{off,Y}} = 6 \times 10^6$ M$^{-1}$s$^{-1}$, $k_{\text{on,Y}} = 36$ s$^{-1}$ (Matveev et al. 2006). Compared with synaptotagmin, the second sensor sites had a higher Ca$^{2+}$ binding affinity and featured slower Ca$^{2+}$ unbinding kinetics with a dwell time of $\sim$30 ms.

In our model we had $k_{\text{on-BAPTA}} > k_{\text{on-syt}} > k_{\text{on-Y}}$ and $k_{\text{off-syt}} > k_{\text{off-EGTA}} > k_{\text{off-Y}}$ (Table 1).

We then proceeded to determine viable numbers of second sensor sites, $n_Y$, on vesicular sites, as well as a vesicle fusion mechanism. Here, a fusion mechanism specifies the manner in which synaptotagmin and second sensor sites had to bind Ca$^{2+}$ for vesicle fusion to be triggered. The initial fusion mechanism we chose was a straightforward extension of that used in our previous excess-calcium-binding-site model. A synaptotagmin molecule was activated once at least two of its five Ca$^{2+}$ binding sites were simultaneously occupied by at least two Ca$^{2+}$ ions. Similarly, second sensor sites were activated as soon as they bound a single Ca$^{2+}$ ion. Vesicles were released once a total of $N$ synaptotagmin and/or second sensor sites were active simultaneously. In particular, both types of Ca$^{2+}$ binding sites could in principle contribute to fusion and facilitation, and their fractional contribution to either process was mainly determined by their Ca$^{2+}$ binding and unbinding kinetics.

To determine a viable fusion mechanism we needed to determine both $n_Y$ and $N$. We simulated a range of models with different $n_Y$ and values for $N$ (see Table 3). Similar to our previous study (Dittrich et al. 2013), we considered synaptotagmin models with two different $k_{\text{on}}$ values ($k_{\text{on}} = 1 \times 10^6$ M$^{-1}$s$^{-1}$ and $4 \times 10^6$ M$^{-1}$s$^{-1}$). Initially, we focused on models that could reproduce the average $n_Y$ per AZ and action potential as well as PPF using a 10-ms ISI. As shown in Table 3, for a given $n_Y$ an increase in $N$ led to a decrease in $n_Y$ and a corresponding increase in the PPF. On the other hand, for fixed values of $N$ an increase in $n_Y$ led to a steep increase in $n_Y$ while leaving the PPF largely unaffected. This nicely illustrates the interplay between the number of available second sensor sites and the required number of active sites $N$. As $N$ was increased, synaptotagmin and Y sites continued to be activated but fewer vesicles were released during the first pulse, leading to increased release during the second pulse and thus enhancing the PPF. For fixed $N$, an increase in $n_Y$ enhanced the number of activated second sensor sites, increasing both the initial release magnitude as well as the PPF.

Table 3 shows that a model with a moderate number of second sensor sites, $n_Y = 28$ (Fig. 4A), and $N = 4$ fit our initial constraints well and also exhibited the proper narrow distribution of release latencies (Fig. 4G). In addition, this 28-Y-site model showed significant tetanic facilitation during a train of stimuli, albeit at a somewhat lower rate than what we measured experimentally (Fig. 4B). Similarly, our model simulations showed the expected relationship between PPF and ISI; however, PPF decayed at a slightly steeper rate as the ISI was increased compared with our experimental observations (Fig. 4C). Furthermore, the computed CRR of 4.84 was slightly higher than the experimentally observed value of 4.2 (Fig. 4D). When examining the model’s response to exogenous buffer we found that adding BAPTA significantly reduced the magnitude of vesicle fusion during a single action potential (Fig. 4E) and also lowered PPF significantly (e.g., by our 70% criterion for block of initial vesicle fusion, PPF was significantly reduced). Furthermore, adding EGTA to the model only moderately (compared with BAPTA) reduced the magnitude of vesicle fusion and significantly decreased PPF (Fig. 4F). These results suggested that the 28-Y-site model with $N = 4$ satisfied most
experimental constraints, with the exception of the slightly elevated value for the CRR. In particular, the introduction of second sensor sites greatly enhanced the model’s sensitivity to exogenous Ca\(^{2+}\) buffer. While this finding was quite satisfactory, we wondered if we could improve the model further to decrease its sensitivity toward external Ca\(^{2+}\) and thus lower the value of the CRR.

