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Brevity of the Ca^{2+} Microdomain and Active Zone Geometry Prevent Ca^{2+}-Sensor Saturation for Neurotransmitter Release

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Shahrezaei, Vahid and Kerry R. Delaney. Brevity of the Ca^{2+} microdomain and active zone geometry prevent Ca^{2+}-sensor saturation for neurotransmitter release. *J Neurophysiol* 94: 1912–1919, 2005. First published May 11, 2005; 10.1152/jn.00256.2005. The brief time course of the calcium (Ca^{2+}) channel opening combined with the molecular-level colocalization of Ca^{2+} channels and synaptic vesicles in presynaptic terminals predict sub-millisecond calcium concentration ($[Ca^{2+}]$) transients of $\geq$100 μM in the immediate vicinity of the vesicle. This $[Ca^{2+}]$ is much higher than some of the recent estimates for the equilibrium dissociation constant of the Ca^{2+} sensor(s) that control neurotransmitter release, suggesting release should be close to saturation, yet it is well known that release is highly sensitive to changes in Ca^{2+} influx. We show that due to the brevity of the Ca^{2+} influx the binding kinetics of the Ca^{2+} sensor rather than its equilibrium affinity determine receptor occupancy. For physiologically relevant Ca^{2+} currents and forward Ca^{2+} binding rates, the effective affinity of the Ca^{2+} sensor can be several-fold lower than the equilibrium affinity. Using simple models, we show redundant copies of the binding sites increase effective affinity of the Ca^{2+} sensor for release. Our results predict that different levels of expression of Ca^{2+} binding sites could account for apparent differences in Ca^{2+} sensor affinities between synapses. Using Monte Carlo simulations of Ca^{2+} dynamics with nanometer resolution, we demonstrate that these kinetic constraints combined with vesicles acting as diffusion barriers can prevent saturation of the Ca^{2+}-sensor(s) for neurotransmitter release. We further show the random positioning of the Ca^{2+}-sensor molecules around the vesicle can result in the emergence of two distinct populations of the vesicles with low and high release probability. These considerations allow experimental evidence for the Ca^{2+} channel-vesicle colocalization to be reconciled with a high equilibrium affinity for the Ca^{2+} sensor of the release machinery.

INTRODUCTION

It is well established that Ca^{2+} influx into the presynaptic nerve terminal through voltage-gated Ca^{2+} channels triggers fusion of the synaptic vesicles and neurotransmitter release. Recent molecular biological studies indicate direct physical and functional interactions between some voltage-gated Ca^{2+} channels and vesicle-associated proteins (Spafford and Zamponi 2003). High-resolution electron microscopic studies at vertebrate neuromuscular junctions provide strong evidence for colocalization, on the order of 10–30 nm between the vesicle and intra-membrane particles (thought to be Ca^{2+} channels), consistent with the separations predicted by binding of channels to synaptic proteins (Harlow et al. 2001). Such extreme colocalization of Ca^{2+} channels with vesicles (and their associated release machinery) leads to high local $[Ca^{2+}]$ of order $\geq$100 μM at the vesicle (Simon and Llinas 1985). Also overlap of the Ca^{2+} microdomains from a few open channels can produce high $[Ca^{2+}]$ of over 100 μM at a region larger than a single-channel microdomain (Yamada and Zucker 1992). Using a low-affinity calcium-dependent photoprotein, high Ca^{2+} microdomains on the order of 200–300 μM close to the plasma membrane have been observed in the squid giant synapse (Llinas et al. 1992).

Neurotransmitter release appears to be triggered by the cooperative action of Ca^{2+} ions on three to five Ca^{2+} binding sites (Bollmann et al. 2000; Dodge and Rahamimoff 1967; Heidelberger et al. 1994; Schneggenburger and Neher 2000). The classical view is that at least one of the binding sites of the Ca^{2+} sensor has a low affinity with a dissociation constant (K_d) of $\geq$100 μM (Zucker 1993). Assuming $[Ca^{2+}]$ of $\sim$100 μM at the Ca^{2+}-sensor, a low affinity is predicted from the fact that the Ca^{2+} sensor is not saturated under normal physiological conditions. Increasing $[Ca^{2+}]$ in the bath increases release and decreasing $[Ca^{2+}]$ (even by small amounts) decreases release substantially (Dodge and Rahamimoff 1967). A low equilibrium binding affinity is consistent with caged Ca^{2+} photolysis experiments performed using goldfish retinal bipolar neurons (Heidelberger et al. 1994). However, this classical view of a low-affinity binding site was challenged by similar experiments in which caged Ca^{2+} was used to stimulate release from the calyx of Held (Bollmann et al. 2000; Schneggenburger and Neher 2000). In this case, $[Ca^{2+}]$ dependence of release for this synapse was best fitted by a model with five Ca^{2+} binding sites with K_d of $\sim$10 μM and two Ca^{2+}-independent steps (Bollmann et al. 2000). The nonsaturation of release by the high local $[Ca^{2+}]$ produced during an action potential is paradoxical if one assumes release is controlled by high-affinity Ca^{2+} binding sites, especially for synapses with highly colocalized channels and vesicles such as vertebrate neuromuscular junctions (Harlow et al. 2001). This problem can be avoided in principle in an active zone with noncolocalized channel vesicles because the $[Ca^{2+}]$ would be lower at the sensor if the vesicles and channels are further apart. Thus in the calyx of Held, a model that assumes a nonuniform channel-vesicle topography with average separations of $\sim$100 nm is successful in describing most aspects of release (Meinrenken et al. 2002). However, because of the steep dependence of release on $[Ca^{2+}]$, it becomes difficult to impossible to accommodate

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observed release probabilities if this separation is reduced by a few tens of nanometer.

Although the affinity of the Ca^{2+} sensors has been actively debated, their chemical kinetics has been given less attention. The on rate of the Ca^{2+}-sensor molecule remains poorly known, although some recent studies suggest it is $\sim 10^7$-10^8 $M^{-1}s^{-1}$ (Davis et al. 1999; Millet et al. 2002). Models of Ca^{2+}-triggered release implemented in different studies have employed a large range of k_{on} values [for example, $\sim 10^6$ $M^{-1}s^{-1}$ (see Bertram et al. 1999), $\sim 10^7$ $M^{-1}s^{-1}$ (see Bennett et al. 2000) and $\sim 5 \times 10^8$ $M^{-1}s^{-1}$ (see Tang et al. 2000)]. The binding kinetics will be crucial to determining the results of release models and release probability if the reaction with Ca^{2+} does not reach equilibrium. In the context of cytoplasmic Ca^{2+} buffering, it has been established that the binding kinetics rather than the affinity determines the effectiveness of a particular buffer for inhibiting Ca^{2+}-triggered release (Adler et al. 1991).

