Time course of transmitter release calculated from simulations of a calcium diffusion model

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ABSTRACT A three-dimensional presynaptic calcium diffusion model developed to account for characteristics of transmitter release was modified to provide for binding of calcium to a receptor and subsequent triggering of exocytosis. When low affinity (20 μM) and rapid kinetics were assumed for the calcium receptor triggering exocytosis, and stimulus parameters were selected to match those of experiments, the simulations predicted a virtual invariance of the time course of transmitter release to paired stimulation, stimulation with pulses of different amplitude, and stimulation in different calcium solutions. The large temperature sensitivity of experimental release time course was explained by a temperature sensitivity of the model's final rate limiting exocytotic process. Inclusion of calcium tail currents and a saturable buffer with finite binding kinetics resulted in high peak calcium transients near release sites, exceeding 100 μM. Models with a single class of calcium binding site to the secretory trigger molecule failed to produce sufficient synaptic facilitation under this condition. When at least one calcium ion binds to a different site having higher affinity and slow kinetics, facilitation again reaches levels similar to those seen experimentally. It is possible that the neurosecretory trigger molecule reacts with calcium at more than one class of binding site.

INTRODUCTION

One of the most striking characteristics of some chemical synapses is the speed of transmitter release. The brevity of the synaptic delay forces the conclusion that release originates from the immediate neighborhood of calcium channel mouths, at a time when internal calcium concentrations are locally quite high (Simon and Llinás, 1985). The short duration of the phasic release period also argues for some process to rapidly terminate release. Measurements of intracellular calcium (Charlton et al., 1982) suggest that it remains high for seconds after presynaptic action potentials, so this appears not to limit the duration of release. And the release process itself can easily be prolonged by prolonging the presynaptic depolarization (Llinás et al., 1981), so release is somehow self limiting. All these results are consistent with the idea that presynaptic potential directly limits the time course of release (Parnas et al., 1986).

An alternative possibility is that transmitter release is triggered by the sharp rise and fall of presynaptic calcium in the immediate neighborhood of open calcium channels (Chad and Eckert, 1984; Fogelson and Zucker, 1985; Simon and Llinás, 1985). This "calcium spike" at release sites would not be detected by fluorimetric or spectrophotometric measures of the average calcium in presynaptic terminals, because as calcium ions rapidly diffuse away from channel mouths after they close, the ions remain in cytoplasm and are still detected until they are eventually taken up into organelles or extruded by active transport pumps. Simulations of calcium diffusion from clusters of calcium channels in presynaptic active zones indicate that the "calcium domains," or clouds of calcium ions surrounding open calcium channels, dissipate within milliseconds after channel closure. Transmitter release depends on up to the fourth or fifth power of calcium concentration, whether measured as external calcium, calcium influx in voltage clamp, or changes in intracellular calcium concentration (Dodge and Rahamimoff, 1967; Augustine and Charlton, 1986; Zucker et al., 1991). Such a highly cooperative calcium-dependent process would terminate more rapidly than the drop in calcium concentration. In fact, theoretical simulations indicate that the calcium concentration in active zones ("active calcium") raised to the fourth or fifth power lasts for less time than phasic transmitter release, suggesting that some step in exocytosis subsequent to the calcium trigger limits the time course of release (Fogelson and Zucker, 1985).

Recent results indicate that the time course of phasic transmitter release is practically invariant under a number of changing conditions. It varies little during pairs of action potentials that elicit facilitated release, or when the amplitude of the presynaptic depolarization is altered, or when the external calcium concentration is varied (Datyner and Gage, 1980; van der Kloot, 1988; Parnas et al., 1989). Only changes in temperature have a marked effect on the synaptic delay and the duration of release (Katz and Miledi, 1965; van der Kloot, 1988; Parnas et al., 1989), pointing again to some process

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other than diffusional collapse of calcium domains in determining the time course of phasic release.

Early simulations (Fogelson and Zucker, 1985) did not consider the effect of steps subsequent to calcium influx and diffusion on the time course of neurosecretion, and included no temperature-sensitive processes. Recent simulations (Parnas et al., 1989) reformulated the "calcium hypothesis of transmitter release" by explicitly adding steps of calcium in active zones binding to a calcium-sensing receptor, with several of these combining to activate exocytosis. These simulations showed that changes in pulse amplitude and calcium concentration, and whether the pulse was the first or second of a pair, caused changes in the predicted time course of release. That such changes were not noted experimentally was taken as proof that some process independent of calcium must govern the duration of release, and that this process is not simply calcium binding or exocytosis acting as a rate-limiting step.

Because these results were presented as irreconcilable with the "calcium hypothesis of transmitter release," it seemed important to explore further the properties of these simulations, to determine whether the mismatch between theory and results is due to a defect in the formulation or application of the theory, or whether it really must be concluded that some other voltage-dependent process independently governs the time course of transmitter release.

METHODS

We have duplicated as closely as possible the Parnas et al. (1989) implementation of the Fogelson and Zucker (1985) and Zucker and Fogelson (1986) presynaptic simulation model. The original model solved the diffusion equation (Fick's Law) in rectilinear coordinates with boundary conditions appropriate to the presynaptic terminal of the giant synapse in the squid stellate ganglion. Calcium influx occurs through arrays of calcium channels in the synaptic face of the terminal. The number of channels opened by a given depolarizing pulse was determined from the ratio of total presynaptic calcium current to single channel currents measured in related molluscan preparations. These channels were distributed in a regular array in active zones disposed in a square pattern in the synaptic face. These considerations lead to 64 channels opening per active zone for pulses to 0 mV, and four channels for pulses to −25 mV (Zucker and Fogelson, 1986). The dimensions of active zones (800 × 800 nm^2) and the spacing between them was based on ultrastructural measurements (Fogelson and Zucker, 1985). Transmitter release was considered to be triggered by calcium acting at the membrane surface at a distance of 56 nm from an open channel mouth, at a putative vesicle release site near the center of the active zone (see Fig. 1). In our initial simulations, pulse-evoked influx was taken to last 1 ms, about the open lifetime of a single channel at resting potential following a brief pulse, and tail current at the end of the pulse was ignored (as in Parnas et al., 1989). Calcium diffused from the array of channel mouths at a rate of 0.6 $\mu m^2 ms^{-1}$ for aqueous solution, which was subsequently damped by rapid binding to an immobile buffer. A binding ratio (of bound to free calcium) of 500 was assumed, as in Parnas et al. (1989), although we regard this figure as a bit higher than what is justified by experimental measurements (see Fogelson and Zucker, 1985). Calcium was extruded at the front and rear terminal surfaces by a pump at the rate of 80 nm ms^{-1}.