Second sensor model with energy-based fusion mechanism further improves agreement with experimental constraints. Previous studies have suggested that to initiate fusion with the

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Fig. 4. Summary of 28-Y-site model. A: schematic view of the bottom of a synaptic vesicle showing the 28 second sensor sites (Y sites, small black triangles forming a ring around the synaptotagmin molecules). The arrangement of synaptotagmin molecules is identical to our original excess-calcium-binding-site model (cf. Fig. 1A). The depicted vesicle is considered released according to a release mechanism with \(N = 4\) since 3 synaptotagmin and 1 second sensor (Y) site are active. B–D: facilitation growth (B), PPF decay (C), and CRR (D) predicted by the 28-Y-site model (cf. Fig. 2 for notational details). E and F: effects of different concentrations of exogenous buffers BAPTA (E) and EGTA (F) on PPF and average \(n_r\). *Statistically significant values (1-way ANOVA followed by Tukey’s post hoc test, \(P < 0.01\)). G: histogram of computed vesicle release latencies in the 28-Y-site model together with the experimentally measured values (Katz and Miledi 1965a).
plasma membrane, the vesicle membrane needs to overcome an energy barrier of roughly $40 \text{ k}_{\text{B}}T$ (Li et al. 2007; Martens et al. 2007). Furthermore, Ca$^{2+}$-bound synaptotagmin has been hypothesized to contribute to lowering this energy barrier (Gao et al. 2012; Malsam et al. 2008; Martens et al. 2007; Sørensen 2009; Wiedenhold and Fasshauer 2009; Young and Neher 2009). Our second sensor model introduced above assumed that activated synaptotagmin and second sensor sites contributed equally to vesicle fusion. However, since the second sensor sites are assumed to be biochemically distinct from synaptotagmin, and may also occupy spatially distinct locations on vesicles, we hypothesized that active synaptotagmin and second sensor sites might contribute differently to vesicle fusion. In particular, in our energy-based fusion mechanism we hypothesized that activated synaptotagmin and second sensor sites each lowered the fusion barrier by increments $\Delta E_S$ and $\Delta E_Y$. To determine if and when a particular vesicle fusion event occurred during a given simulation run, we computed the time series of total energies summed over all instantaneously active synaptotagmin ($\Delta E_S$) and second sensor ($\Delta E_Y$) sites and then used the Metropolis-Hastings algorithm (Hastings 1970; Metropolis et al. 1953) to decide whether or not vesicle fusion took place (see METHODS).

We first considered a 28-Y-site-energy model (described above) in which both synaptotagmin and second sensor sites contributed equally to vesicle fusion ($\Delta E_S = \Delta E_Y = 10 \text{ k}_{\text{B}}T$). This model corresponded closely to our previous 28-Y-site model with $n = 4$, with the exception that because of the Metropolis sampling, release was now possible even for an occupancy of less than four, albeit with low probability. As shown in Table 4, the results for both models were very similar, as one would have expected. In particular, the CRR of the 28-Y-site-energy model was 4.68 and thus again somewhat larger than our experimental constraint (4.2).

