Relationship between transmitter release and presynaptic calcium influx when calcium enters through discrete channels

(synapse/Ca current/Ca channels/neural models)

ROBERT S. ZUCKER* AND AARON L. FOGELSON†

Departments of *Physiology-Anatomy and †Mathematics, University of California, Berkeley, CA 94720

ABSTRACT We have used a three-dimensional diffusion model of calcium entering the presynaptic nerve terminal through discrete channels to simulate experiments relating transmitter release to presynaptic calcium current. The relationship will be less than linear, or will curve downward, if calcium channels are well separated. It will resemble a power-law function with exponent less than the cooperativity of calcium action if channels are clustered closer together. Large presynaptic depolarizations elicit more release than small depolarizations admitting the same calcium influx. This occurs because large pulses open more channels near each other, with the result that the calcium concentration near release sites is greater, due to overlap of calcium diffusing from adjacent channels.

The phasic release of neurotransmitter by an action potential requires the entry of calcium ions into the presynaptic terminal (1, 2). The mechanism by which calcium ions trigger transmitter release remains a mystery. The relationship between calcium influx and transmitter release provides one clue to this mechanism. The best measurements of this relationship come from studies of the giant synapse in the stellate ganglion of the squid (3–8). These studies show a power-law relationship between calcium influx and transmitter release, with an exponent of about 3 in the most carefully controlled experiments (7, 8). It is generally thought that this provides a measure of the degree of cooperativity between calcium ions and other presynaptic agents involved in neurosecretion. In particular, it is imagined that three calcium ions bind simultaneously to a release site, organelle, or reactant to elicit release of transmitter. In contrast to these results, the relationship between transmitter release and external calcium may be more nonlinear, with exponents of 4 or higher (9, 10). Moreover, these latter measurements are likely to significantly underestimate the calcium cooperativity of release (11, 12). Thus, there is a quantitative discrepancy between these estimates of calcium cooperativity in the release process.

Both presynaptic calcium current and transmitter release rise with increasing depolarization as calcium channels open and then fall as the calcium equilibrium potential is approached (4, 7, 8, 13, 14). When transmitter release, measured as postsynaptic response, is plotted vs. presynaptic calcium current, the points corresponding to the rising and falling limbs of the presynaptic potential do not always lie on the same curve. Rather, calcium currents elicited by large presynaptic potentials often release more transmitter than do the same calcium currents elicited by small depolarizations. This has been interpreted as evidence that the rate of transmitter release by calcium-activated release sites has a direct voltage dependence (4).

Attempts to understand the action of presynaptic calcium have often relied on one-dimensional diffusion simulations, in which calcium enters uniformly through the presynaptic membrane and diffuses radially inward from the surface where transmitter is released (15). In such models no distinction is made between identical influxes caused by large and small presynaptic depolarizations. However, Simon and Llinás (16) and Chad and Eckert (17) have pointed out that calcium influxes during large and small depolarizations are not identical. Small depolarizations open few calcium channels, while larger depolarizations open more channels with less influx per channel as the calcium equilibrium potential is approached. Thus, the number of release sites and the local intracellular calcium concentration at such sites might be quite different in the two situations, leading to different levels of synaptic transmission.

We have investigated this behavior with a model of presynaptic calcium diffusion in which calcium enters the terminal through discrete channels and diffuses away from each channel mouth into the terminal in three dimensions (18). We have explored the effect of discrete calcium entry on the relationship between transmitter release and calcium current. Our simulations reveal two new results. (*i*) We observe an approximately third-power relationship between transmitter release and calcium current, even though the actual cooperativity between calcium and release sites was assumed to be higher; and (*ii*) we see more transmitter release for calcium currents elicited by large depolarizations than for similar currents elicited by small depolarizations, even though no step in the transmitter release process is intrinsically voltage dependent.