Among the vesicle-associated proteins, synaptotagmin is the best Ca^{2+}-sensor candidate for Ca^{2+}-triggered release (Augustine 2001; Chapman 2002). Yet the role of different binding sites of synaptotagmin, their chemical affinity, kinetics, position relative to the vesicle and Ca^{2+} channel, and whether all the Ca^{2+}-binding sites needed for release are on the same molecule or on different molecules continue to be debated (Bai and Chapman 2004; Koh and Bellen 2003). The cooperation of three to eight SNARE complexes may be needed for fusion (Han et al. 2004; Hua and Scheller 2001). There is a synaptotagmin associated with each SNARE complex, and evidence suggests their interaction plays a role in fusion (Bai et al. 2004). If we assume up to five binding sites on each synaptotagmin molecule and three to eight synaptotagmin molecules per vesicle, the potential number of binding sites could be well above the observed Ca^{2+} cooperativity. Consequently, the Ca^{2+} binding sites that need to bind to Ca^{2+} to trigger release could be distributed around the vesicle (Stewart et al. 2000). Release models with Ca^{2+} ions binding to a subset of a larger number (>5) of potential binding sites to cause release have not yet been considered.

In this study, we address the possibility of nonsaturation of release in a synapse with colocalized active zone topography and high affinity Ca^{2+} sensor(s) using a detailed assessment of the temporal and spatial aspects of neurotransmitter release. In the first part of this study, our analysis relies primarily on the fact that the Ca^{2+} sensor(s) are exposed to high [Ca^{2+}] for only a fraction of a millisecond during and immediately after the channel opening (Llinas et al. 1982, 1995; Simon and Llinas 1985). Therefore chemical equilibrium may not be achieved, and the forward binding kinetics of the sensor, rather than the equilibrium affinity, becomes the critical variable determining the extent of Ca^{2+} binding. Next, we introduce two simple models that allow for the possibility of more than five binding sites for release consistent with biochemical evidence as discussed in the preceding text. We explore the effect of extra binding sites on release probability using simple rate equations. Then using a Monte Carlo simulation of Ca^{2+} dynamics with nanometer spatial resolution, we assess the effect of the vesicle as a barrier to free diffusion of Ca^{2+} ions on release. Finally using the Monte Carlo analysis we explore the effect of the position and number of potential binding sites around the docked vesicle on release.

METHODS

Invasion of the nerve terminal by an action potential opens voltage-gated Ca^{2+} channels. Ca^{2+} ions enter and diffuse within the nerve terminal where they bind reversibly to fixed and mobile endogenous buffers. Remaining free Ca^{2+} ions can bind to the Ca^{2+}-sensor sites on the release machinery to initiate fusion of synaptic vesicles. The release follows the onset of the brief influx of Ca^{2+} with a delay of a fraction of millisecond (Llinas et al. 1981; Sabatini and Regehr 1996).

For most of this study, following Bollmann et al. (2000), we used a general release model with five independent Ca^{2+} binding sites and two Ca^{2+}-independent fusion steps

$$X \underset{k_{off}}{\overset{5k_{on}[Ca]}{\rightleftharpoons}} XCa_1 \underset{2k_{off}}{\overset{4k_{on}[Ca]}{\rightleftharpoons}} XCa_2 \underset{3k_{off}}{\overset{3k_{on}[Ca]}{\rightleftharpoons}} XCa_3 \underset{4k_{off}}{\overset{2k_{on}[Ca]}{\rightleftharpoons}} XCa_4 \underset{5k_{off}}{\overset{k_{on}[Ca]}{\rightleftharpoons}} XCa_5 \underset{\delta}{\overset{\gamma}{\rightleftarrows}} XCa_5^* \overset{\rho}{\rightarrow} F \quad (1)$$

where X represents the Ca^{2+} sensors, which comprise five Ca^{2+} binding sites, XCa_5^* is an intermediate state prior to fusion, and F represents a complete fusion event. k_{on} and k_{off} are the on rate and off rate of the reaction. The fusion rates, γ, δ, and ρ were fixed to 30,000, 8,000, and 40,000 s^{-1}, respectively, as estimated in Calyx of Held (Bollmann et al. 2000). We call this release model 1.

The number of potential Ca^{2+} binding sites per docked vesicle could exceed the observed Ca^{2+} cooperativity of release because several synaptotagmin molecules, each with five potential binding sites, may comprise each docking complex. One of the simplest generalizations of release model 1 is to assume several ($m > 5$) identical binding sites per vesicle, with binding to any five sites allowing fusion to proceed. In this case the release model presented in *Eq. 1* will be slightly modified

$$X \underset{k_{off}}{\overset{mk_{on}[Ca]}{\rightleftharpoons}} XCa_1 \underset{2k_{off}}{\overset{(m-1)k_{on}[Ca]}{\rightleftharpoons}} XCa_2 \underset{3k_{off}}{\overset{(m-2)k_{on}[Ca]}{\rightleftharpoons}} XCa_3 \underset{4k_{off}}{\overset{(m-3)k_{on}[Ca]}{\rightleftharpoons}} XCa_4 \underset{5k_{off}}{\overset{(m-4)k_{on}[Ca]}{\rightleftharpoons}} XCa_5 \underset{\delta}{\overset{\gamma}{\rightleftarrows}} XCa_5^* \overset{\rho}{\rightarrow} F \quad (2)$$

where the forward rates are now dependent on m, the number of available binding sites. Release model 1 is a special case of release model 2 for which the number of binding sites is equal 5 ($m = 5$).

Another possibility is to assume five clusters of binding sites each comprising k independent sites. Release proceeds only if at least one Ca^{2+} is bound to each cluster. In this model (release model 3), there are more than five binding sites (5k in total), but unlike model 2, more than five Ca^{2+} ions will likely bind before the conditions required to initiate fusion are achieved.

In the first part of the study (Figs. 1 and 2), we assume the [Ca^{2+}] at the sensor is constant and that it persists for either 0.3 or 1 ms during the opening of the channel and briefly afterward. These are good approximations because [Ca^{2+}] reaches its steady state in the immediate vicinity of the channel within microseconds of the opening of the channel and dissipates rapidly after closing (Llinas et al. 1995). The release probability predicted for a given set of rate constants can be calculated from the rate equations describing the reaction scheme in *Eq. 1* (or *Eq. 2*). Figure 1 is produced using this procedure for 10,000 different choices of k_{on} [Ca^{2+}] and K_d ($=k_{off}/k_{on}$). Figure 2 is produced by solving *Eq. 1* for different values of [Ca^{2+}] and k_{on} and *Eq. 2* for different values of [Ca^{2+}] and m.

Our analysis of the effect of Ca^{2+} binding kinetics or number of binding sites on release is independent of whether the Ca^{2+} domain from a single highly colocalized channel or overlapping domains from a few less tightly colocalized channels combine to produce [Ca^{2+}] ≥ 100 μM. For the second part of this study, we assumed influx through a single channel was sufficient to trigger fusion. Although this assumption probably does not strictly apply at all synapses (Stanley

1997), there is evidence that such a situation applies at some synapses (Gentile and Stanley 2005; Wachman et al. 2004), and it simplifies our analysis.