We next turned to models with lower (8, 12, and 16) and higher (68 and 144) numbers of second sensor sites over a range of values for both $\Delta E_S$ and $\Delta E_Y$. Similar to our earlier approach, we initially selected models based on two experimental constraints: the average $n_i$ per AZ and action potential and PPF with a 10-ms ISI. The simulation results are listed in Table 4, and several general trends are apparent. Overall, the number of fused vesicles per action potential and AZ increased as either $\Delta E_S$ or $\Delta E_Y$ was increased. On the other hand, an increase in $\Delta E_S$ or $\Delta E_Y$ led to a decrease in PPF, with the latter being significantly more sensitive to changes in $\Delta E_S$ compared with $\Delta E_Y$. As shown in Table 4, several models provided good agreement based on average number of fusion events and PPF ($n_i = 0.41–0.59$, PPF = 1.45–1.75). In particular, using $k_{\text{on}} = 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for synaptotagmin’s Ca$^{2+}$ binding sites, models with $n_Y = 12$ ($\Delta E_S/\Delta E_Y = 10/15 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 10/16 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 11/14 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 11/15 \text{ k}_{\text{B}}T$) and $n_Y = 16$ ($\Delta E_S/\Delta E_Y = 10/13 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 10/14 \text{ k}_{\text{B}}T$) fit our two initial constraints. On the other hand, for models with $n_Y = 8$, 28, 68, and 144 binding sites, we could not find suitable $\Delta E_S$ and $\Delta E_Y$ values that agreed with our experimental constraints. We also examined models with a synaptotagmin $k_{\text{on}}$ value of $4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. Here again, models with $n_Y = 16$ ($\Delta E_S/\Delta E_Y = 9/11 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 9/12 \text{ k}_{\text{B}}T$; $\Delta E_S/\Delta E_Y = 9/13 \text{ k}_{\text{B}}T$, $n_Y = 28$ ($\Delta E_S/\Delta E_Y = 9/8 \text{ k}_{\text{B}}T$), and $n_Y = 68$ ($\Delta E_S/\Delta E_Y = 9/4 \text{ k}_{\text{B}}T$) provided good agreement with our two initial experimental constraints. Interestingly, when including the CRR as a constraint, only the 16-Y-site-energy model with $k_{\text{on}} = 4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for synaptotagmin and $\Delta E_S/\Delta E_Y = 9/11 \text{ k}_{\text{B}}T$ provided a good match with a CRR value of 4.40 (Fig. 5C). This model also satisfied all of our additional constraints, namely, the narrow vesicle release latency (Fig. 5D), experimentally observed tetanic facilitation (Fig. 5A), and PPF decay as ISI was increased (Fig. 5B). Similar to our 28-Y-site model above, the magnitude of tetanic facilitation magnitude was slightly lower compared with our experimental measurements, and the PPF decayed more rapidly as the ISI was increased. The 16-Y-site-energy model also showed the expected sensitivity toward addition of exogenous Ca$^{2+}$ buffer (Fig. 5, $E$ and $F$): addition of BAPTA reduced vesicle release rapidly (Fig. 5E) and also lowered PPF significantly (e.g., using our 70% criterion for block of vesicle fusion during a single action potential). Similarly, addition of EGTA only moderately reduced vesicle release (compared to BAPTA) and PPF (Fig. 5F).

Thus, among all models we examined, the 16-Y-site-energy model with $k_{\text{on}} = 4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for Ca$^{2+}$ binding sites on synaptotagmin and energy contributions $\Delta E_S/\Delta E_Y = 9/11 \text{ k}_{\text{B}}T$ toward overcoming the energy barrier for vesicle fusion matched our experimental constraints best. Together with the 28-Y-site model above, our results indicate that within the context of a second sensor model the number of Y binding sites may be on the order of the number of available synaptotagmin Ca$^{2+}$ binding sites or less. Table 5 summarizes the main properties of all viable models examined in this study.