MATERIALS AND METHODS

Three-Dimensional Diffusion Model. We use a diffusion model in which calcium enters the presynaptic terminal through discrete channels. The channels are organized in regular arrays in separate active zone patches on the synaptic face of the terminal, as indicated by ultrastructural observations (19, 20). Calcium diffuses from each channel mouth in three dimensions into a rectangular cylinder with dimensions similar to the presynaptic terminal. The model includes extrusion of calcium by a surface membrane pump at the rate 20 nm/ms and instantaneous, immobile, and nonsaturable cytoplasmic calcium binding with a ratio of bound to free calcium of 250. This high degree of calcium binding is similar to some estimates for molluscan neurons (21) and reflects the possibility that calcium-binding proteins are more concentrated in the peripheral axoplasm beneath the plasma membrane (22). Such a buffer slows calcium diffusion and extrusion and reduces the proportion of entering calcium that remains free (16, 18). Transmitter release is imagined to occur 50 nm from channel mouths, a distance that allows enough time for the synaptic delay yet prevents vesicle fusion from obliterating calcium channels during exocytosis (see also ref. 23). The rate of transmitter release is assumed to depend on

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the calcium concentration at these release sites raised to an integral power, reflecting the cooperative action of several calcium ions in vesicle exocytosis. Possible effects of saturation of the release mechanism are not included. The degree of calcium cooperativity in the release process is a variable in our simulations. We use linear analytic solution methods to solve the diffusion equation. Our results, therefore, apply to increments in internal calcium above the resting level, which is not stipulated. The full set of equations comprising this model, our methods of solution, and more detailed justification for our parameter choices are given in ref. 18.

RESULTS

Simulations of Transmitter Release vs. Calcium Current. We consider the case of calcium influx and transmitter release occurring in response to brief depolarizing pulses lasting 2.5 ms. During such a pulse, calcium channels will have time to open only once, with few exceptions, and the open time will be about 2.0 ms and will be virtually independent of voltage (24). The sigmoid rise in macroscopic calcium current recorded from presynaptic terminals during a pulse (3–5, 7, 13, 14) corresponds to the rise in probability of calcium channel opening. On the average, the calcium channels that will open during a pulse do so after about 0.5 ms. Calcium channels remain open an average of 0.4 ms after the end of a brief depolarizing pulse, as indicated by the decay of macroscopic calcium tail current (4, 5, 7, 13, 14, 25). Thus, the "typical" channel opens about 0.5 ms after the beginning of the pulse, and remains open for the remaining 2.0 ms of the pulse and another 0.4 ms afterwards. During the pulse, the open channel current is less than after the pulse, because of the change in driving force. Fig. 1A shows the relationship between macroscopic calcium current and "typical" single channel current during a brief depolarization.

As the amplitude of depolarization rises, more calcium channels open until a maximum near 0 mV (7, 13, 14). The number of calcium channels open can be estimated from the macroscopic current at the end of the pulse and the single channel current at the membrane potential of the pulse (26). Table 1 shows the single channel current, the macroscopic current, and the number of open channels at membrane potentials between −25 and +60 mV used in these simulations. The single channel current at the holding potential after the pulse was taken as 0.84 pA (26).

Open calcium channels were distributed evenly among 10,000 active zones in the presynaptic terminal. As more calcium channels open, the average spacing between open
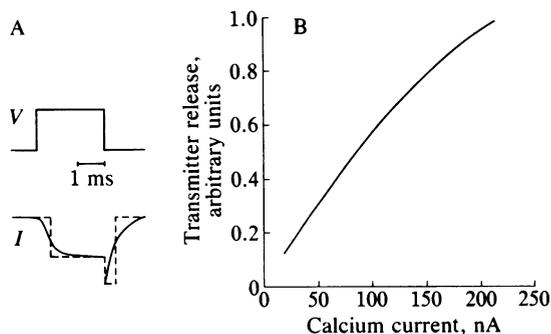
Table 1. Properties of calcium channels used in the simulations

Membrane potential, mV	Macroscopic calcium current, nA*	Single channel current, fA†	Open channels, no. × 10⁻⁵‡	Average distance between channels, nm§
−25	19	525	0.36	441
−20	72	490	1.47	218
−15	125	455	2.75	160
−10	175	420	4.17	130
−5	216	385	5.61	112
0	240	350	6.86	101
10	185	270	6.86	101
20	144	210	6.86	101
30	127	185	6.86	101
40	103	150	6.86	101
50	85.7	125	6.86	101
60	68.6	100	6.86	101

*R.S.Z., unpublished data.
†From ref. 26.
‡Ratio of macroscopic to single channel currents.
§From arrays similar to those of Fig. 2.

channels in an active zone decreases. The average distance between neighboring open channels at each potential is given in Table 1, and our dispositions of open channels in active zones at selected potentials are illustrated in Fig. 2.