We used the numerical approximation to the diffusion equation and its boundary conditions represented by Eqs. 1–4 and 5–8 in Parnas et al. (1989), rather than the analytical solution developed by Fogelson and Zucker (1985). The numerical approach is more flexible in allowing the incorporation of kinetics and saturation in processes such as calcium buffering and extrusion. The numerical solution requires that the nerve terminal be divided into a large number of cells or compartments. Sufficient resolution is provided by making the cell length equal to ½ the 100-nm separation between calcium channel mouths (at 0 mV), or 20 nm. Then, calcium concentration will be represented as an array of 2.2 × 10^{11} cells in a terminal of 50-μm diameter and 700-μm long. Cell [0, 0] was centered on the origin.

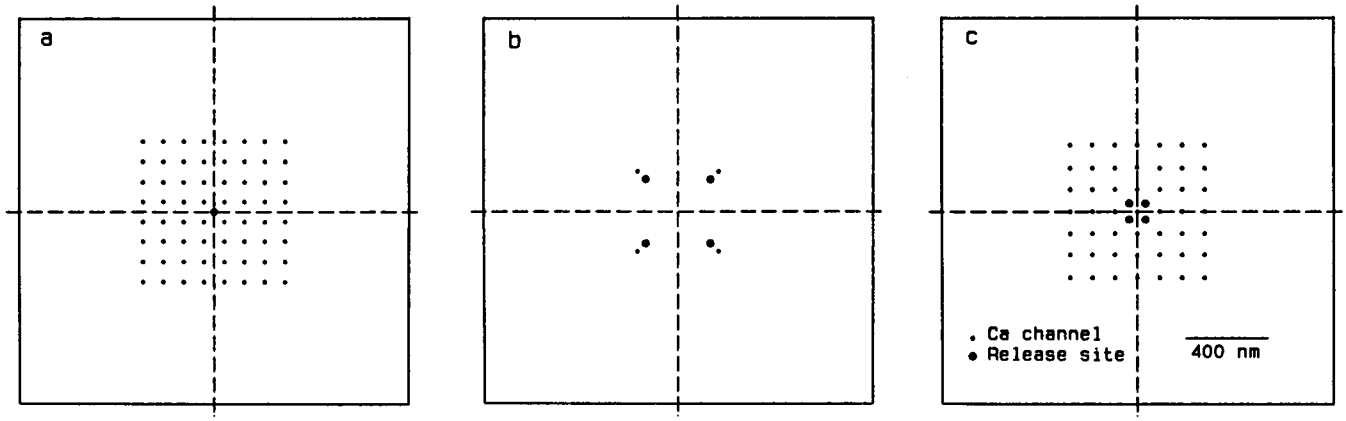

FIGURE 1 Disposition of open calcium channels (*small dots*) and exemplary transmitter release sites (*filled circles*) in an active zone at three pulse potentials: 64 channels at 0 mV in *a*, four channels at −25 mV in *b*, and 49 channels at −7.5 mV in *c*. The typical release site is centrally located, 56 nm from a calcium channel. The dotted lines show *x* and *z* axes, and the four-fold symmetry of an active zone.

Such large arrays are difficult to work with. To simplify the problem, it may be noted that a typical active zone, centrally located, is surrounded by identically behaving active zones. In that case, each active zone, and the region surrounding and separating it from adjacent zones, may be taken as an element of the terminal, across whose lateral boundaries no calcium will pass due to symmetry. A further reduction may be achieved by recognizing that each active zone displays a four-fold symmetry, so that an element may be taken as one quadrant of an active zone, which would then contain 5.8×10^6 cells.

This is still a formidable array, but the problem can be further simplified by recognizing that sharp calcium gradients are present only near the surface with calcium channels. The greater the distance from this surface, the shallower the gradients, and the greater the minimum cell size needed to resolve them. Thus, after the first 10 sheets of cells near the membrane (at a depth of 0.2 μm), we increased the cell size to 40-nm cubes. After another 10 sheets (0.6 μm), we doubled cell length again to 80 nm. At 1.4 μm, we increased the cell size to a 160-nm cube, until we reached the rear surface. Each element now contained only 4.1×10^4 cells. This change in cell dimension requires care at the interfaces between sheets of different cell size, where the *dx*, *dy* and *dz* of Eq. 6 in Parnas et al. (1989) are different on each side of the interface, and *dy* separating cells across an interface is the average of the *dy* on each side. The diffusion equation must be solved using a *dt* of 100 μs or less to satisfy Crank's Rule (Crank, 1975) for the small cell size at the synaptic surface, with $D = 1/500$ of 0.6 $\mu m^2\ ms^{-1}$. We normally used $dt = 10$ μs.

One additional problem was the representation of the spatial pattern of calciun influx through an open channel. Parnas et al. (1989) represented a calcium channel as a two-dimensional normal distribution of surface flux, with a standard deviation of 0.1 μm. This means that calcium influx occurred through a channel mouth which was greater than the separation between channels. In the analytical solution of Fogelson and Zucker (1985), calcium influx through a channel was confined to a surface flux within 2 nm^2. Such a flux, as well as that through a real channel, would fall completely into a single surface cell, so we represented an open channel as simply a flux of 2.7×10^{-9} fM μs^{-1} into one cell at −25 mV, and half that of 0 mV.

For simulations lasting 10 ms, we had to generate a calcium concentration array with 0.6 billion time and space elements, and perform about 7×10^8 operations. Our initial Fortran 77 code required ~3 min per 10-ms simulation on a Cray (Fagan, MN) X-MP/14 supercomputer. Subsequent optimization and vectorization reduced this to ~15 s.

We tested this new implementation of our presynaptic calcium diffusion model by replicating the results of earlier simulations. For this purpose, we calculated the calcium profile that would underlie two channels separated by either 100 or 218 nm, appropriate for depolarizations to 0 and −20 mV respectively. We used an influx duration of 2.4 ms to match the simulations performed with our earlier analytical model, and obtained submembrane calcium concentration profiles very similar to those of Fig. 4 in Zucker and Fogelson (1986) and Fig. 6 in Parnas et al. (1989). Our calculations of calcium at release sites during twin pulse stimulations to −25 or 0 mV also resemble closely those of Parnas et al. (1989), as may be seen in Fig. 2, *a* and *b*.