Effect of second sensor site grouping on facilitation. In our 16-Y-site-energy model the second sensor sites were activated once bound by a single Ca$^{2+}$ ion. Hence, we wanted to explore how grouping of second sensor sites, analogous to the manner in which synaptotagmin binding sites are grouped in our model, would impact the behavior of our facilitation model. Since the nature of the second sensor protein is currently unknown, we do not know how many Ca$^{2+}$ sensor sites are on each individual protein and how many need to bind for it to be activated. Starting from our 16-Y-site-energy model we initially grouped Y sites into groups of two and four while still requiring that binding of only a single Ca$^{2+}$ binding site per group was sufficient for activation. This modification left both release and facilitation basically unchanged (see Table 6). However, once simultaneous binding of two (or more) Ca$^{2+}$ ions was required for activation of second sensor proteins, only models with a larger total number of Y binding sites were viable. In addition, the number of Y binding sites per second sensor protein (grouping) had to be at least six to provide the proper level of release and facilitation. For example, a model with 144 Y binding sites and 6 Ca$^{2+}$ binding sites per second sensor protein, 2 of which had to be bound simultaneously, agreed well with our release and facilitation constraints (see Table 6). The need for larger numbers of Y binding sites derives from the fact that the total number of Ca$^{2+}$ ions that bind to second sensor sites is small (<6). Thus, in order to activate sufficient second sensor proteins (required for facilitation) given the requirement for simultaneous binding of 2 Ca$^{2+}$ ions per protein, ~20 second sensor proteins with at least 6
Ca\textsuperscript{2+} binding sites each were needed to provide the required level of release and facilitation.

Facilitation is enhanced under low external calcium conditions. With our second sensor model in hand, we wondered how variations in the external Ca\textsuperscript{2+} concentration would affect PPF within our models. We reduced the external Ca\textsuperscript{2+} concentration in our 16-Y-site-energy model and then determined the PPF in each case. The results are depicted in Fig. 6 and show that PPF increases significantly as the external Ca\textsuperscript{2+} concentration is lowered. We observed similar behavior for the 28-Y-site-energy and -nonenergy models and also the 68-Y-site-energy model (data not shown).

Table 5. Comparison of selected models matching our experimentally measured average \( n_r \) and PPF

<table>
<thead>
<tr>
<th>Model</th>
<th>( \Delta E_s )</th>
<th>( \Delta E_y )</th>
<th>( k_{on} ) M\textsuperscript{-1}s\textsuperscript{-1}</th>
<th>( n_r )</th>
<th>PPF</th>
<th>CRR</th>
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<tbody>
<tr>
<td>Persistent-binding-site model</td>
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<td>N/A</td>
<td></td>
<td>1 \times 10^6</td>
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<td>1.45</td>
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<tr>
<td>28-Y-site model</td>
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<td>N/A</td>
<td>1 \times 10^6</td>
<td>0.50</td>
<td>1.74</td>
<td>4.84</td>
</tr>
<tr>
<td>16-Y-site-energy model</td>
<td>10 k_BT</td>
<td>14 k_BT</td>
<td>1 \times 10^6</td>
<td>0.51</td>
<td>1.64</td>
<td>2.77</td>
</tr>
<tr>
<td>16-Y-site-energy model</td>
<td>9 k_BT</td>
<td>11 k_BT</td>
<td>4 \times 10^6</td>
<td>0.47</td>
<td>1.68</td>
<td>4.40</td>
</tr>
</tbody>
</table>

See Table 4 for additional parameter definitions.
Such a PPF increase under low external Ca\textsuperscript{2+} concentration was observed in earlier studies using experimental and computational methods (Holohean and Magleby 2011; Magleby and Zengel 1982). Fundamentally, this is due to a dramatic decrease in initial vesicle fusion under low Ca\textsuperscript{2+} conditions during the first stimulus, and correspondingly enhanced release during the second and subsequent pulses due to the effects of residual Ca\textsuperscript{2+}.

Nanodomain coupling of VGCCs to synaptic vesicles persists during repeated stimuli. Because of the particle-based nature of our MCell simulations we were able to track which and how many VGCCs contributed to the release of individual vesicles during repeated stimulation. To this end, Fig. 6B depicts the fractional contribution of different numbers of Ca\textsuperscript{2+} channels to the release of individual synaptic vesicles during a five-pulse tetanic stimulation event using our 16-Y-site-energy model.