Before discussing responses of our model to the above arrays of open calcium channels, we consider two extremes. In the case of uniform calcium influx across the presynaptic membrane, without regard to calcium channels, there is a nearly linear relationship between calcium influx and submembrane calcium activity at the end of a pulse (4, 15). Deviations from linearity are due to saturable binding and removal processes. In this case, the biochemical cooperativity of calcium action in neurosecretion should be reflected by the exponent of the power-law relationship between postsynaptic response and presynaptic calcium current. If five calcium ions act at some site to release one quantum of transmitter (trigger exocytosis of one synaptic vesicle), then transmitter release will be proportional to the fifth power of presynaptic calcium current (assuming a linear relation between calcium influx and the accumulation of active intracellular calcium).

In the opposite extreme, calcium might enter through discrete channels that are widely separated, in which case a very different result is anticipated. Increased calcium current reflects the opening of additional calcium channels and the formation of additional separate nonoverlapping calcium domains surrounding each calcium channel. The calcium concentration near each channel mouth will not rise; on the contrary, it will fall as the calcium equilibrium potential is
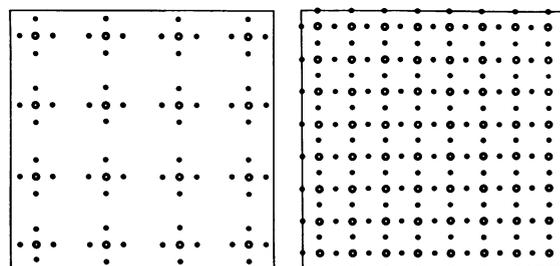

FIG. 1. (A) Time course of single channel current (dashed line, *I*) used in simulations of responses to brief depolarizations (*V*). The time course of macroscopic calcium current is also shown (solid line, *I*). (B) Predicted relationship between peak rate of transmitter release and presynaptic calcium current magnitude at the end of a 2.5-ms pulse, for the case of completely nonoverlapping calcium domains and widely separated calcium channels.

FIG. 2. Location of open calcium channels (circles) and release sites (dots) in an active zone at two voltages, −20 mV (*Left*) and 0 mV (*Right*). Release sites are 50 nm from calcium channels. The active zone is 800 nm on a side.

approached. If transmitter release near each calcium channel is proportional to the nth power of calcium activity at release sites, then total release will be proportional to this calcium raised to the nth power times the number of open channels. We calculated submembrane calcium 50 nm from a single channel mouth, and then simulated the behavior of such a system of nonoverlapping channel domains. Fig. 1B shows the result: there is a less-than-linear (concave downward) dependence of release on calcium current (cf. ref. 16), despite a postulated high degree of cooperativity in calcium action.

Our model lies between these extremes, in that a large depolarization opens a sufficient number of calcium channels that some overlap of adjacent calcium domains occurs. Fig. 3 shows what happens when our model is used to predict release triggered 50 nm from calcium channels in active zones in which several calcium channels open, as expected from the numbers in Table 1 and sketches in Fig. 2. Since the calcium activity depends on where the calcium channel is located in the active zone, and on the position of the point at which calcium is measured, we use an average at several locations of calcium activities raised to a power to predict the average effect on transmitter release (see Fig. 2). We assume a calcium cooperativity of 5, because this provides a good fit to the time course of facilitation and termination of phasic transmitter release in simulations of synaptic transmission at squid giant synapses and frog neuromuscular junctions (18) and accounts well for the relationship between posttetanic decay of facilitation and miniature postsynaptic potential frequency at crayfish neuromuscular junctions (27).

Meaning of the Slope of the Logarithm–Logarithm Plot. Two key results emerge from Fig. 3; namely, (i) the mid-range slope of log (release) vs. log (calcium current) is not 5; it is about 3, as observed experimentally (5, 7) and (ii) the calcium currents corresponding to large depolarizations elicit more release than identical currents corresponding to small depolarizations, as observed experimentally (4, 7, 8).

We explain the first result as follows. As more calcium channels open, the domains of calcium accumulation surrounding channel mouths move closer together and begin to

overlap (Fig. 4). The calcium concentration 50 nm from each channel mouth rises as calcium enters from adjacent calcium channels. This rise in active calcium more than compensates for the decrease in single channel current, in the case of small pulses. Since depolarization not only recruits additional channel domains but also increases the concentration of calcium at release sites, transmitter release increases more than linearly with increasing calcium influx, except at low potentials, where channel domains overlap very little. For the parameters chosen in the simulation of Fig. 3, the slope of logarithm of release vs. logarithm of calcium current is similar to experimental observation.