Additional simulations were performed in which the binding to the cytoplasmic calcium buffer was neither instantaneous nor nonsaturable. For these simulations, calcium was allowed to bind to, and unbind from, the cytoplasmic buffer at each time step and in each cell. This required the use of spatial arrays for free and calcium-bound forms of the buffer in addition to the presynaptic calcium concentration array. Because free calcium now diffused at the full rate of 0.6 $\mu m^2\ ms^{-1}$, the time step *dt* had to be reduced to 0.0625 μs to satisfy Crank's Rule. These changes increased the number of operations per simulation to ~1.5×10^{11}, and each simulation required ~2 h of Cray processing time after optimization.

RESULTS

Parnas et al. (1989) reported that the time course of calcium concentration at release sites was slightly different for two 1-ms pulses separated by 5 ms. Depolarizations to −25 mV and 0 mV also led to slightly different calcium concentration profiles, and these differences were reflected in raising the active calcium concentration to the fourth power to account for the cooperativity of calcium action.

ADDING A CALCIUM BINDING STEP

Such calculations ignore the binding of calcium to its receptor responsible for triggering transmitter release (X), which would be a saturable process, and also neglect the kinetics of subsequent steps in exocytosis. Parnas et al. (1989) proposed two schemes for connecting changes in calcium concentration, C, to transmitter release. In one, a single calcium ion binds to a releasing molecule X to activate it to form Q, and n such molecules combine to promote release at rate dR/dt, the rate of formation of species R. In the other scheme, n calcium ions bind to a releasing molecule X to form an activated quantum or vesicle Q, and transmitter is release at rate dR/dt following additional steps. Because they found that both models generated identical results, we chose to implement the former scheme as they did, to more easily allow comparison of results:

$$C + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Q, \qquad nQ \overset{k_2}{\rightarrow} R,$$

$$\frac{dQ}{dt} = k_1 \cdot C \cdot X - k_{-1} \cdot Q - nk_2 \cdot Q^n,$$

$$\frac{dR}{dt} = k_2 \cdot Q^n, \qquad X = X_T - Q.$$

$$\text{Rate of Release} = dR/dt. \tag{1}$$

This scheme (which corrects an error in Eq. 13 of Parnas et al. 1989) has the disadvantage that the kinetics of Q and therefore R are affected by the value of X_T. Because there is no precedent for selecting this value, we adopted the Parnas et al. (1989) convention of $X_T = 1$ M.

In our simulations, $C(t)$ was calculated from the diffusion equation with boundary conditions, and then used to calculate $dR(t)/dt$ from the above equations. This assumes that $C(t)$ is unperturbed by the binding of calcium ions to releasing molecules. Because each open