The Monte Carlo simulation employed for the second part of this study is adopted from Shahrezaei and Delaney (2004). In the Monte Carlo simulation method, the motion of each individual Ca^{2+} or buffer molecule is followed as it diffuses inside the nerve terminal. This is not done at the level of actual Brownian motion but rather at a coarser level, using random walk theory. Ca^{2+} ions bind to the binding sites of the Ca^{2+} sensor with some probability if they get closer than a certain distance. Therefore in this part the forward reaction rate is diffusion limited.

Because of the stochastic nature of the Monte Carlo method, repeated trials are required to assess the average behavior of the system. The steady-state concentration profile in this study (Fig. 3*B*) is the result of averages of 1,000 1-ms-long Monte Carlo runs corresponding to $\sim 10^8$ independent sampling events.

For calculating release probabilities in Figs. 3 and 4, we produce a Ca^{2+} current of 0.3 pA into the terminal for 0.3 ms, which is equivalent of $\sim$280 Ca^{2+} ions (Stanley 1993) and see if the release machinery reaches the fusion step within 0.6 ms after the closure of the channel. We repeat this 1,000 times for each choice of the position for the Ca^{2+} sensor. Release probability for a single vesicle is the fraction of the Monte Carlo trials for which fusion was achieved.

RESULTS

Kinetics and effective affinity

Due to brevity of the Ca^{2+} influx, the binding kinetics of the Ca^{2+} sensor can be critical to determining release probability. To address the effect of binding kinetics on release, we use release model 1, which has five independent Ca^{2+} binding sites and two final Ca^{2+}-independent fusion steps (*Eq. 1*). We examine the release probability in response to a single-channel opening for a wide range of k_{on} and K_d of the Ca^{2+} sensor. The release probability is dependent on the equilibrium affinity of the site ($1/K_d$) and independent of the on rate, if equilibrium between free Ca^{2+} and Ca^{2+} binding sites is achieved. Figure 1 shows that for a given $[Ca^{2+}]$, if k_{on} is greater than a critical value, equilibrium will be largely achieved. The regime, where binding between Ca^{2+} and the sensor is at equilibrium, is evident where the constant release probability contours are parallel to the $k_{on}[Ca^{2+}]$ axis (Fig. 1). Importantly, though, if the on rate is smaller than this critical value, the release probability will depend strongly on the on rate and will be almost independent of the K_d. This corresponds to the portion of Fig. 1 where the constant release probability contours are parallel to the $K_d/[Ca^{2+}]$ axis. The forward rate of reaction is proportional to both k_{on} and $[Ca^{2+}]$ (*Eq. 1*). Consequently a site that experiences lower $[Ca^{2+}]$ needs a higher k_{on} to achieve equilibrium. For example, for a set of sensors with $K_d = 10$ μM that transiently experience $[Ca^{2+}] = 200$ μM, equilibrium conditions are achieved for k_{on} of $\sim 3 \times 10^8$ $M^{-1}s^{-1}$. If the same set of sensors experience $[Ca^{2+}] = 15$ μM, equilibrium would be achieved for k_{on} of $\sim 2 \times 10^9$ $M^{-1}s^{-1}$.

The time constant for the exponential approach to equilibrium for the reaction between a single Ca^{2+} binding site and free Ca^{2+} is described by the following

$$\tau_{eq} = 1/(k_{on}[Ca^{2+}] + k_{off}) \quad (3)$$

Although the cooperativity between the binding sites for fusion makes *Eq. 1* more complicated than a simple collection of five equal and independent binding sites, τ_{eq} provides a good approximation of the approach to equilibrium. A good rule of thumb is that the kinetic rates of the Ca^{2+}-sensor are important if the duration of the current (T_{Ca}) is comparable to or smaller than the equilibrium time constant of the reaction (τ_{eq}).

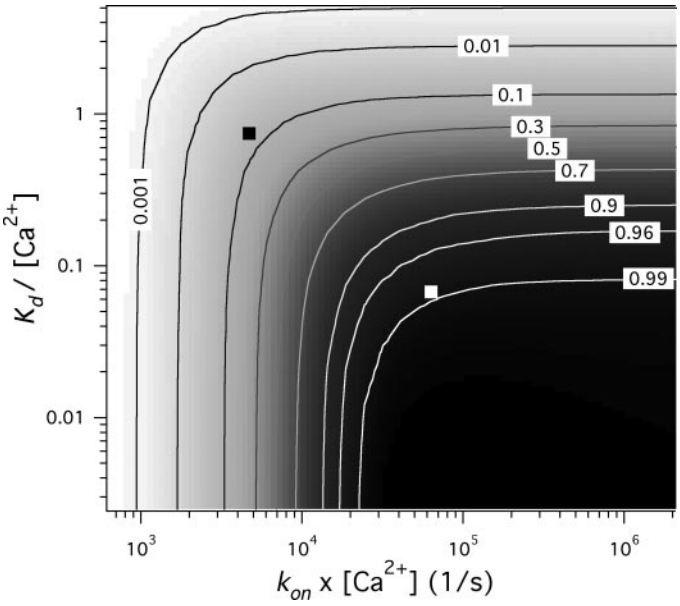

FIG. 1. Release probability as a function of on rate and affinity of the Ca^{2+} sensor in response to a single channel opening that lasts 0.3 ms for release model 1. The $[Ca^{2+}]$ dependence of the rate of forward reaction in *Eq. 1* makes it natural to plot release probability as a function of the K_d (dissociation constant of the sensor) divided by $[Ca^{2+}]$ against k_{on} (on rate of the sensor) times $[Ca^{2+}]$. So for a given $[Ca^{2+}]$, this graph specifies the minimum k_{on} that is required for release probability to be dependent only on K_d. This condition is indicated by the regions where the constant release probability curves become parallel to the $k_{on} \times [Ca^{2+}]$ axis. Estimated k_{on} and K_d values for Calyx of Held in the presence of 15 μM (■) or 200 μM (□) $[Ca^{2+}]$.

To investigate the effect of the on rate of the Ca^{2+} sensor, we fixed the K_d at 10 μM and looked at the release probability as a function of the $[Ca^{2+}]$ at the sensor. We did this for two durations of Ca^{2+} current (T_{Ca}) 0.3 and 1 ms (Fig. 2, *A* and *B*) and five different values for the on rate between 10^6 and 10^{10} $M-^1s^{-1}$. It should be noted that in reality, there is a limit to the on-rate because the arrival of new molecules at the binding site is limited by diffusion. This effect is absent from our analysis in this section because we are just solving the rate equations (*Eq. 1*). The diffusion-limited rate for Ca^{2+} reactions in the cytoplasm is thought to be $\sim 5 \times 10^8$ to 10^9 $M^{-1}s^{-1}$ and that limits how fast the binding site can function and also the effect of multiple binding sites. For $k_{on} = 10^{10}$ $M^{-1}s^{-1}$ (which is well above the diffusion limited rate) the Ca^{2+} reaction is close to equilibrium because higher k_{on} values do not increase the release probability. So for Ca^{2+} durations of 0.3–1 ms, the release probability for $k_{on} = 10^{11}$ $M^{-1}s^{-1}$ or higher would lie almost on top of the line for $k_{on} = 10^{10}$ $M^{-1}s^{-1}$. At lower on rates, release probability is reduced significantly. Although this effect is larger for the shorter T_{Ca} of 0.3 ms (Fig. 2*A*), it is still significant for T_{Ca} of 1 ms (Fig. 2*B*). Because there is more time for the Ca^{2+} reaction to fully equilibrate and there is more time for the final Ca^{2+}-independent fusion step to proceed, release probabilities for the same equilibrium affinity and binding kinetics are higher for longer duration currents. Therefore for a given equilibrium affinity the release probability is

