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The kinetics of nerve-evoked quantal secretion

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Current views on quantal release of neurotransmitters hold that after the vesicle migrates towards release sites (active zones), multiple protein interactions mediate the docking of the vesicle to the presynaptic membrane and the formation of a multimolecular protein complex (the 'fusion machine') which ultimately makes the vesicle competent to release a quantum in response to the action potential. Classical biophysical studies of quantal release have modelled the process by a binomial system where n vesicles (sites) competent for exocytosis release a quantum, with probability p, in response to the action potential. This is likely to be an oversimplified model. Furthermore, statistical and kinetic studies have given results which are difficult to reconcile within this framework. Here, data are presented and discussed which suggest a revision of the biophysical model. Transient silencing of release is shown to occur following the pulse of synchronous transmitter release, which is evoked by the presynaptic action potential. This points to a schema where the vesicle fusion complex assembly is a reversible, stochastic process. Asynchronous exocytosis may occur at several intermediate stages in the process, along paths which may be differentially regulated by divalent cations or other factors. The fusion complex becomes competent for synchronous release (armed vesicles) only at appropriately organized sites. The action potential then triggers (deterministically rather than stochastically) the synchronous discharge of all armed vesicles. The existence of a specific conformation for the fusion complex to be competent for synchronous evoked fusion reconciles statistical and kinetic results during repetitive stimulation and helps explain the specific effects of toxins and genetic manipulation on the synchronization of release in response to an action potential.

Keywords: synaptic transmission; neurotransmitter release; neuromuscular junction; vesicle fusion; neuronal secretion; fusion machine

1. INTRODUCTION

It is generally accepted that quantal release of neurotransmitters involves exocytotic fusion of synaptic vesicles (Ceccarelli & Hurlbut 1980). During intense activation of quantal secretion, synaptic vesicles undergo multiple cycles of exo-endocytosis. Although the precise sequence of events has not been fully clarified, it is generally agreed that vesicles may be mobilized from a reserve pool—vesicles reversibly associated to the actin-based cytoskeleton of the nerve terminal through the membrane-associated phosphoprotein synapsin I—and cluster near specialized sites on the presynaptic membrane (active zones). Here, the specific interaction among vesicle-membrane proteins (synaptobrevin, synaptotagmin), soluble factors (N-ethylmaleimide-sensitive fusion protein or NSF, soluble NSF attachment protein or SNAP, and other modulatory proteins) and plasmalemmal proteins (syntaxins, synaptosome associated protein 25 or SNAP-25, calcium channels) promotes the formation of a multimolecular complex (SNAP receptor protein complex or SNARE complex) which mediates exocytotic fusion (Calakos & Sheller 1996; Hanson *et al.* 1997). The vesicle membrane may then be retrieved on the spot (Ceccarelli *et al.* 1973; Fesce *et al.* 1994; Palfrey & Artalejo 1998) or via coated pits and coated vesicles (Heuser & Reese 1973; Cremona & De Camilli 1997).

Morphologically, some vesicles appear in close contact with the presynaptic membrane at the active zones ('docked' vesicles). However, the precise molecular features of vesicle docking are not clear. Attempts have been made at defining whether each protein involved in exocytosis comes into play before or after docking, and vesicles ready for exocytosis have sometimes been indicated as 'cocked'—or docked and competent for release. The term 'readily releasable quanta' has also been used in the literature; this implicitly suggests that vesicles appropriately positioned and primed by protein interactions might undergo spontaneous exocytosis and also display a defined probability of releasing their quantum in response to the action potential.

Actually, no precise functional meaning can be attributed to any of these terms. In particular, competence for fusion has been evaluated using several different means of stimulation—hypertonic solutions, elevated K^+, toxins, drugs or electrical stimulation—although no evidence is available to indicate that the various treatments act on the same vesicle conformation(s) and population(s).

Here we shall discuss how the concurrent analysis of asynchronous quantal release and synchronous release evoked by the presynaptic action potential might help in dissecting functionally defined stages in the exocytotic cycle, based on the statistical and kinetic properties of the secretory activity.