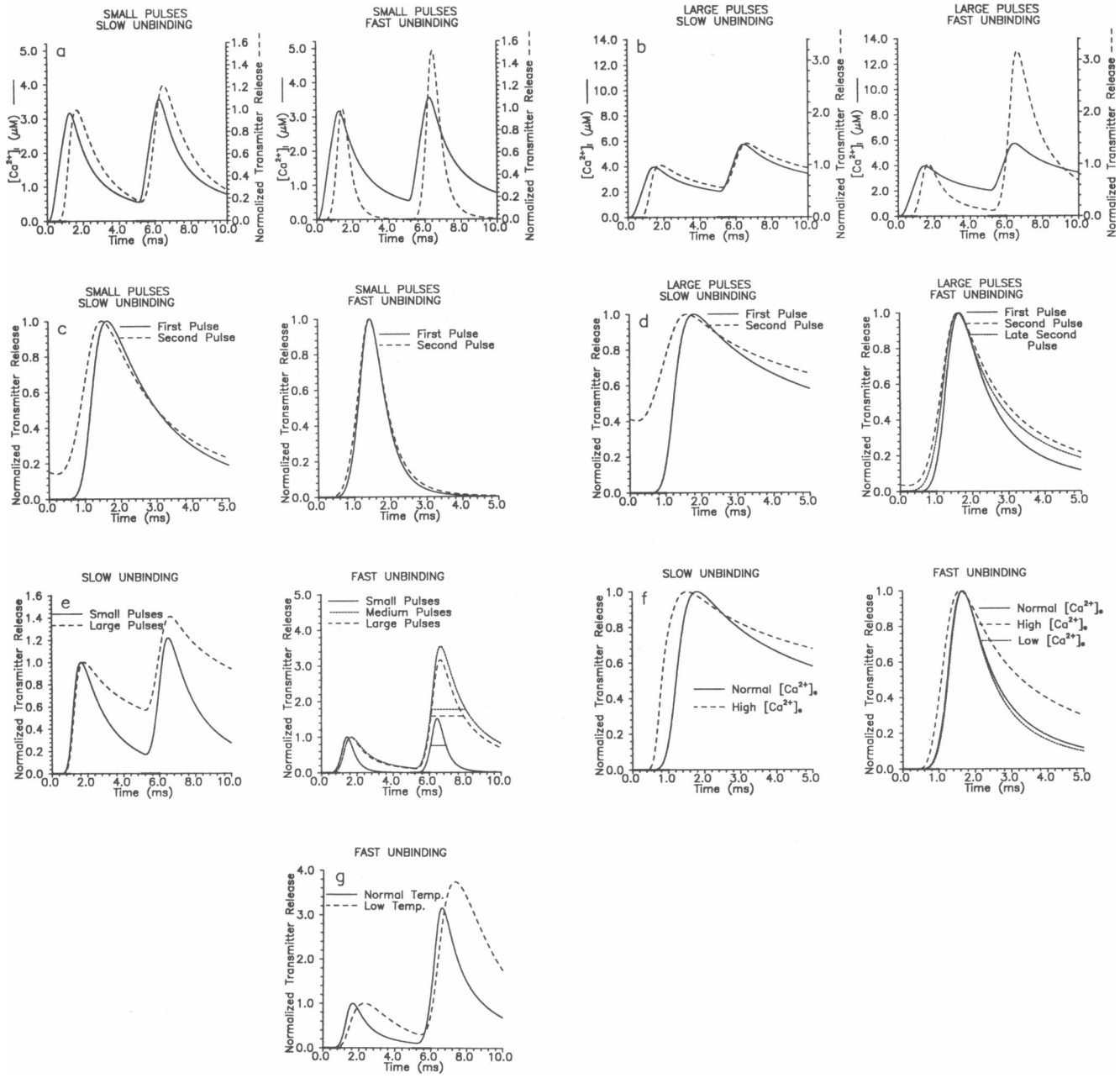

FIGURE 2 Simulations of the time courses of calcium concentration at a release site (*solid lines* in *a*, *b*) and of transmitter release (all remaining traces) to various patterns of stimulation. In all panels, the left-hand graph used a high affinity slow unbinding of calcium from its receptor (Scheme 1 with k_{-1} = 0.4 ms^{-1}) and the right-hand graph shows low affinity fast unbinding with rate-limiting exocytosis (Scheme 2 with k_{-1} = 10 ms^{-1}). (*a*) "active calcium" and transmitter release to paired small (−25 mV) pulses lasting 1 ms and separated by 5 ms. (*b*) responses to large (0 mV) pulses. (*c*) comparison of transmitter release to the first and second pulses to 0 mV. (*d*) release evoked by the first and second pulses to −25 mV; the right panel includes release to a late second pulse that follows the first by 15 ms (*lowest dotted line*). (*e*) comparison of responses to the −25 mV and 0 mV pulses; the right panel includes a response to −7.5 mV medium pulses (*uppermost dotted line*). The horizontal lines indicate half-widths. (*f*) responses in normal [Ca^{2+}] and five times normal [Ca^{2+}] in the medium; the right panel includes release in a half-normal [Ca^{2+}] medium. (*g*) transmitter release to large pulses at normal and low temperature, using Scheme 2 with low affinity fast unbinding of calcium from its receptor.

calcium channel admits ~1,600 ions during its 1 ms open time (twice as many at −25 mV), and each vesicle binds only four ions, and only a few vesicles could be influenced by calcium entering any one channel, this assumption seems justified. Experimentally, dR/dt may be compared to histograms of quantal release times following presynaptic action potentials.

Simulations of the behavior of dR/dt continued to show differences between release activated by each of the paired pulses, and between pulses to different voltage levels. We have repeated these simulations using the same parameter values as Parnas et al. (1989): k_1 = 0.5 ms^{-1} μM^{-1}, k_{-1} = 0.4 ms^{-1}, and k_2 = 1 $ms^{-1}M^{n-1}$. Our results are shown in the left-hand column of Fig. 2, and they resemble those reported previously (cf Fig. 13 of Parnas et al., 1989). Slight differences appear in the case of small pulses, probably because we chose a different position for the location of the transmitter release site near an open channel, and also reflecting our correction in the equations for the release scheme.

The following properties are evident from the left-hand graphs in Fig. 2: (1) Transmitter release is similar in time course to that of calcium at release sites (*a* and *b*), despite the cooperativity of calcium action. (2) For small pulses, release has nearly terminated by 2 ms after the peak, but for large pulses, release has dropped to only about half its peak even 4 ms later (*e*). This does not match experimental results obtained at crayfish neuromuscular junctions, where the half-width of release is only ~1 ms at 19°C (Parnas et al., 1989). (3) Release to the second pulse rises somewhat earlier and decays somewhat slower (*c* and *d*), especially to large pulses. Experimentally, release time course is similar for two pulses. (4) Release to the second pulse was only slightly facilitated (*a* and *b*: 20% for small pulses, 40% for large ones resembling action potentials), whereas spike-evoked release is actually facilitated by ~400% at 5-ms intervals (Zucker, 1974). (5) Release rises slightly faster, and decays substantially more gradually for large pulses than small pulses (*e*), also contradicting the experimentally observed similar time courses of release. (6) Finally, high external calcium, represented by scaling up the influx 5× and therefore multiplying the accumulation of intracellular calcium 5×, evokes release which rises sooner and lasts much longer (*f*), again contradicting experimental observation.

A RATE-LIMITING EXOCYTOTIC STEP

These results were surprising for a scheme in which steps subsequent to calcium entry and diffusion were supposed to be rate limiting. However, the time course of transmitter release in Scheme 1 is actually determined entirely by the time course of calcium at release sites and the calcium binding rates, k_1 and k_{-1}. The rate of transmitter release, dR/dt, is merely proportional to the number of activated calcium complexes, Q, raised to the power n; k_2 has no effect on the time course of release. For exocytosis to be a rate limiting step, as Parnas et al. (1989) said they intended, it is necessary to either make the conversion between nQ and R reversible, or allow R to be terminated by inactivation (or perhaps depletion) to a nonreleasable state I. We have chosen the latter approach, and modify the reaction scheme as follows:

$$C + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Q, \qquad nQ \overset{k_2}{\rightarrow} R \overset{k_3}{\rightarrow} I$$

$$\frac{dQ}{dt} = k_1 \cdot C \cdot X - k_{-1} \cdot Q - nk_2 \cdot Q^n,$$

$$\frac{dR}{dt} = k_2 \cdot Q^n - k_3 R, \qquad X = X_T - Q.$$

$$\text{Rate of Release} = R. \tag{2}$$

In this formulation, calcium (C) binds to release sites (X) to form activated sites (Q); n of these combine to form a release promoter (R) which inactivates at rate k_3 to state I. The rate of transmitter release is proportional to the level of R. The rate constant of the step terminating exocytosis, k_3, was chosen to be 10/ms to provide a time course of transmitter release similar to that observed experimentally.

With an instantaneous, nonsaturable cytoplasmic calcium buffer, calcium concentration at release sites is ~2 μM for much of the phasic release period, whereas the affinity of the calcium receptor is only 800 nM for the values chosen by Parnas et al. (1989). This means that these receptors would be nearly fully occupied with calcium during action potentials or depolarizing pulses, so that transmitter release would be almost fully saturated. Under these conditions, the time course of changes in Q, and hence of dR/dt in Scheme 1, is similar to the time course of the calcium transient. This makes the simulated time course of transmitter release sensitive to pulse number, pulse amplitude, and external calcium level.

This postulated saturation of calcium binding sites is not consistent with the very high sensitivity of transmitter release to external calcium concentration (Dudel, 1981), or to the large amounts of facilitation (up to 100-fold) attainable with tetanic stimulation (Zucker, 1974). These characteristics of transmission, at least at the crayfish neuromuscular junction, indicate that transmitter release to one or two pulses at normal calcium levels is far from saturated. To better agree with these properties of transmitter release, we have selected a value of 10 ms^{-1} for k_{-1}. This reduces the affinity of the

calcium binding site to 20 μM, and speeds the time constant of the binding reaction (at 2 μM calcium) from 1.4 ms to 48 μs, so that excytosis is truly rate limiting. Recent results of Adler et al. (1991) confirm that the calcium receptor for exocytosis has low affinity.