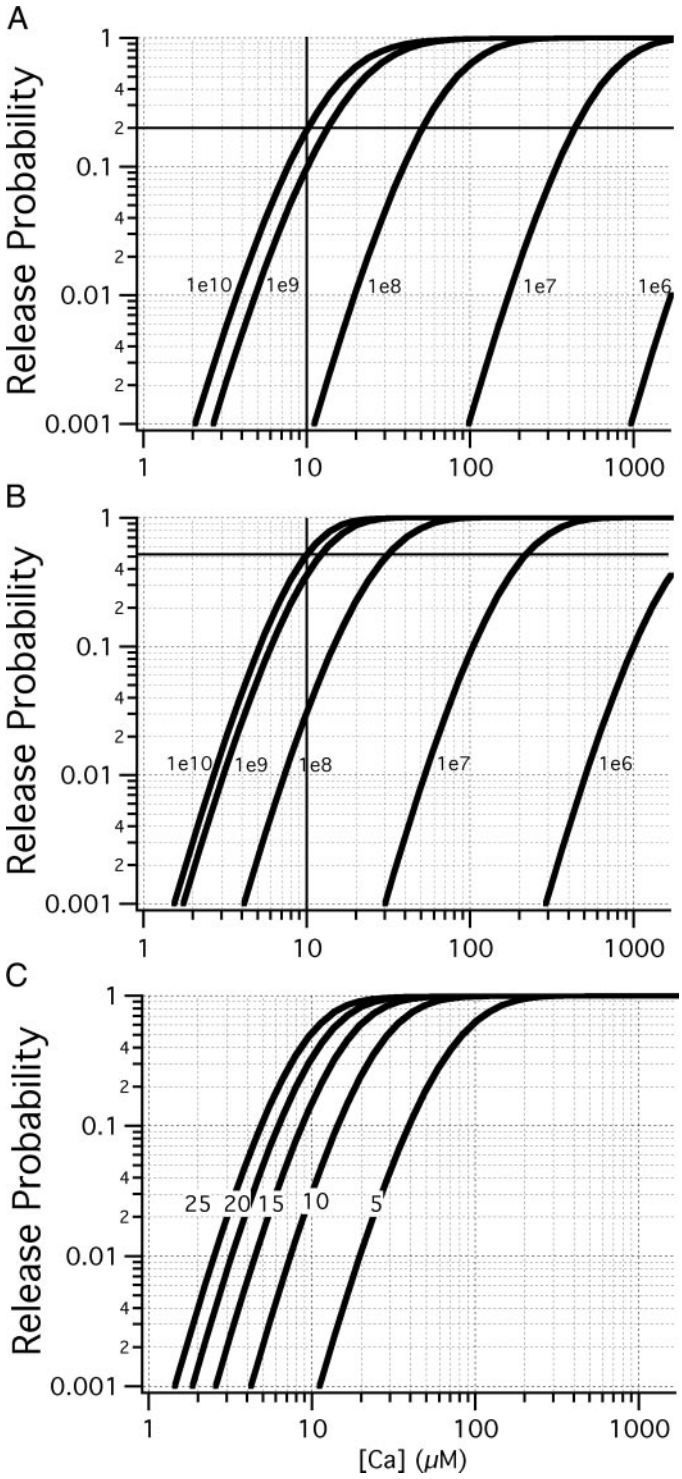

FIG. 2. Release probability as a function of $[Ca^{+2}]$ for a Ca^{2+} sensor with $K_d = 10$ μM. Release probability using release model 1 for different values of the on-rate with Ca^{2+} current durations of 0.3 ms in (*A*) and 1 ms in (*B*). In *A* and *B*, k_{on} values are indicated on the graph beside each trace in $M^{-1}s^{-1}$. The intersection of the vertical bold line and the trace of $k_{on} = 10^{10}$ $M^{-1}s^{-1}$ defines the P_{eq} for each panel. The intersection of the horizontal bold line and each trace indicates the effective affinity associated with each combination of on-rate and T_{Ca}. *C*: release probability using release model 2 with a Ca^{2+} current duration of 0.3 ms assuming the number of binding sites varies from 5 to 25 with $k_{on} = 10^8$ $M^{-1}s^{-1}$.

TABLE 1. *Effective dissociation constant of the Ca^{2+} sensor as a function of Ca^{2+} current duration (T_{Ca}) and on rate of the binding sites*

k_{on} (M^{-1} s^{-1})	10^6, μM	10^7, μM	10^8, μM	10^9, μM	10^{10}, μM
$T_{Ca} = 0.3$ ms	4300	440	50	14	~10
$T_{Ca} = 1$ ms	2000	220	30	12	~10

invariant under the following scaling of the parameters by a constant (c): $T_{Ca} \rightarrow T_{Ca} \times c$, $k_{on} \rightarrow k_{on}/c$, $\gamma \rightarrow \gamma/c$, $\delta \rightarrow \delta/c$, and $\rho \rightarrow \rho/c$. In the limit of large fusion rates (γ, δ, and ρ), this scaling freedom simplifies to the scaling of the duration of the Ca^{2+} influx and the on rate of the sensor. The estimates for the Ca^{2+}-independent fusion rates under physiological conditions (Bollmann et al. 2000) are not fast enough to put us in the preceding mentioned simplified scaling regime with the result that Fig. 2*B* is not just a simple scaling transformation of Fig. 2*A*.

The on rate of the Ca^{2+} binding and the duration of the $[Ca^{2+}]$ transient combine to create an "effective affinity" that is lower than the true equilibrium affinity. To compare the results of our release model under equilibrium and nonequilibrium conditions, we define "P_{eq}" as the release probability at $[Ca^{2+}] = K_d$ for a fast Ca^{2+} sensor (that is where equilibrium between Ca^{2+} and its binding sites is rapidly achieved). P_{eq} depends on the duration of the Ca^{2+} current transient. For example, for $K_d = 10$ μM and Ca^{2+} current duration of 0.3 ms, P_{eq} is ~0.2, whereas for a current duration of 1 ms, it increases to 0.5 (Fig. 2, *A* and *B*). Using P_{eq} we define the effective dissociation constant of a Ca^{2+} sensor with a finite on rate as the $[Ca^{2+}]$ that produces a release probability equal to P_{eq}. By this definition, the effective dissociation constant of a Ca^{2+} sensor with $K_d = 10$ μM and $k_{on} = 10^8$ $M^{-1}s^{-1}$ is ~50 μM if $T_{Ca} = 0.3$ ms, which is fivefold larger than its equilibrium dissociation constant (Table 1).