In this situation, the apparent cooperativity of calcium, as indicated by the relationship between transmitter release and calcium current, underestimates the true cooperativity of the underlying calcium-dependent secretory reaction. That this is generally true is evident from Fig. 5. For several values of assumed biochemical cooperativity between 2 and 20, the apparent mid-range slope of the logarithm–logarithm plot of transmitter release vs. calcium current is always less than the real cooperativity.

We might expect that the amount by which the apparent cooperativity of calcium action, as assessed by plots such as Fig. 3, differs from the true cooperativity would depend on the shape of the calcium domain surrounding each channel. In particular, reducing the cytoplasmic binding of calcium will speed its diffusion and broaden the calcium concentration profile near channel mouths. This should heighten the power of the relationship between release and calcium current. Indeed, the bulk cytoplasm calcium-binding ratio has been measured to be as low as 40 in squid axoplasm (28). Using this value caused the apparent cooperativity in the mid-range of the plot, where the slope reached about 4.8, to approach the assumed real cooperativity.
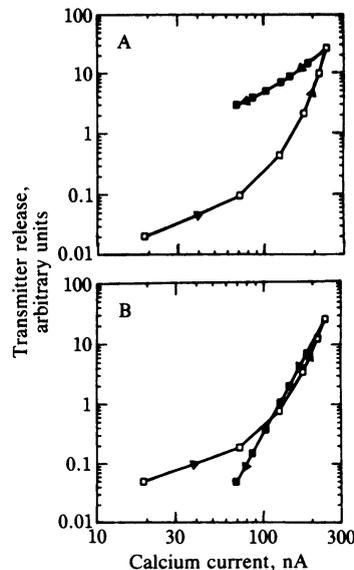

FIG. 3. Predicted relationship between peak rate of transmitter release and presynaptic calcium current at the end of a 2.5-ms pulse. The pulse voltage corresponding to each point is shown. The arrows indicate the direction of increasing pulse amplitude. Filled symbols correspond to the descending limbs of the release—voltage and calcium current—voltage relationships. (A) Responses to single channel currents including tail currents, as in Fig. 1A. (B) Responses to single channel currents without tail currents.
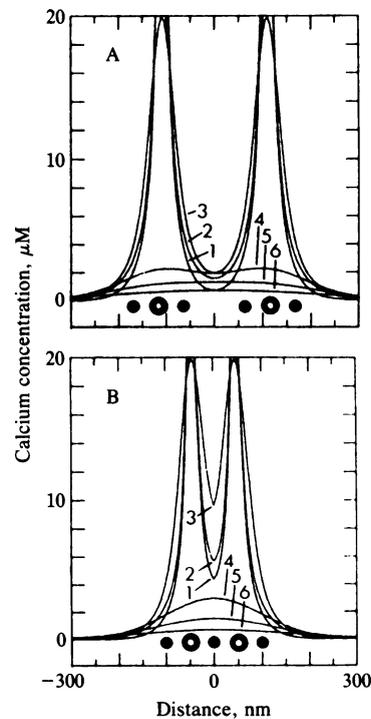

FIG. 4. Spatial distribution of submembrane free calcium concentration near open calcium channels. The curves lie in the plane perpendicular to the presynaptic membrane and through two open channels. The curves labeled 1 to 6 are for the following times, measured from the beginning of a pulse: 1.0, 2.0, 2.4, 3.4, 4.4, and 6.4 ms. Open channels are separated by 218 nm in a pulse to −20 mV (A) or by 101 nm in a pulse to 0 mV (B). o, channels; •, release sites.

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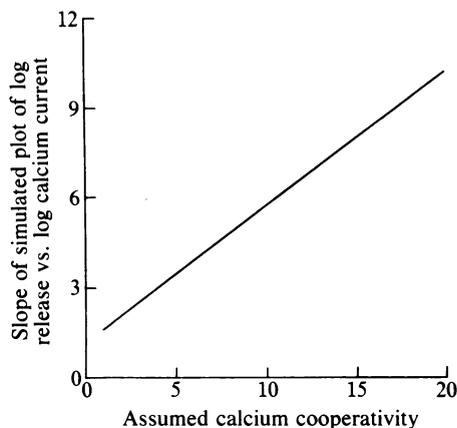*Proc. Natl. Acad. Sci. USA 83 (1986)*   3035

FIG. 5. The degree of calcium cooperativity was varied from 1 to 20, and the simulated peak rate of transmitter release was plotted versus presynaptic calcium current at the end of a 2.5-ms pulse, using logarithmic coordinates. The mid-range slope of this curve is plotted here as a function of the assumed calcium cooperativity.