The right-hand graphs of Fig. 2 show our results when the off rate for calcium binding was increased to 10 ms^{-1}. These simulations using Scheme 2 show the following differences from those obtained with slow unbinding of calcium from its receptor and Scheme 1: (1) Transmitter release is now triggered by the very rapid changes in Q^n and is substantially faster than the calcium transient (*a* and *b*), but it now really is rate-limited primarily by the final exocytotic step, rather than by the formation and dissipation of calcium-activated release sites. (2) In particular, release is almost complete after 4 ms, even for large pulses. (3) The differences in release time course between first and second pulses are much reduced (*c* and *d*), and for small pulses would be undetectable experimentally. (4) Release to the second pulse is much more facilitated (*a* and *b*); in particular, release to large spike-like depolarizations resembles levels observed experimentally. (5) The difference in release time course between small and large pulses (*e*) is also greatly reduced, but not eliminated. (6) Finally, the differences in release time course at calcium levels differing by five-fold (*f*) are reduced to the point that they are unlikely to be detectable experimentally in release time histograms (Datyner and Gage, 1980).

We found that the differences in rise time in Scheme 1 (*c* and *d*) were eliminated when the release promoter *R* was made to inactivate, allowing exocytosis to become a rate-limiting step. Decreasing the affinity of the calcium binding site by speeding the off-rate was responsible for desaturating this site, speeding the time course of release (*a* and *b*), and reducing differences in release time course between facilitated and unfacilitated release (*c* and *d*), between large and small pulses (*e*), and at different levels of external calcium (*f*).

ADJUSTING SIMULATIONS TO MATCH EXPERIMENTS

It is important to recognize that some differences in release time course still persist in these simulations. For example, Fig. 2 *e* compares release time course to pulses to 0 and −25 mV. The simulations show a 50% increase in release duration for the first pulse, and nearly a doubling for the second pulse (compare small and large pulse traces for Scheme 2 in Fig. 2 *e*). But for these pulses, the total calcium influx differs by over 10-fold, and transmitter release differs by three orders of magnitude when extrapolated from experimental results (Augustine et al., 1985). The large and small depolarizing pulses used in the experiments of Parnas et al. (1989) differed by only 20% of the larger pulse, and the large pulses released only twice as much transmitter as small pulses.

For simulations to match the conditions of the experiment, we used an array of calcium channels and typical release site shown in Fig. 1 *c*. This corresponds to a pulse to −7.5 mV, which admits 80% as much calcium as a pulse of 0 mV. This released about half as much transmitter in experiments on the squid giant synapse (Augustine et al., 1985), and so is comparable to the small pulses of Parnas et al., 1989). Fig. 2 *e* includes a prediction of the time course of transmitter release for such a medium pulse, which releases 50% as much transmitter as the large pulse in the figure. The time course is virtually indistinguishable from that evoked by the larger pulses, in agreement with experimental results.

The simulations also show a 28% increase in release duration to the second of two large pulses (compare first and second pulses of Scheme 2 in Fig. 2 *d*), which one might expect to be detectable. However, pulses in these simulations were separated by only 5 ms, whereas the experimental results to which they were compared were for pairs of pulses separated by 15 ms (Parnas et al., 1989). When we performed simulations using large pulses separated by 15 ms (Fig. 2 *d*, Scheme 2, "Late Second Pulse") to match the experimental conditions, release time course was more similar to that of the first pulse, again in agreement with experimental results of Parnas et al. (1989) and Datyner and Gage (1980). Now release to the second pulse was facilitated about two-fold, also in agreement with experimental observation (Zucker, 1974; Parnas et al., 1989).

It is remarkable that in experiments in which a train of five pulses separated by 10 ms followed a first pulse by 20 ms, the duration of release to the last pulse in the train was almost doubled (Fig. 3 of Parnas et al., 1989). Apparently, conditioning stimulation is in fact capable of prolonging transmitter release to a pulse, much as predicted in the simulation of Fig. 2 *b* to large paired pulses separated by a very brief interval.

Fig. 2 *f* shows that increasing the calcium unbinding rate nearly eliminated the differences in rise time seen when calcium concentration, and therefore influx through calcium channels, is raised five-fold. The falling phase of release was slowed sufficiently in high calcium medium that this effect might be detectable (compare "Normal $[Ca^{2+}]_e$" with "High $[Ca^{2+}]_e$" traces). However, experimental measurements of the time course of transmitter release are available for only a two-fold reduction in calcium concentration (Datyner and Gage, 1980, Fig. 8). Simulations of this condition (Fig. 2 *g*, Scheme 2, "Low $[Ca^{2+}]_e$") predict no distinguishable change in time

course of release for a halving of external calcium concentration. It is not surprising that no data exist for the time course of release for a five-fold change in calcium level: Increasing external calcium this much has little effect on transmitter release (Dodge and Rahamimoff, 1967), due to saturation of influx through calcium channels (Hagiwara and Byerly, 1981); decreasing external calcium to one-fifth would entail measuring release that is reduced to ~0.15% of normal, where an accurate estimate of release time course would be difficult to obtain.

Another property of the time course of transmitter release is that it is very sensitive to temperature (Katz and Miledi, 1965; Parnas et al., 1989). Scheme 2 easily accounts for this sensitivity by assigning the temperature sensitivity of duration of release to rate constants k_2 and k_3, the rates of the final exocytotic steps. We have found that reducing these from 10 ms^{-1} to 1 ms^{-1} doubles the half width of the release time course (Fig. 2 *g*). Increases in the minimum synaptic delay at low temperature may also arise from delays in one or more steps separating calcium binding from transmitter release. Cooling also increased somewhat the magnitude of facilitation. Such an effect has been observed experimentally (compare Zucker, 1973 to Zucker, 1974).

Fig. 2 *e* indicates that the magnitude of facilitation experienced by the second pulse depends on the amplitude of the pulses, with moderate pulses displaying the most facilitation. Such behavior has been observed experimentally (Dudel, 1986, 1989), and was previously attributed to effects of resting calcium levels and saturation of transmitter release. However, in our simulations with Scheme 2, this behavior is mirrored in the calcium transients triggering release (data not illustrated). It arises from the fact that as the membrane is depolarized, the single-channel current is reduced, but more open channels contribute faster calcium transients as they open nearer to a typical release site. The first factor reduces the calcium gradient near release sites, but the second factor sharpens the gradient at very large voltages. This leads to the shallowest $[Ca^{2+}]_i$ profiles near channel mouths occurring at moderately large pulse amplitudes, which then collapse at the slowest rate. This results in the largest fraction of peak calcium remaining as residual calcium at the time of the second pulse, and hence moderately large pulses show the largest degree of facilitation.