Another way to change the effective affinity is by changing the number of potential binding sites (m in *Eq. 2*). To illustrate this, we fixed the K_d to 10 μM and the k_{on} to 10^8 $M^{-1}s^{-1}$ [a rather fast on rate comparable to the on rate of bis-(*o*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid (BAPTA)] and changed the number of binding sites (from 5 to 25) in the release model 2 (Fig. 2*C*). As we increase the number of binding sites, the effective affinity (or effective on rate) and consequently the release probability will increase.

Release probability is proportional to $[Ca^{2+}]^n$ for small $[Ca^{2+}]$ and n, the power of this $[Ca^{2+}]$ dependence, reflects the Ca^{2+} cooperativity of release. Because in the models we used five Ca^{2+} ions are needed for release, the Ca^{2+} cooperativity (n, the limiting slope seen in Fig. 2 for low $[Ca^{2+}]$) is exactly 5. This slope for low $[Ca^{2+}]$, where the relationship in a log-log plot is linear, is independent of the duration of the current, the number of potential binding sites (m) and whether the Ca^{2+} reaction is at equilibrium or not. This is consistent with the fact that measurements performed under conditions where the time scales for $[Ca^{2+}]$ experienced at the Ca^{2+} sensor are different obtain similar results for the Ca^{2+} cooperativity of release (Dodge and Rahamimoff 1967; Heidelberger et al. 1994). However, if the range of concentrations used is close to saturation, then longer durations of current

could result in an apparently lower cooperativity. This phenomenon has been observed in the context of glutamate binding to *N*-methyl-D-aspartate receptors (Chen et al. 2001).

Geometry of active zone

The micro-geometry of the vesicle channel Ca^{2+} sensor(s) complex is critical for release because Ca^{2+} influx through the channel produces a very steep [Ca^{2+}] gradient in the vicinity of the channel (Fogelson and Zucker 1985; Simon and Llinas 1985). To use the results of our kinetic analysis in the context of a spatially realistic model of neurotransmitter release, we employ a Monte Carlo method with nanometer resolution to simulate the Ca^{2+} microdomain of a Ca^{2+} channel in the presence of a colocalized docked vesicle (Shahrezaei and Delaney 2004). We assume the docked vesicle is in contact with the presynaptic membrane and 20 nm separated from the channel by virtue of its interaction with SNARE proteins. We assume the Ca^{2+} sensor(s) are situated at a radius of 10 nm around the contact point, which is a plausible model considering our current knowledge of the structure of the release machinery complex (Fig. 3*A*). A mobile endogenous buffer (0.5 mM) with a rather high affinity (K_d = 2 μM), fast kinetics (k_{on} = 3 × 10^8 $M^{-1}s^{-1}$) and slow diffusion (diffusion coefficient for free and bound buffer is 27.5 $\mu m^2 s^{-1}$) is included (Burrone et al. 2002). The [Ca^{2+}] profile at the immediate vicinity of the channel and around the vesicle is not strongly dependent to the choice of the buffer for moderate concentrations of the buffer (Shahrezaei and Delaney 2004). This is the case as long as the buffer binding kinetics are Ca^{2+} diffusion limited. We assume a Ca^{2+} current of 0.3 pA through the channel that lasts for 0.3 ms (~280 Ca^{2+} ions) (Stanley 1993). The [Ca^{2+}] profile close to the channel reaches to >90% of its steady-state value within tens of microseconds. Because the vesicle diameter is comparable with the channel-vesicle dis-