Table 6. Effect of grouping of Y binding sites on model release and facilitation

<table>
<thead>
<tr>
<th>Model</th>
<th>N</th>
<th>n_c</th>
<th>ΔE_s</th>
<th>ΔE_Y</th>
<th>k_on, M\textsuperscript{-1}s\textsuperscript{-1}</th>
<th>n_v</th>
<th>PPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-Y-site-energy model</td>
<td>2</td>
<td>1</td>
<td>9 k_BT</td>
<td>11 k_BT</td>
<td>4 \times 10^8</td>
<td>0.45</td>
<td>1.74</td>
</tr>
<tr>
<td>16-Y-site-energy model</td>
<td>4</td>
<td>1</td>
<td>9 k_BT</td>
<td>11 k_BT</td>
<td>4 \times 10^8</td>
<td>0.46</td>
<td>1.73</td>
</tr>
<tr>
<td>144-Y-site-energy model</td>
<td>6</td>
<td>2</td>
<td>12 k_BT</td>
<td>15 k_BT</td>
<td>1 \times 10^8</td>
<td>0.55</td>
<td>1.58</td>
</tr>
</tbody>
</table>

Models in which different numbers N of Y binding sites were grouped into second sensor proteins are listed. Proteins were activated once n_c Y binding sites per grouping were simultaneously bound by Ca\textsuperscript{2+}. Only models that matched our experimentally measured average n_v and PPF are shown. See Table 4 for additional parameter definitions.

Fig. 6. Contribution of VGCC, synaptotagmin, and second sensor sites to vesicle release. Data shown are for the 16-Y-site-energy model with k_on = 4 \times 10^8 M\textsuperscript{-1}s\textsuperscript{-1} for synaptotagmin at 100 Hz. A: PPF as a function of external [Ca\textsuperscript{2+}] shows an increase in PPF as [Ca\textsuperscript{2+}] is decreased. B: fractional contribution of different numbers of VGCCs to release during repeated stimuli. Numbers over bars for each stimulation event are the average number of VGCCs contributing to vesicle release during that stimulus. C and D: average number of bound synaptotagmin (C) and Y (D) sites per vesicle as a function of time under control conditions and in the presence of different BAPTA concentrations (average over n = 260,000 vesicles). E and F: average number of bound synaptotagmin (E) and Y (F) sites per vesicle as a function of time under control conditions and in the presence of different EGTA concentrations (average over n = 260,000 vesicles).
model. As we observed previously (Dittrich et al. 2013), during the first stimulus the majority of vesicle release events were triggered by Ca\(^{2+}\) ions from a single (29%) or two (44%) VGCCs, and only a smaller fraction (27%) were derived from three channels or more. No release events were triggered by more than five channels. On average, 2.06 channels contributed to each vesicle fusion event during the first stimulus, in agreement with our previous results (Dittrich et al. 2013) and values reported in the literature (Shahrezaei et al. 2006). This suggests that at the frog NMJ only a small number of nearby channels are responsible for vesicle release in a nanodomain fashion (Tarr et al. 2013). During repeated stimulation, the number of channels contributing to vesicle fusion increased as shown in Fig. 6B. For example, the fraction of vesicle fusion events triggered by Ca\(^{2+}\) from only one or two VGCCs dropped from 73% during the first pulse to 44% during the fifth stimulus. Correspondingly, the contribution to fusion of three or more channels grew from 27% in the first stimulus to 56% during the fifth stimulus. Therefore, on average, more VGCCs contributed to vesicle release during later stimuli in the train (from 2.06 during the first stimulus to 2.69 during the fifth). However, this increase was mainly due to additional sampling of available nearby channels during repeated stimuli as opposed to recruitment of more distant channels (data not shown). Thus vesicle release continued to be triggered by Ca\(^{2+}\) ions from one up to four VGCCs and nanodomain coupling was retained during a short train of five stimuli.