2. APPROACHES TO STATISTICAL AND KINETIC ANALYSIS OF QUANTAL RELEASE

The history of electrophysiology of quantal release is half a century long. During these decades the initial hope that the mechanisms of transmitter release and the properties of the synaptic secretory machinery might be clarified and understood by means of quantitative statistical and kinetic analysis of postsynaptic responses has gradually surrendered to the ever-growing awareness of the complexity of transmitter release modulation by a multitude of functional, biochemical and pharmacological factors.

A classical approach to the study of quantal release has been the statistical analysis of fluctuations in quantal content of synaptic responses evoked by presynaptic stimulation at low frequency. The statistics of quantal content in successive responses to electrical stimulation can be described by binomial statistics at many synapses and this has suggested that a defined number n of entities (be they releasable vesicles, release sites or active zones) can produce the release of a quantum of transmitter, with probability p, in response to a presynaptic stimulus. However, estimated values and changes of n and p under various experimental conditions could not be easily reconciled with a comprehensive and consistent interpretation. Most difficulties in this approach presumably arise from the unreliability of the estimated binomial parameters in the presence of heterogeneous probability of release and temporal variability during repetitive stimulation, which cannot be spotted by standard binomial analysis but markedly affect the estimates (see Brown *et al*. 1976). Although these problems might, in principle, be overcome by more sophisticated approaches to statistical analysis of quantal content (Courtney 1978; Miyamoto 1986; Fesce 1990), this path has not been systematically explored.

A conceptually different, kinetic approach was also employed in investigating the question: the probability of release was estimated from the decrease in endplate potential (EPP) amplitude (or in quantal content) in response to pairs or trains of stimuli delivered at short intervals (Liley & North 1953, Thies 1965; Elmqvist & Quastel 1965). Mallart & Martin (1967) combined the regression of quantal content on total past release with ensemble averaging over several trains of stimuli, to reduce fluctuations and increase the reliability of the approach. In the absence of recycling (i.e. at sufficiently short intervals between successive stimuli), the fractional decrease in quantal content between the stimuli should reflect the fraction of available quanta released (or release sites activated) by the first stimulus, i.e. p, and the regression of quantal content ($m = n \times p$) on total past release (which should parallel the decrease in n, in the absence of recycling) should also yield an estimate of p. Although this approach seemed self-evident, a series of undemonstrated (or even false) assumptions were implicit in it. Even accepting that n entities with probability p of determining release of a quantum are sufficient to describe the system and no recycling occurs in short time intervals, no evidence indicates that p will remain unaltered between successive stimuli. Indeed, Betz (1970) and Christensen & Martin (1970) demonstrated that the decrease in quantal content of the second EPP is not linearly but rather quadratically related to the size of the first EPP, thereby suggesting that p is decreased to about the same extent as n, as a consequence of release in response to the first stimulus; the authors proposed that preferential release of high-probability quanta in response to the first stimulus might explain this finding. However, it appears that this approach also is unable to yield clear-cut and consistent answers to the question of what statistical and kinetic parameters determine the quantal content of evoked responses.