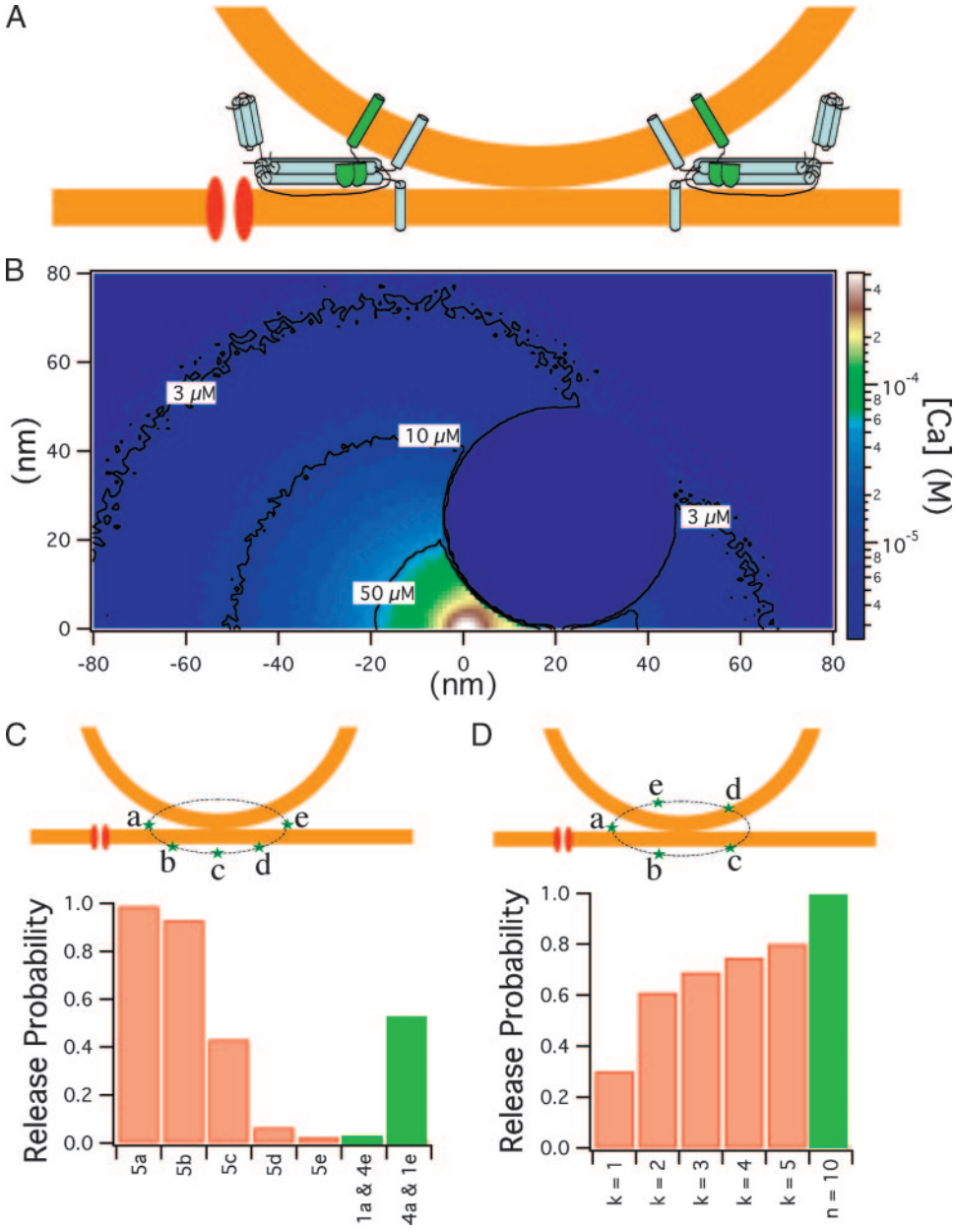

FIG. 3. Effect of geometry of release site on release probability. *A*: schematic view of the arrangement of Ca^{2+} channel (red), vesicle (orange), the Ca^{2+}-sensor(s) molecule synaptotagmin (green) and SNARE complex (light blue). Association of the channel and the SNAREs allows for possible functional interactions between them. *B*: steady-state [Ca^{2+}] after opening of the Ca^{2+} channel and constant [Ca^{2+}] contour profiles for a cross section through the channel at (0, 0), the center of the vesicle at (20 nm, 25 nm) and the docking site at (20 nm, 0). *C*: schematic view of possible Ca^{2+}-sensor site positions a to e around the vesicle (*top*). Release probability for release model 1 for a single-channel opening for different choices for the position of 5 Ca^{2+} binding sites. Results for 5 binding sites clustered at a single point, a, b, c, d, or e (red bars) or some binding sites at point a with the rest at e (green bars) are shown. *D*: schematic view of a uniform distribution of 5 clusters of Ca^{2+}-sensor sites at positions a to e around the vesicle (*top*). Red bars are release probabilities assuming k binding sites (k = 1 to 5) located at each position with binding of at least one Ca^{2+} at each cluster required for release (model 3). Green bar is release probability assuming 10 binding sites, equivalent to k = 2 but with binding to any 5 sites being sufficient to cause release (model 2).

tance, the synaptic vesicle acts as a diffusion barrier and modifies the shape of the steady-state Ca^{2+} microdomain in its vicinity (Fig. 3*B*). [Ca^{2+}] on the channel side of the vesicle is higher than the corresponding [Ca^{2+}] in the absence of the vesicle due to the reflection of Ca^{2+} by the vesicle. Conversely blocking of Ca^{2+} diffusion by the vesicle reduces [Ca^{2+}] on the opposite side of the vesicle from the channel. Therefore the Ca^{2+} sensor is potentially exposed to markedly different [Ca^{2+}] depending on its position around the vesicle, differing by as much as 13-fold (from ~15 to ~200 μM) (Shahrezaei and Delaney 2004).

To explore the effect of the position of the Ca^{2+} sensor on its saturation, we fixed the parameters of our release models to those measured in the calyx of Held ($K_d = 10$ μM, $k_{on} = 3 \times 10^8$ $M^{-1}s^{-1}$) (Bollmann et al. 2000). For release model 1, with all Ca^{2+} binding sites on the channel side of the vesicle, the release probability for a single-channel opening is 0.99 and release is highly saturated (the reaction in this case has reached to an equilibrium). Shielding only one of the binding sites from the channel by placing it behind the vesicle protects the release from saturation and reduces the release probability to 0.53 (Fig. 3*C*). With all the Ca^{2+} binding sites on the other side of the vesicle, the release probability drops to 0.02 even though the [Ca^{2+}] level is still higher than the K_d (equilibrium is not achieved due to briefness of the Ca^{2+} transient and [Ca^{2+}] is lower than the effective affinity). These results show that release can be extremely sensitive to the position of the Ca^{2+}-sensor(s).

Uniform distribution of five Ca^{2+} binding sites around the vesicle (every 72° as illustrated in Fig. 3*D, top*) results in a release probability of ~0.3. To test the effect of more than five binding sites, we employed models 2 and 3. When we consider five clusters of k (2–5) binding sites release probability increases by an amount depending on the number of extra binding sites (k) and type of release model (Fig. 3*D*). For model 3 where binding to at least one binding site of each cluster around the vesicle is needed, release probability is smaller than model 2 where binding to any five binding sites is sufficient to trigger release. Also for model 3, the effectiveness of adding more binding sites is reduced at the point where the diffusion-limited rate is approached. Thus increasing from 5 binding sites to 10 has a more significant effect on release than going from 10 to 20 (Fig. 3*D*).

An unexpected result is the emergence of two populations of vesicles with distinct release probabilities when one allows a variable position for the Ca^{2+}-binding sites (Fig. 4). We consider two cases, first, all five binding sites clustered at a single random position and second, five binding sites at different random positions around the vesicle. The average release probability is slightly greater for the multi-site case (0.55) than the single clustered site case (0.49), but the variance is almost the same. Interestingly the histogram for both geometries is bimodal; most of the time the release probability for the vesicle is either very high or very low (Fig. 4*A*). Therefore if the position of the Ca^{2+}-sensor sites around the vesicle is not constrained to the channel, then vesicles are roughly divided into two populations with high and low release probabilities. This result is quite robust against small variations of parameters and as long as the effective dissociation constant for the Ca^{2+} sensor is between the ranges of concentrations that occur around the vesicle then the histogram of release probabilities

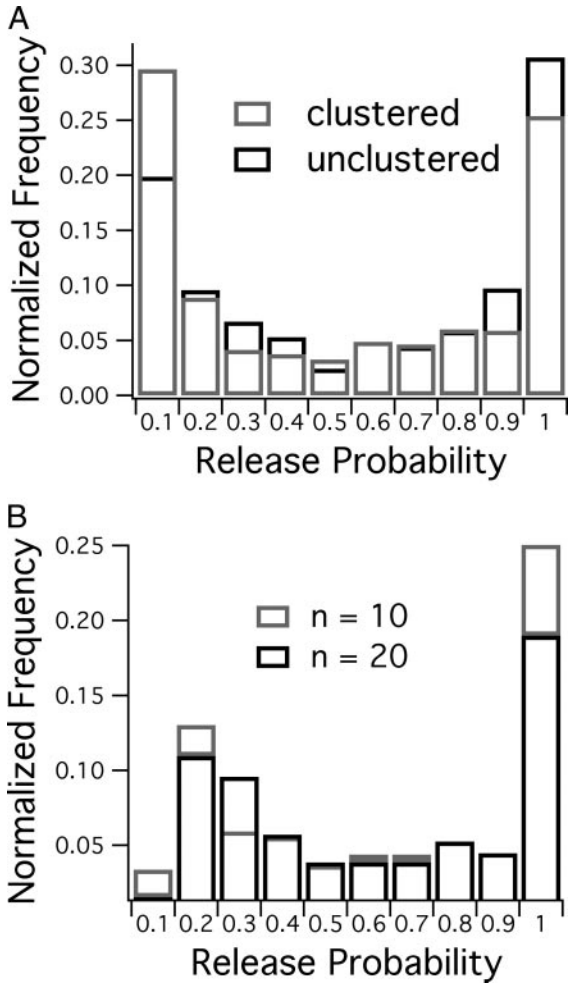

FIG. 4. Two populations of vesicles. *A*: histogram of release probabilities for release model 1 assuming random choices for the position of the Ca^{2+}-sensor molecule(s) for 2 cases of clustered and unclustered binding sites. *B*: histogram of release probabilities for release model 2 assuming random choices for the position of the Ca^{2+}-sensor molecule(s)'s binding sites (unclustered) with 10 or 20 binding sites.

remains bimodal. This bimodal distribution can be understood from a simple geometrical perspective. The gradient of [Ca^{2+}] with respect to position around the circumference of the circle defining potential binding sites is the smallest at positions close to and far from the channel (because at these positions the circumference is almost perpendicular to the channel-vesicle axis). Therefore there will be disproportionately more locations corresponding to large or small [Ca^{2+}] because the Ca^{2+} sensors are assumed to be distributed randomly around the vesicle.