Effect of exogenous buffer on Ca\(^{2+}\) binding to synaptotagmin and the second sensor site. In our simulations, BAPTA significantly reduced binding of Ca\(^{2+}\) to both synaptotagmin and second sensor sites (Fig. 6, C and D, for 16-Y-site-energy model), while EGTA mainly reduced Ca\(^{2+}\) binding to second sensor sites but not to synaptotagmin (Fig. 6, C and D, for 16-Y-site-energy model). Fundamentally, this was due to the competition of Ca\(^{2+}\) binding to either exogenous buffer or binding sites on vesicles. Since BAPTA is a fast buffer (\(k_{\text{off}} = 4 \times 10^{7} \text{M}^{-1}\text{s}^{-1}\)), large concentrations outcompeted Ca\(^{2+}\) for binding to both synaptotagmin (\(k_{\text{off}} = 4 \times 10^{7} \text{M}^{-1}\text{s}^{-1}\)) and second sensor sites (\(k_{\text{off}} = 1 \times 10^{8} \text{M}^{-1}\text{s}^{-1}\)), thus reducing both vesicle release and facilitation. On the other hand, the slow buffer EGTA (\(k_{\text{off}} = 1 \times 10^{7} \text{M}^{-1}\text{s}^{-1}\)) competed effectively only with second sensor sites and thus affected facilitation significantly more than initial release. Based on this observation our model suggests that the \(k_{\text{off}}\) for Ca\(^{2+}\) binding to the yet unknown second sensor sites is likely to be lower than or on the order of the \(k_{\text{off}}\) of EGTA (\(1 \times 10^{7} \text{M}^{-1}\text{s}^{-1}\)).

DISCUSSION

Synaptic facilitation is a key element of neural activity and underlies many important physiological processes. At the frog NMJ, the synaptic response typically grows severalfold during repeated stimulation over the course of several tens to hundreds of milliseconds (Fig. 1C). A wide range of possible mechanisms have been hypothesized to underlie short-term synaptic facilitation (summarized in Zucker and Regehr 2002), but none has so far been conclusively identified to underlie this important aspect of synaptic function. In fact, different synapses (in different organisms) may employ different facilitation mechanisms altogether (Atwood and Karunanithi 2002; Dittman et al. 2000; Pan and Zucker 2009). We do know, however, that facilitation primarily arises presynaptically (Fisher et al. 1997; Worden et al. 1997; Zucker 1989) and that Ca\(^{2+}\) ions play a critical role. Several proposed facilitation mechanisms derive from this key insight, most prominently perhaps the so-called residual calcium hypothesis, which posits that Ca\(^{2+}\) remaining in the terminal after a stimulation event contributes productively to future vesicle release events (Bennett et al. 1997; Delaney and Tank 1994; Matveev et al. 2006; Tank et al. 1995; Zucker and Regehr 2002). At the calyx of Held, there is mounting evidence that Ca\(^{2+}\)-triggered facilitation of P/Q-type VGCCs might underlie facilitation of transmitter release (Catterall et al. 2013; Catterall and Few 2008; Mochida et al. 2008). However, since the NMJ features N-type VGCCs, the relevance of Ca\(^{2+}\)-triggered channel facilitation is unclear.