Apparent Voltage-Dependence of Transmitter Release. The second result concerns the apparent enhancement of transmitter release by large depolarizations (Fig. 3). This is due to two effects. (*i*) Large depolarizations open more channels than small depolarizations admitting the same total calcium influx. Open calcium channels are, therefore, closer together in large depolarizations, calcium channel domains overlap more, and so calcium concentration at release sites is higher due to summation of calcium from neighboring channels. Due to the cooperativity of calcium action, this leads to a greater release for a given calcium current when more channels are open. (*ii*) In large depolarizations, the tail currents predominate in releasing transmitter. These tail currents remain constant for all pulses above 0 mV, and this leads to a release which approaches constancy for very large depolarizations, where the calcium influx during the pulse becomes negligible. Fig. 3A shows that these effects combine to produce a substantial "hysteresis" in the curves relating release to calcium influx (figure 4A of ref. 4). This does not reflect voltage sensitivity in any aspect of the release process *per se*, other than the voltage sensitivity of calcium channels.

The effect of increased overlap of calcium channel domains can be seen in isolation by simulating responses to pulses without tail current (Fig. 3B). The "hysteresis" is still apparent, but now greatly reduced. Increasingly large pulses cause a uniform reduction in calcium current through all open channels, which simply scales down the influx. Thus, the upper arm of the curve in Fig. 3B has a slope approaching 5. The two arms cross at low response amplitudes. These results should be compared to more recent measurements, where an effort is made to eliminate the effects of tail currents from the postsynaptic responses by measuring presynaptic current and postsynaptic response magnitudes before the end of the depolarizing pulse (7, 8). The small augmentation of release to equal presynaptic calcium currents at higher presynaptic potentials (Fig. 3B) is similar to that in figure 3 of ref. 7 and figures 4 and 6 of ref. 8. The intersection of the two branches of Fig. 3B occurs at responses less than 2% of the amplitudes of the largest responses. Such responses are at the noise level of the data presently available; therefore, this prediction remains to be tested.

DISCUSSION

The present results underscore the utility of the three-dimensional calcium diffusion model as an aid in interpreting the properties of synaptic transmission in terms of underlying

mechanism. The relationship between transmitter release and macroscopic calcium current is often supposed to reflect the cooperativity of calcium action in the release process. Because of the incomplete overlap of calcium channel domains, this is an imperfect measure of the real calcium cooperativity in the underlying chemical reactions.

If calcium channels were scattered uniformly in the presynaptic membrane, channel domains would not overlap, and the relationship between transmitter release and calcium current would be sublinear. Calcium channels and the release machinery may be concentrated into active zones at chemical synapses to permit cooperative calcium action from overlapping domains to enhance transmitter release.

It had also been thought that the release of more transmitter by calcium currents elicited by large depolarizations indicated a direct effect of voltage in modulating the release machinery. This apparent voltage dependence may actually be a consequence of the overlap of adjacent calcium domains at large positive potentials. Thus, the hysteresis in release vs. calcium current curves does not necessarily provide evidence for a direct voltage dependence of release.

Limitations of the Present Model. Rarely can complex biological processes be represented perfectly by a tractable model. Our simulations are subject to numerous simplifications and limitations. To list just a few: (*i*) Calcium channels are not organized into regular arrays. The spacing and overlap between neighboring open channel domains are quite variable, both within and between active zones. We have ignored this variability. (*ii*) Calcium channels open and close stochastically. The time to first opening and the open time are neither constant from trial to trial nor uniform among channels. Our model does not treat this probabilistic nature of channel behavior. (*iii*) The site of vesicle fusion is not likely to be exactly 50 nm from channel mouths, but rather depends on the distance between calcium channels and nearest release sites or synaptic vesicles, which will also be a spatial random variable. (*iv*) It is not known what the relation is between calcium and rate of neurosecretion, but an integral power relation is certainly an oversimplification. The apparent stoichiometry may vary with calcium concentration and may be reduced at high release levels by saturation of the release process. (*v*) Many simplifications were made for analytic or computational simplicity, such as rectangular terminal cross section, constant channel open time, instantaneous, nonsaturable, immobile, and uniform cytoplasmic calcium binding, nonsaturable first-order calcium extrusion only at the surface, no consideration of depletion of releasable stores, or tortuosity of cytoplasmic diffusion pathways, etc.