To see the effect of having more binding sites randomly situated around the vesicle on the population of vesicles with low and high release probability, we use model 2 with 10 and 20 binding sites (Fig. 4*B*). The population of vesicles with low release probability will decrease, but interestingly, there still exist two distinct populations of vesicles with low and high release probability. Also doubling the number of binding sites from 10 to 20 does not change the average release probability (~0.7) and the shape of the histogram significantly. This is because the reaction approaches the diffusion-limited rate of

arrival of Ca^{2+} ions to the potential sensor sites around the vesicle.

DISCUSSION

In summary we suggest that it is possible to reconcile the colocalized active zone topography with a high-affinity Ca^{2+} sensor for transmitter release to produce release probabilities less than unity with a high sensitivity to changes in Ca^{2+} influx. This is a direct consequence of the fact that during an action potential the Ca^{2+} sensor is exposed to a brief Ca^{2+} transient, and in general the sensor does not reach chemical equilibrium with free Ca^{2+} ions. In addition to the kinetics of the reaction, the geometry of some active zones may help to prevent saturation of the sensor since the sensor may not be exposed to $[Ca^{2+}]$ levels as high as traditionally assumed due to the effect of vesicles acting as local diffusion barriers. This blocking effect would be particularly relevant in linear active zones like those of amphibian or mammalian neuromuscular junction.

The effective affinity of the Ca^{2+} sensor under physiological conditions could be different from the actual affinity of the sensor molecule, depending on the duration of the Ca^{2+} transient and number of binding sites involved in the release. For a brief, sub-millisecond Ca^{2+} transient, it is likely that the Ca^{2+} binding sites of the release machinery do not reach chemical equilibrium. Consequently, the on rate, not the equilibrium affinity, of the site may be the relevant quantity in determining effective affinity and as a result the release probability (Fig. 1). Efforts to characterize the Ca^{2+}-sensor molecules controlling release should closely consider their Ca^{2+} binding kinetics because these are expected to play an essential role in determining the release properties under physiological conditions. Also, the number of binding sites involved and their relationship to release need to be examined to be able to construct a realistic model of Ca^{2+}-triggered release.

Ca^{2+} uncaging experiments that employ $[Ca^{2+}]$ elevations longer than the action-potential-evoked Ca^{2+} influx reveal the true Ca^{2+} cooperativity of release and the functional equilibrium affinity of the Ca^{2+} sensor (Bollmann et al. 2000; Heidelberger et al. 1994; Schneggenburger and Neher 2000), but it is more difficult to obtain a reliable estimate of the Ca^{2+}-sensor molecule on rate and its affinity from these experiments. k_{on} is estimated from the dependence of the delay to release onset on the $[Ca^{2+}]$ after the flash. Although the delay can be measured directly, $[Ca^{2+}]$ within a few milliseconds after the flash must be modeled using the binding kinetics of the indicator dyes, which have been reasonably well characterized. Attempts to derive the $[Ca^{2+}]$ experienced by a typical Ca^{2+} sensor during an action potential (Bollmann et al. 2000; Schneggenburger and Neher 2000) are limited by the quality of the estimate of the on rate because for a brief Ca^{2+} transient, the release probability depends directly on the product of the on rate and $[Ca^{2+}]$ (Fig. 1). Consequently, if the estimate of k_{on} was high by some factor, then the estimated $[Ca^{2+}]$ at the Ca^{2+} sensor would be underestimated by the same amount. Recently, using rapidly decaying $[Ca^{2+}]$ elevations generated by Ca^{2+} uncaging technique, the estimate of kinetic properties of the Ca^{2+} sensor has improved, and a lower on rate has been suggested (Bollman and Sakmann 2005). The new technique provides a more reliable method of studying kinetic properties of the Ca^{2+} sensor of release machinery.

The binding rates are the limiting factor in any reaction that has limited time to progress. A relevant biological example is the nonsaturation of *N*-methyl-D-aspartate (NMDA) receptors by action-potential-evoked glutamate release in hippocampal synapses that occurs despite the high affinity of the receptor for glutamate (McAllister and Stevens 2000). Consistent with the ideas presented here, Chen et al. (2001) demonstrated that NMDA receptors due to their slow kinetics have relatively lower potency under brief application of glutamate than under equilibrium conditions.

It appears that cooperation of a few SNARE complexes, along with their associated synaptotagmin molecules, is needed for Ca^{2+} triggered neurotransmitter release (Bai et al. 2004; Han et al. 2004). It is apparent that because each synaptotagmin molecule has five Ca^{2+} binding sites, the total number of binding sites potentially available for release is much more than the observed Ca^{2+} cooperativity of release. We introduced two simple models for release (models 2 and 3) that despite having extra binding sites produce the same Ca^{2+} cooperativity as model 1 with five binding sites. We showed that extra binding sites for the release model increase the effective on rate of the Ca^{2+} sensors, but still the functional on rate is constrained by the diffusion-limited rate for a Ca^{2+} binding site, which is generally accepted to be $\sim 5 \times 10^8$-10^9 $M^{-1}s^{-1}$. This result suggests the apparent high affinity observed in Calyx of Held compared with retinal bipolar cell could be due to the number of available binding sites rather than the affinity of the binding site itself.

It is possible that Ca^{2+} binding sites are distributed around the docking point of the vesicle on a few sensor molecules (Stewart et al. 2000). Using a Monte Carlo simulation with nanometer spatial resolution, we show that shielding even one of the binding sites from the Ca^{2+} source by placing it behind the vesicle can significantly reduce the probability of release. This is because the vesicle acts as barrier to free diffusion of Ca^{2+} and modifies the shape of the Ca^{2+} microdomain. If more than one channel is contributing to release, the microdomain of the closest channel would be the most affected and binding sites opposite the closest channel would be the hardest to occupy (Shahrezaei and Delaney 2004).