SATURABLE CYTOPLASMIC BUFFER AND CALCIUM TAIL CURRENT

Our simulations up to this point suffer from a number of limitations. Perhaps the most serious is that they ignore the characteristics of a real cytoplasmic calcium buffer. Such a buffer must have finite binding and unbinding rates, just as the calcium binding site for transmitter release. One might expect this buffer to be temporarily saturated in the vicinity of calcium channel mouths. Also, its rate of calcium binding might not be fast enough to compete with calcium binding to the release sites, and might not be able to keep up with the rapid calcium transients at channel mouths. Then peak calcium concentration during depolarizing pulses would reach much higher levels than in the simulations of Fig. 2.

Our calculations of calcium transients were modified to account for the action of such a saturable cytoplasmic buffer. We used 2 mM of a buffer with a binding rate of 0.5 ms^{-1} μM^{-1}, similar to that of the T-sites of calcium-binding protein in muscle (Robertson et al., 1981). The unbinding rate was set to 25 ms^{-1}, to yield a dissociation constant of 50 μM. This is similar to the affinity of the main calcium binding component of squid axoplasm (Alemà et al., 1973), and gives a buffer capacity similar to that measured in molluscan neurons (Smith and Zucker, 1980).

Our simulations thus far treat only the time course of normalized transmitter release. When the relative magnitudes of release to pulses of different sizes are considered, another failing of the model immediately becomes evident. In simulations with Scheme 2 and with the calcium transients calculated thus far, the large pulses evoked transmitter release per release site at a maximum rate of only two times that of small pulses, or five times after correction for the increase in number of release sites per active zone in large pulses (see Fig. 1). Experimentally, pulses to 0 mV release over one thousand times as much transmitter as pulses to −25 mV (extrapolated from Augustine et al., 1985). We believe this defect is due largely to ignoring the effects of the calcium tail currents. Most of the calcium influx actually enters as a tail current following action potentials or brief depolarizing pulses (Llinás et al., 1981, 1982). After an action potential or depolarizing pulse, the membrane potential returns to ~−75 mV. At this potential, the single channel flux should increase to $\sim 4.6 \times 10^{-9}$ fM μs^{-1} (extrapolated from Zucker and Fogelson, 1986). In the following simulations, we represent calcium tail currents as a flux of this magnitude lasting 0.4 ms and following the 1-ms flux of calcium through channels open during the depolarization.

Fig. 3, *a* and *b*, shows the calcium transients at putative release sites near calcium channels during pulses to −25 mV and 0 mV. The peak calcium concentration to one pulse reaches 16 and 115 μM, respectively. These are much higher levels than those achieved when buffer kinetics and tail currents are ignored (Fig. 2, *a* and *b*).