Residual free Ca\(^{2+}\) does not lead to facilitation. One facilitation mechanism proposed early on relies on an increase in the presynaptic background free Ca\(^{2+}\) concentration due to the accumulation of Ca\(^{2+}\) ions from previous stimuli. The idea was that this residual free Ca\(^{2+}\) would add to the Ca\(^{2+}\) ions entering the AZ through VGCCs during subsequent stimuli, act on the synaptotagmin sensors that trigger vesicle fusion (Bennett et al. 1997; Kanz and Miledi 1968), and lead to increased vesicle fusion during repeated trials. We could test this hypothesis directly with our excess-calcium-binding-site model (Dittrich et al. 2013) previously developed under a single-pulse paradigm by applying repeated stimuli. As shown in Fig. 1C, our simulations showed no facilitation at all, and instead exhibited minor depression caused by the decrease in numbers of available synaptic vesicles due to prior fusion events. We could trace the lack of facilitation to two fundamental underlying causes. First, the accumulation of residual Ca\(^{2+}\) ions in the terminal was too small to contribute productively to subsequent release events. This observation is consistent with previous modeling efforts, which found that accumulation of free residual Ca\(^{2+}\) in the AZ was insufficient for facilitation (Blundon et al. 1993; Zucker and Regehr 2002). As shown in Fig. 3B, in a model in which Ca\(^{2+}\) ions were removed when they encountered the edge of the AZ, the Ca\(^{2+}\) concentration quickly saturated at \(\sim 0.07 \mu\text{M}\). Interestingly, using the peak Ca\(^{2+}\) concentration after the first pulse (\([\text{Ca}]_{\text{loc}}\)), the residual Ca\(^{2+}\) (\([\text{Ca}]_{\text{res}}\)) measured in our simulations (Fig. 3B), and the relationship (\([\text{Ca}]_{\text{loc}} + [\text{Ca}]_{\text{res}}\)^{4}/([\text{Ca}]_{\text{loc}}^{4}) proposed by Zucker and coworkers (Magleye and Zengel 1982; Zucker and Regehr 2002) predicted values for facilitation of 1.8 for the fifth stimulus event, while none was observed in our simulations. This discrepancy between predicted facilitation based on whole terminal residual Ca\(^{2+}\) and the observed lack of facilitation in our actual simulations emphasizes the need for a highly localized Ca\(^{2+}\) nanodomain to productively contribute Ca\(^{2+}\) ions to vesicle fusion (Dittrich et al. 2013). In fact, in our simulations we did not observe any significant localized accumulation of residual free Ca\(^{2+}\) ions near vesicular sensors (Fig. 1D) despite the presence of free Ca\(^{2+}\) within the whole terminal. Such a nanodomain is only transiently established by the VGCCs closely associated with synaptic vesicles during an action potential. In contrast, the residual Ca\(^{2+}\) ions distributed across the whole terminal at the concentrations observed in our simulations are not effective in contributing to this nanodomain and thus vesicle fusion.
A second factor in the observed lack of facilitation in our excess-calcium-binding-site model was the binding kinetics of Ca²⁺ ions to synaptotagmin on synaptic vesicles. In particular, the dwell time required to keep the latency distribution narrow (Dittrich et al. 2013) was too short for a significant number of ions to remain bound to synaptotagmin during a typical ISI and thus to contribute to subsequent release events. In principle, increasing the dwell time of Ca²⁺ ions on synaptotagmin could enhance facilitation. Indeed, previous work (Atluri and Regehr 1996) reported a single Ca²⁺ binding site model with high binding affinity (167 nM), which generated facilitation. However, such a large value for the Ca²⁺ binding affinity does not match the reported values for the high-affinity synaptotagmin Ca²⁺ binding site (60 µM) (Radhakrishnan et al. 2009). Our results suggest that facilitation likely derives from a more complex scenario than is provided by a simple accumulation of free Ca²⁺ acting on synaptotagmin.