Our Monte Carlo simulation reveals a novel result that in the presence of a nonuniform $[Ca^{2+}]$ around the vesicle variation in the position of the sensor around the vesicle can produce two distinct populations of vesicles with high and low release probabilities. This heterogeneity of release probabilities does not require different types of Ca^{2+}-sensor molecules or distinct populations of colocalized and noncolocalized vesicle-channel populations; it arises solely from the micro-geometry of the vesicle-channel-Ca^{2+} sensor(s) complex. The two distinct populations would exist even if the number of available binding sites changes, although the size of one population versus another could change. Conceivably, Ca^{2+}-dependent interactions between the release machinery and the Ca^{2+} channels (Spafford and Zamponi 2003) could cause small rearrangements of the Ca^{2+} sensor(s) relative to the channel to increase the size of the high-release-probability vesicle population. This is a potential mechanism for activity-dependent enhancement of neurotransmitter release.

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REFERENCES

Adler EM, Augustine GJ, Duffy SN, and Charlton MP. Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *J Neurosci* 11: 1496–1507, 1991.

Augustine GJ. How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* 11: 320–326, 2001.

Bai J and Chapman ER. The C2 domains of synaptotagmin–partners in exocytosis. *Trends Biochem Sci* 29: 143–151, 2004.

Bai J, Wang CT, Richards DA, Jackson MB, and Chapman ER. Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. *Neuron* 41: 929–942, 2004.

Bennett MR, Farnell L, and Gibson WG. The probability of quantal secretion near a single calcium channel of an active zone. *Biophys J* 78: 2201–2221, 2000.

Bertram R, Smith GD, and Sherman A. Modeling study of the effects of overlapping Ca^{2+} microdomains on neurotransmitter release. *Biophys J* 76: 735–750, 1999.

Bollmann JH and Sakmann B. Control of synaptic strength and timing by the release-site Ca(2+) signal. *Nat Neurosci* 8: 426–434, 2005.

Bollmann JH, Sakmann B, and Borst JG. Calcium sensitivity of glutamate release in a calyx-type terminal. *Science* 289: 953–957, 2000.

Burrone J, Neves G, Gomis A, Cooke A, and Lagnado L. Endogenous calcium buffers regulate fast exocytosis in the synaptic terminal of retinal bipolar cells. *Neuron* 33: 101–112, 2002.

Chapman ER. Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? *Nat Rev Mol Cell Biol* 3: 498–508, 2002.

Chen N, Ren J, Raymond LA, and Murphy TH. Changes in agonist concentration dependence that are a function of duration of exposure suggest *N*-methyl-D-aspartate receptor nonsaturation during synaptic stimulation. *Mol Pharmacol* 59: 212–219, 2001.

Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, and Chapman ER. Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes. *Neuron* 24: 363–376, 1999.

Dodge FA Jr and Rahamimoff R. Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J Physiol* 193: 419–432, 1967.

Fogelson AL and Zucker RS. Presynaptic calcium diffusion from various arrays of single channels. Implications for transmitter release and synaptic facilitation. *Biophys J* 48: 1003–1017, 1985.

Gentile L and Stanley EF. A unified model of presynaptic release site gating by calcium channel domains. *Eur J Neurosci* 21: 278–282, 2005.

Han X, Wang CT, Bai J, Chapman ER, and Jackson MB. Transmembrane segments of syntaxin line the fusion pore of Ca^{2+}-triggered exocytosis. *Science* 304: 289–292, 2004.

Harlow ML, Ress D, Stoschek A, Marshall RM, and McMahan UJ. The architecture of active zone material at the frog's neuromuscular junction. *Nature* 409: 479–484, 2001.

Heidelberger R, Heinemann C, Neher E, and Matthews G. Calcium dependence of the rate of exocytosis in a synaptic terminal. *Nature* 371: 513–515, 1994.

Hua Y and Scheller RH. Three SNARE complexes cooperate to mediate membrane fusion. *Proc Natl Acad Sci USA* 98: 8065–8070, 2001.

Koh TW and Bellen HJ. Synaptotagmin I, a Ca^{2+} sensor for neurotransmitter release. *Trends Neurosci* 26: 413–422, 2003.

Llinas R, Steinberg IZ, and Walton K. Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys J* 33: 323–351, 1981.

Llinas R, Sugimori M, and Silver RB. Microdomains of high calcium concentration in a presynaptic terminal. *Science* 256: 677–679, 1992.

Llinas R, Sugimori M, and Silver RB. The concept of calcium concentration microdomains in synaptic transmission. *Neuropharmacology* 34: 1443–1451, 1995.

Llinas R, Sugimori M, and Simon SM. Transmission by presynaptic spike-like depolarization in the squid giant synapse. *Proc Natl Acad Sci USA* 79: 2415–2419, 1982.

McAllister AK and Stevens CF. Nonsaturation of AMPA and NMDA receptors at hippocampal synapses. *Proc Natl Acad Sci USA* 97: 6173–6178, 2000.

Meinrenken CJ, Borst JG, and Sakmann B. Calcium secretion coupling at calyx of held governed by nonuniform channel-vesicle topography. *J Neurosci* 22: 1648–1667, 2002.

Millet O, Bernado P, Garcia J, Rizo J, and Pons M. NMR measurement of the off rate from the first calcium-binding site of the synaptotagmin I C2A domain. *FEBS Lett* 516: 93–96, 2002.

Sabatini BL and Regehr WG. Timing of neurotransmission at fast synapses in the mammalian brain. *Nature* 384: 170–172, 1996.

Schneggenburger R and Neher E. Intracellular calcium dependence of transmitter release rates at a fast central synapse. *Nature* 406: 889–893, 2000.

Shahrezaei V and Delaney KR. Consequences of molecular-level Ca^{2+} channel and synaptic vesicle colocalization for the Ca^{2+} microdomain and neurotransmitter exocytosis: a Monte Carlo study. *Biophys J* 87: 2352–2364, 2004.

Simon SM and Llinas RR. Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys J* 48: 485–498, 1985.

Spafford JD and Zamponi GW. Functional interactions between presynaptic calcium channels and the neurotransmitter release machinery. *Curr Opin Neurobiol* 13: 308–314, 2003.

Stanley EF. Single calcium channels and acetylcholine release at a presynaptic nerve terminal. *Neuron* 11: 1007–1011, 1993.

Stanley EF. The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci* 20: 404–409, 1997.

Stewart BA, Mohtashami M, Trimble WS, and Boulianne GL. SNARE proteins contribute to calcium cooperativity of synaptic transmission. *Proc Natl Acad Sci USA* 97: 13955–13960, 2000.

Tang Y, Schlumpberger T, Kim T, Lueker M, and Zucker RS. Effects of mobile buffers on facilitation: experimental and computational studies. *Biophys J* 78: 2735–2751, 2000.

Wachman ES, Poage RE, Stiles JR, Farkas DL, and Meriney SD. Spatial distribution of calcium entry evoked by single action potentials within the presynaptic active zone. *J Neurosci* 24: 2877–2885, 2004.

Yamada WM and Zucker RS. Time course of transmitter release calculated from simulations of a calcium diffusion model. *Biophys J* 61: 671–682, 1992.

Zucker RS. Calcium and transmitter release. *J Physiol Paris* 87: 25–36, 1993.