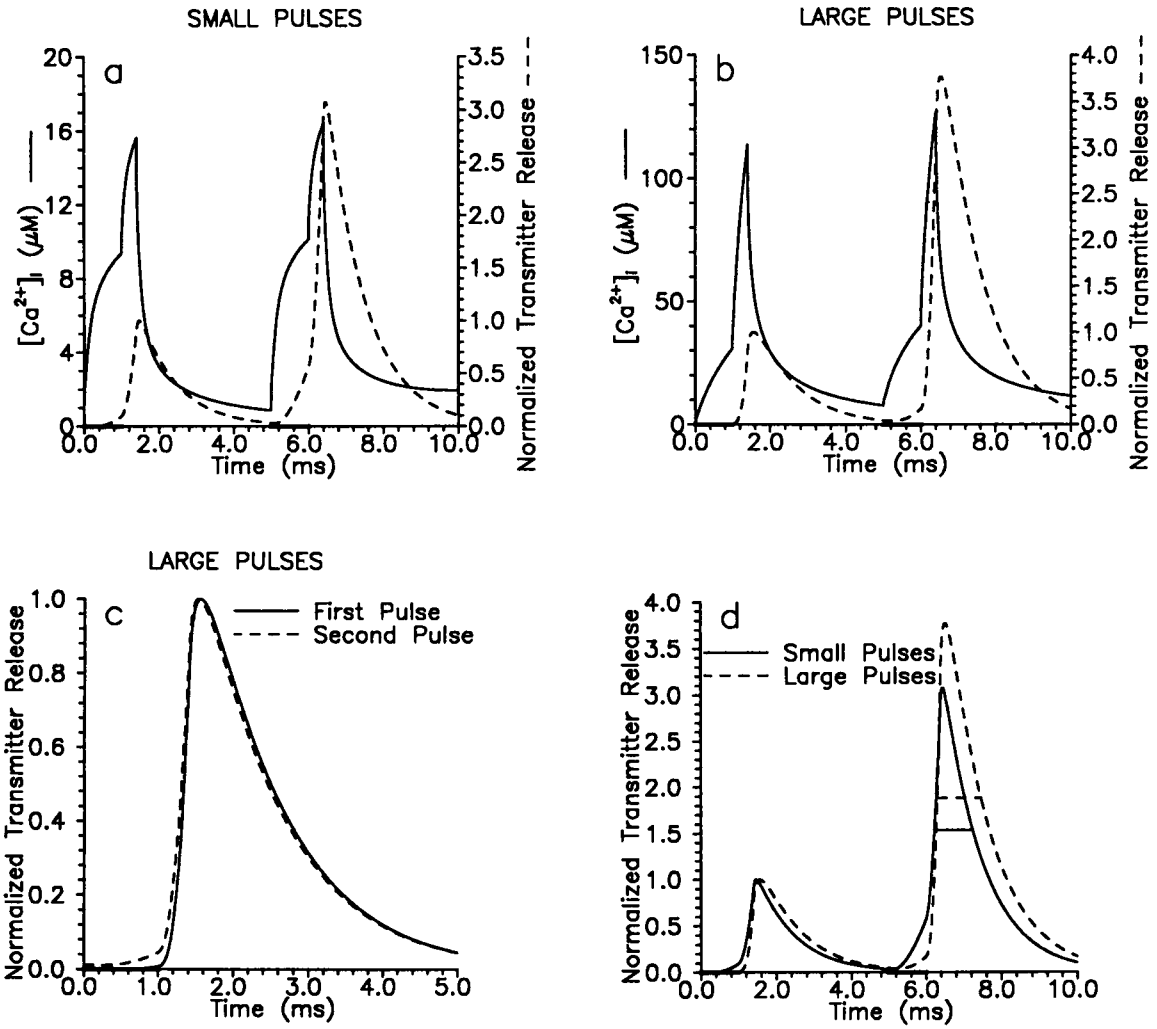

FIGURE 3 Simulations of calcium transients and transmitter release using Scheme 3. The pulses now evoke a tail current, and the cytoplasm contains a saturable calcium buffer with finite binding kinetics. (*a* and *b*) the solid lines show calcium concentration at release sites for small (−25 mV) and large (0 mV) paired pulses. The dashed lines show transmitter release when calcium binds to two different classes of sites on the exocytotic trigger molecule. (*c*) responses to the first and second large (0 mV) pulses separated by 5 ms. (*d*) transmitter release to small and large pulses. The horizontal lines indicate half widths.

For small pulses, most of the additional calcium is due to the incomplete binding of calcium to the cytoplasmic buffer near calcium channels while the channels remain open. For large pulses, the tail current at the end of the pulse is chiefly responsible for the high levels of calcium reached, and for the much bigger difference between small and large pulses than the simulations of Fig. 2, *a* and *b*.

TWO CALCIUM BINDING SITES

We next attempted to use Scheme 2 to predict the time course of transmitter release from the calcium transients of Fig. 3, *a* and *b*. To accommodate the high calcium levels without saturating the calcium binding sites of the release mechanism, its affinity was reduced to 200 μM by increasing k_{-1} to 100 ms^{-1}. Transmitter release terminated rapidly, and its time course was relatively independent of pulse number or amplitude. However, facilitation exhibited by the second pulse was reduced to only 150%, significantly less than that observed experimentally (Zucker, 1974). This result is a consequence of the greatly increased peak calcium concentration when a saturable buffer and tail currents are included, whereas the residual calcium, even that bound to release sites, is little affected by buffer kinetics or tail currents. This leads to a reduced residual calcium expressed as a

fraction of the peak calcium, and therefore facilitation is reduced. This situation persisted despite manipulation of the parameters of Scheme 2.

To address this problem, we devised a third scheme for calcium action in evoking transmitter release. In this model, calcium binds at two sites, X and Y, on the transmitter releasing trigger molecule. Site X is a rapidly equilibrating low affinity site, whereas site Y is a more slowly equilibrating higher affinity site. Release only occurs when calcium has bound to n X-sites and to m Y-sites:

$$X + C \underset{k_{-x}}{\overset{nk_{+x}}{\rightleftharpoons}} CX + C \underset{2k_{-x}}{\overset{(n-1)k_{+x}}{\rightleftharpoons}} \cdots \underset{nk_{-x}}{\overset{k_{+x}}{\rightleftharpoons}} C_nX$$

$$Y + C \underset{k_{-y}}{\overset{mk_{+y}}{\rightleftharpoons}} CY + C \underset{2k_{-y}}{\overset{(m-1)k_{+y}}{\rightleftharpoons}} \cdots \underset{mk_{-y}}{\overset{k_{+y}}{\rightleftharpoons}} C_mY$$

$$C_nX + C_mY \xrightarrow{k_2} R \xrightarrow{k_3} I$$

$$\frac{d(C_iX)}{dt} = (n - i + 1) \cdot k_{+x} \cdot C \cdot C_{i-1}X - i \cdot k_{-x} \cdot C_iX - (n - i) \cdot k_{+x} \cdot C \cdot C_iX + (i + 1) \cdot k_{-x} \cdot C_{i+1}X, \quad i = 0, \ldots, n$$

$$\frac{d(C_iY)}{dt} = (m - i + 1) \cdot k_{+y} \cdot C \cdot C_{i-1}Y - i \cdot k_{-y} \cdot C_iY - (m - i) \cdot k_{+y} \cdot C \cdot C_iY + (i + 1) \cdot k_{-y} \cdot C_{i+1}Y, \quad i = 0, \ldots, m$$

$$\frac{dR}{dt} = k_2 \cdot C_nX \cdot C_mY - k_3 \cdot R,$$

$$\sum_{i=0}^{n} C_iX + \sum_{i=0}^{m} C_iY = X_T,$$

$$\text{Rate of Release} = R. \tag{3}$$

Simulations of Scheme 3 for calcium transients in the presence of a saturable buffer and tail currents are shown in Fig. 3. We use four rapidly equilibrating low-affinity calcium binding sites with $k_{+x} = 0.5$ ms^{-1} μM^{-1} and $k_{-x} = 100$ ms^{-1} (200 μM dissociation constant), and one slowly equilibrating high-affinity calcium binding site with $k_{+y} = 0.01$ ms^{-1} μM^{-1} and $k_{-y} = 0.15$ ms^{-1} (15 μM dissociation constant). Panels *a* and *b* show transmitter release for small and large pulses (to −25 and 0 mV, respectively). Transmitter release starts with a delay after the beginning of the pulse, and is terminated rapidly, within 2–3 ms after the end of the pulse. Facilitation is robust, reaching levels of over 300% for both small and large pulses. The time course of transmitter release is virtually identical for the first and second of two large pulses separated by 5 ms (*c*), and the duration of release is almost the same for large and small pulses (*d*).

When the relative (not normalized) magnitudes of transmitter release in panels *a* and *b* were compared, the large pulses released 1,700 times as much transmitter per release site as the small pulses. After correction for the increased number of release sites per active zone (see Fig. 1), the large pulses released 3,800 times as much transmitter, consistent with experimental results (Augustine et al., 1985). The success of this aspect of the new simulation, in contrast to the failure of the earlier simulations, is due to inclusion of the tail current in calculating the calcium concentration transients.

The parameters of Scheme III were modified to get some idea of the range of values that would behave like Fig. 3. It did not matter much how many of the five calcium binding sites were type X vs type Y. The low affinity rapidly equilibrating X sites are responsible for triggering phasic release to the peak calcium concentration. The kinetics of this site must be fast, or the time course of release will not be invariant for different pulse amplitude and number. The affinity must be low to avoid saturation and the problems of Scheme 1. The high affinity slowly equilibrating Y site(s) is responsible for generating facilitation. The magnitude of the affinity is not critical, but the speed is. Moderately slow kinetics make this site a low pass filter which integrates the effect of the calcium transient in the first pulse and "stores" its effect. If the kinetics are too slow, the integration continues for several milliseconds, and facilitation grows in the interpulse period. This is not a property of experimentally recorded facilitation (Zucker, 1974).