Some of the cited problems do not affect our simulations but would severely limit other applications. For example, the stochastic nature of channel opening is a serious problem in using this model to study the response to long depolarizations. A persistent calcium current reflects many channels opening and closing, with important fluctuations among old and newly formed calcium domains, so that the local accumulation of calcium becomes difficult to predict without considering these stochastic properties. Fortunately, this does not affect the use of the model to treat short pulses or action potentials that open most channels only once.

Other limitations may have serious effects on the quantitative form of our results. For example, saturation of the release mechanism would affect the behavior of the model at high release rates. A noninstantaneous, mobile, and saturable buffer and a nonuniform disposition of calcium channels will have important effects on the peak calcium concentration reached at release sites and the maximum degree of overlap that can occur between neighboring open calcium channel domains. We do not think that this model can be used to deduce the values of parameters of the physiological processes in the model. We do not believe, for example, that our

fit of Fig. 3 to experimental data using a bound-to-free calcium buffer ratio of 250 implies that this is really the magnitude of submembrane calcium buffering at the squid giant synapse. If this ratio is closer to 40 (28), the greater overlap between adjacent channel domains could be compensated by somewhat less crowding of calcium channels. Our choice of active zone dimensions is based on ultrastructural estimates subject to errors such as tissue shrinkage during fixation. The model is, therefore, too elastic to draw conclusions about parameter values. Rather, its utility lies in its ability to explain qualitatively observations that had been regarded as implying a voltage modulation of transmitter release that may not actually exist.

Comparison to Other Studies. Simon and Llinás (16) have also considered the effect of diffusion from calcium channels on the relationship between transmitter release and presynaptic calcium current. Their model is conceptually similar to ours, although numerical approximation methods rather than analytic solution techniques were used in the simulations, and there are numerous detailed differences in parameter choices and boundary conditions. For example, they treat the effects of a mobile, saturable, and noninstantaneous buffer (with binding ratio similar to ours), and a fixed array of channels whose members are activated stochastically by depolarization.

Their model predicts an apparent stoichiometry between transmitter release and calcium current of less than one, the more so the higher the degree of calcium cooperativity assumed (similar to our Fig. 1B). This result is due primarily to one key assumption that minimizes the effect of overlap of adjacent calcium channel domains—release occurs at the intense peaks of submembrane calcium concentration at calcium channel mouths (open circles in Fig. 4). The reduction in these peaks with increasing depolarization is too great to be compensated by calcium spreading from additional open channels. Furthermore, the peak calcium concentration at calcium channel mouths is too great to summate sufficiently with residual calcium from prior activity to account for synaptic facilitation.

We have constrained exocytosis to occur at least one vesicle radius away from calcium channels (filled circles of Fig. 4), to avoid obliterating the channels and to be consistent with anatomical observations (18, 23). At these locations the effects of domain overlap and residual calcium are far more substantial. The former can lead to a high power in the release vs. calcium current relationship (Fig. 3), and the latter can cause synaptic facilitation resembling that observed experimentally (18).

To explain a two-branched power-law relationship between release and calcium current, Simon and Llinás (16) propose two possibilities. (i) Release sites may become refractory after an exocytotic event, limiting release rates especially when few calcium channels open, and (ii) exocytosis requires the simultaneous opening of multiple calcium channels, raising the calcium concentration at widely separated binding sites on a vesicle. Such effects may indeed contribute to the form of the release vs. calcium current curve. We would only point out that in their simulations, the effect of vesicle depletion could not generate an apparent stoichiometry as high as 3 (7). The second proposal, that calcium acts at separate binding sites, could account for the curves of release vs. calcium current and release vs. calcium concentration, and for synaptic facilitation. It would still be necessary, however, for transmitter release to occur some distance from calcium channel mouths for such a model to work. This model differs from ours in assigning cooperativity to multiple vesicular binding molecules, rather than to multiple ionic binding sites on one molecule. However, ultra-

structural evidence suggests that each synaptic vesicle binds one or more calcium ions at only one site, not multiple widely separated sites (29).

In conclusion, we favor a model of calcium entering through discrete channels located near but not at transmitter release sites, with a high degree of calcium cooperativity at one vesicle or plasma membrane attachment site and with significant overlap of calcium diffusing from neighboring calcium channels to release sites in active zones during presynaptic depolarization. Such a model can most easily explain the qualitative features of phasic transmitter release, synaptic facilitation, and presynaptic calcium accumulation (18), the dependence of release on extracellular calcium, and the form of the release vs. calcium current relationship. Other more complex models (16) can also account for these properties and cannot be excluded at this time.

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