Facilitation via a second Ca²⁺ sensor on synaptic vesicles. The involvement of multiple spatially and kinetically distinct Ca²⁺ binding sites in triggering vesicle fusion and facilitation has been proposed previously (Bennett et al. 2004; Matveev et al. 2006; Tang et al. 2000; Yamada and Zucker 1992). One early facilitation model (Bertram et al. 1996) used four independent Ca²⁺ binding sites with distinct binding affinities ranging from 100 nM to >1,000 µM and suggested that residual Ca²⁺ existed in bound rather than free form. A similar conclusion was reached based on a different model (Matveev et al. 2006) also with four Ca²⁺ binding sites (2 with high and 2 with low unbinding rates). A series of computational studies on the crayfish NMJ using a finite-difference method (Matveev et al. 2002; Tang et al. 2000) and on the amphibian NMJ via a Monte Carlo approach (Bennett et al. 2004) also used four Ca²⁺ binding sites (3 with high and 1 with low unbinding rates). In the latter study the two types of Ca²⁺ binding sites were segregated in space (>150 nm) to avoid saturation of the high-affinity binding site. Given a typical vesicle diameter of 50 nm, this would place the high-affinity Ca²⁺ binding sites sufficiently far away from the vesicle and, at least for the frog NMJ it is not clear what the structural correlate would be. A more recent publication reported a model of vesicle release and facilitation, which included vesicle mobilization, priming, and two release pools in addition to the Ca²⁺ binding/unbinding kinetics (Pan and Zucker 2009). Despite this model’s more comprehensive nature, its Ca²⁺ binding/unbinding kinetics were similar to earlier models (Miliar et al. 2005; also adopted in Nadkarni et al. 2010) in which all five cooperative binding sites needed to bind Ca²⁺ to trigger fusion. In these models, addition of bound Ca²⁺ ions reduces the dissociation rate, which gradually turns the synaptotagmin binding sites into sites similar to the second sensor sites in our model. All the above models rely on a cooperative scheme for Ca²⁺ binding. In contrast, our second sensor model assumes no ad hoc cooperativity and builds on our previously developed excess-calcium-binding-site model (Dittrich et al. 2013) by explicitly accounting for the spatial arrangement of Ca²⁺ sites on synaptic vesicles. Our model includes distinct second sensor sites in close vicinity to synaptotagmin molecules on synaptic vesicles (Fig. 4A). Similar to our synaptotagmin model, these second sensor sites bound Ca²⁺ with simple on and off kinetics ($K_D = 6 \text{ µM, } k_{on} = 6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}, k_{off} = 36 \text{ s}^{-1}$) chosen to be similar to previous models (Matveev et al. 2006). We found that a model with 16–28 second sensor sites agreed well with our experimental constraints, including short-term facilitation and response to the addition of exogenous buffers BAPTA and EGTA. Interestingly, while the main role of the second sensor sites within our model was in facilitation, they also contributed to the initial fusion event. In fact, during a train of five stimuli at 100 Hz the average number of second sensor sites that contributed to fusion increased from 1.3 during the first pulse to 1.9 during the last. These data show that the need for an excess of Ca²⁺ binding sites on both synaptotagmin and the (unknown) protein responsible for facilitation (only a small subset of which have to bind Ca²⁺ for release to take place) continues to underlie our model’s Ca²⁺ binding dynamics. Thus, while the precise molecular nature of the protein hosting the second Ca²⁺ sensor sites is currently unknown, our simulations predict the number of second sensor binding sites to be on the order of the number of Ca²⁺ sites present on synaptotagmin.

Persistent binding of synaptotagmin leads to facilitation. Recent biochemical and structural studies indicate that Ca²⁺-bound synaptotagmin associates with the lipid membrane and engages in a longer-lived persistent state (~80 ms) (Bai et al. 2002; Hui et al. 2006; Lynch et al. 2007; Paddock et al. 2011). We reasoned that such a persistent state could provide the “memory” required for facilitation that was lacking from our original excess-calcium-binding-site model (Dittrich et al. 2013). Indeed, adding such a persistent state to our model led to facilitation in good agreement with our experimental constraints. This is to our knowledge the first model correlating synaptotagmin/membrane interactions with short-term facilitation. While a reaction scheme that included activation of a bound Ca²⁺ sensor had been proposed earlier (Atluri and Regehr 1996), those authors did not actually implement and test their scheme. Given the simplicity of our persistent-binding model it is rather remarkable that it agrees so well with most of our experimental constraints. Nevertheless, because of a lack of Ca²⁺ exchange once in the persistent state our model did not fully capture the effect of an exogenous Ca²⁺ buffer such as BAPTA on facilitation. More complex persistent binding schemes, which are outside the scope of the present investigation, will likely be required to faithfully model buffer effects.

Conclusions. In this study we were able to show that both the second sensor and persistent-binding models are good candidates for mediating facilitation at the frog NMJ. In a real biological context it is quite likely that facilitation will be due to a combination of both mechanisms and others not considered here. In fact, experiments have revealed several distinct phases of short-term facilitation (Magleby 1979; Zucker and Regehr 2002), which may be due to distinct facilitation mechanisms or combinations thereof. In the present study we did not consider facilitation mechanisms that have been shown to be important at synapses other than the NMJ. Examples are facilitated Ca²⁺ entry through P/Q-type VGCCs at the calyx of Held (Catterall et al. 2013; Mochida et al. 2008) and saturation of local buffer (Blatow et al. 2003). Detailed investigation of these mechanisms within the context of our modeling approach will require additional experimental data at the NMJ and may be the subject of a future study.
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