DISCUSSION

This paper explores the capability of the "Calcium Hypothesis" of explaining the properties of transmitter release evoked by brief depolarizing pulses. It had previously been shown that simulations of calcium transients at sites of transmitter release near presynaptic calcium channels displayed different time courses to pulses of different amplitude and to the two pulses of paired-pulse stimulation (Parnas et al., 1989). Using a model of transmitter release with a high affinity calcium binding site to trigger exocytosis, similar changes in time course of release were found to different pulse amplitudes, facilitated and unfacilitated pulses, and to pulses in different external calcium concentrations. These changes in simulated time course of transmitter release stood in contrast to measurements of constant release time course to paired pulses, pulses of different amplitude, and responses to action potentials in different calcium concentrations. The discrepancies between simulations and experimental results were taken as evidence against the calcium hypothesis of transmitter release.

We have found that the results of the earlier simulations depended on the choice of model parameters used. When a low affinity calcium binding site was substituted for the previously saturated site, and when a separate rate-limiting exocytotic step was added, the time course of release became less influenced by pulse number and amplitude, and by changes in external calcium level. When the stimulation parameters of the simulation were adjusted to match those of experiments, in terms of the relative sizes of pulses of different amplitude, the interval between paired pulses, and the differences in external calcium concentration, the release time courses under different conditions became so similar that they would not be experimentally distinguishable. Thus the objections raised to the calcium hypothesis vanish when the affinity of the calcium receptor is reduced and the model is adjusted to fit experimental conditions.

We have refined the simulations of transmitter release to include a saturable cytoplasmic calcium buffer with finite binding rate constants, and have modified the influx of calcium through open channels to account for the calcium tail current at the end of a brief pulse or action potential. We found that calcium concentration at release sites could exceed 100 μM in a pulse, whereas the residual calcium at release sites following a pulse was much less affected. This caused the model to underpredict facilitation of release in paired-pulse stimulation. This discrepancy could be eliminated by revising the release scheme to include two classes of calcium binding sites: rapidly equilibrating low affinity sites, and slowly equilibrating high affinity sites. In this scheme, the latter are responsible for facilitation, but both must be fully occupied by calcium ions before transmitter release can occur.

There is good experimental support for several aspects of this new scheme of calcium action. First, that the endogenous calcium buffers do not bind calcium instantaneously and without saturation even in the vicinity of calcium channel mouths can hardly be doubted. Second, that transmitter release occurs in the neighborhood of such channels, where and when the local calcium concentration is quite high, is indicated by the submillisecond delay between opening of calcium channels and the commencement of transmitter release (Simon and Llinás, 1985). Third, that calcium rises and falls very rapidly at transmitter release sites during and after a depolarization is a direct consequence of the diffusional collapse of the sharp concentration gradients that must exist at release sites near calcium channel mouths (Zucker and Fogelson, 1985). Fourth, that calcium concentration reaches a level of 100 μM at release sites near calcium channels is confirmed by the use of calcium-dependent calcium current to calibrate the level of calcium reached near clusters of calcium channels at hair cell terminals (Roberts et al., 1990). Fifth, that calcium tail currents contribute much of the calcium triggering release is shown by the fact that release can be delayed to the end of a pulse, when the membrane is repolarizing and the calcium current suddenly increases (Katz and Miledi, 1967*a*,*b*; Llinás et al., 1981). Sixth, that calcium ions act cooperatively to evoke release is indicated by the highly nonlinear relationship between release and external calcium concentration (Dodge and Rahamimoff, 1956; Dudel, 1981), presynaptic calcium current (Augustine and Charlton, 1986), or intracellular calcium accumulation on repetitive stimulation (Zucker et al., 1991). Seventh, that calcium binds rapidly to a low affinity receptor to trigger transmitter release is indicated by the effects on transmission of exogenous buffers injected into the squid giant presynaptic terminal (Adler et al., 1991), as well as by the high sensitivity of release to changes in extracellular calcium concentration (Dodge and Rahamimoff, 1967; Dudel, 1981). Eighth, that calcium remains bound to other sites after stimulation is suggested by the observation that the apparent cooperativity of calcium action is lower for facilitated responses (Stanley, 1986). A more complicated model, in which calcium triggers subsequent changes in the release-promoting calcium receptor that relax at different rates following the unbinding of calcium from its various binding sites, cannot be excluded by either experimental results or simulations at this time. And finally, that the rate of transmitter release is limited by a late exocytotic step is indicated by the high temperature sensitivity of the time course of release (Katz and Miledi, 1965; Datyner and Gage, 1980; van der Kloot, 1988).

Nevertheless, the model of calcium concentration changes and transmitter release developed here is still quite rudimentary. In the model, calcium channels are disposed in a regular array and assumed to open simultaneously and for a fixed time. In reality, calcium channels will flutter open asynchronously and remain open for different times. Channels are randomly dispersed in active zones, and transmitter release is likely to occur from vesicles that happen to be docked near where a number of clustered calcium channels open together, rather than at a fixed distance from open calcium channel mouths. Thus we probably underestimate the real peaks of calcium achieved in active zones, and certainly ignore the stochastic complexity of calcium channel locations and openings and vesicle position. We have neglected the effects of vesicle depletion and mobilization. We oversimplify the calcium removal process by placing membrane pumps only at the front and rear surfaces of the nerve terminal, and neglect internal uptake processes and the effects of mobility of the cytoplasmic buffer. It is inappropriate to expect a close

quantitative agreement between simulations of such a simplified model, most of whose parameters are based on measurements at squid giant synapses, and results from crayfish neuromuscular junctions. Rather, the purpose of such modeling is to explore the consequences of certain notions of how synapses work, and to see what general characteristics of experiments they can explain. Major discrepancies between predictions and observations indicate the need to refine the model. Only when it can be shown that no version of the calcium hypothesis for transmitter release qualitatively resembles experimental results will it be warranted to throw out the hypothesis and formulate a new one. In particular, the idea that the time course of transmitter release is determined mainly by presynaptic potential acting independently of calcium (Dudel et al., 1986; Hochner et al., 1989) is now refuted by a large body of direct experimental evidence (Zucker and Haydon, 1988; Delaney and Zucker, 1990; Mulkey and Zucker, 1991).

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