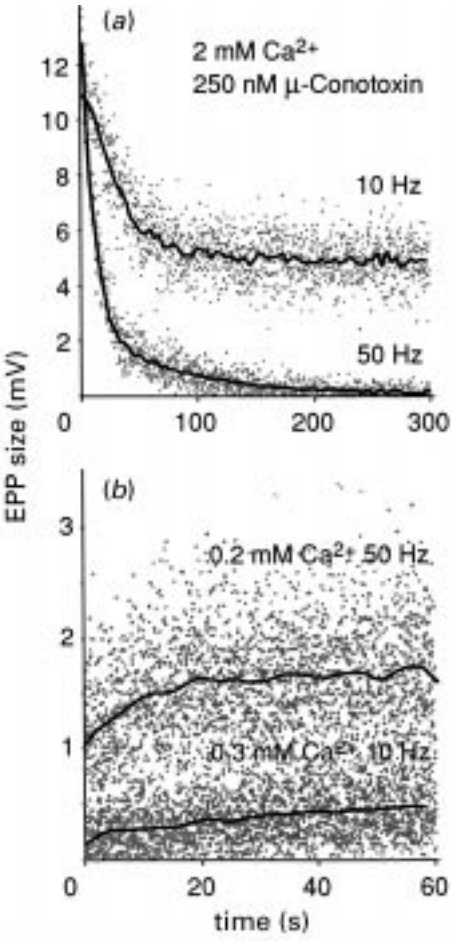

Figure 1. The dots illustrate the peak size of successive EPPs recorded at motor endplates in the mouse *extensor digitorum longus* (EDL) muscle during trains of stimuli delivered to the sciatic nerve at various stimulation frequencies and under the different experimental conditions indicated. The lines display momentary average values smoothed over time. (*a*) 250 nM μ-conotoxin added at normal Ca^{2+} concentration (2 mM) to prevent muscle action potential; (*b*) 10 Hz, 0.3 mM Ca^{2+}; 50 Hz, 0.2 mM Ca^{2+}.

3. BINOMIAL ANALYSIS DURING SUSTAINED ELECTRICAL STIMULATION

At the neuromuscular junction, markedly heterogeneous time-courses can be induced in EPP size and quantal content by repetitive stimulation at relatively high frequency (10–100 Hz). Figure 1 illustrates the declining responses observed during repetitive stimulation at 10 and 50 Hz in physiological extracellular

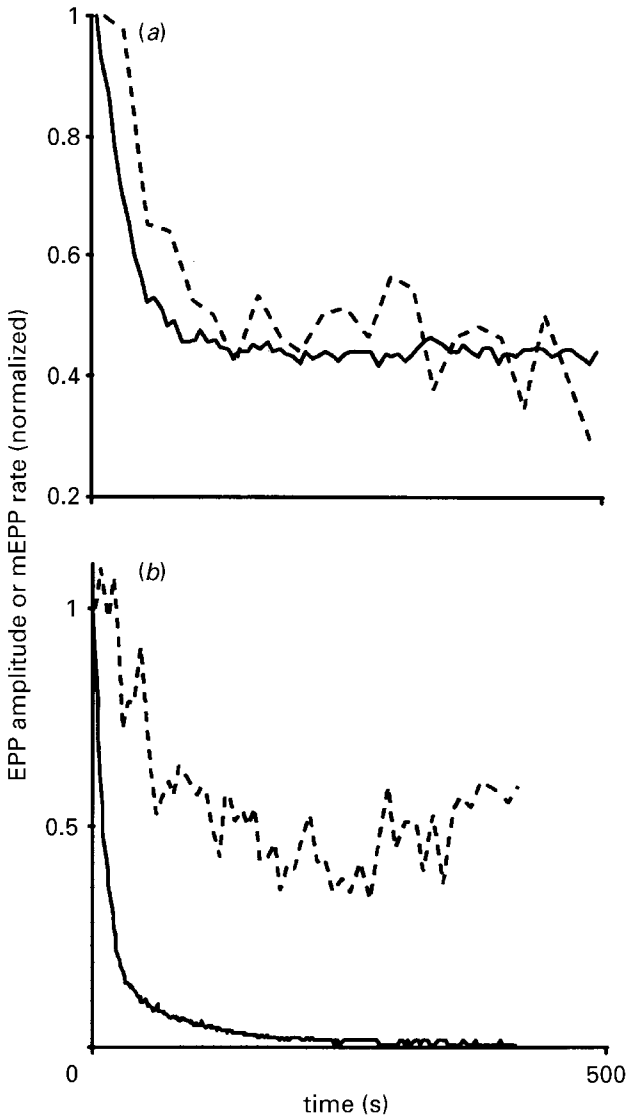

Figure 2. Smoothed time-courses of EPP size (solid line) and mEPP rate of occurrence between EPPs (dashed line) during the two trains of stimuli illustrated in figure 1. Calcium concentration, 2 mM; 250 nM μ-conotoxin. Notice the delay in depression of asynchronous release at 10 Hz (*a*), and the initial facilitation and less prominent depression at 50 Hz (*b*).

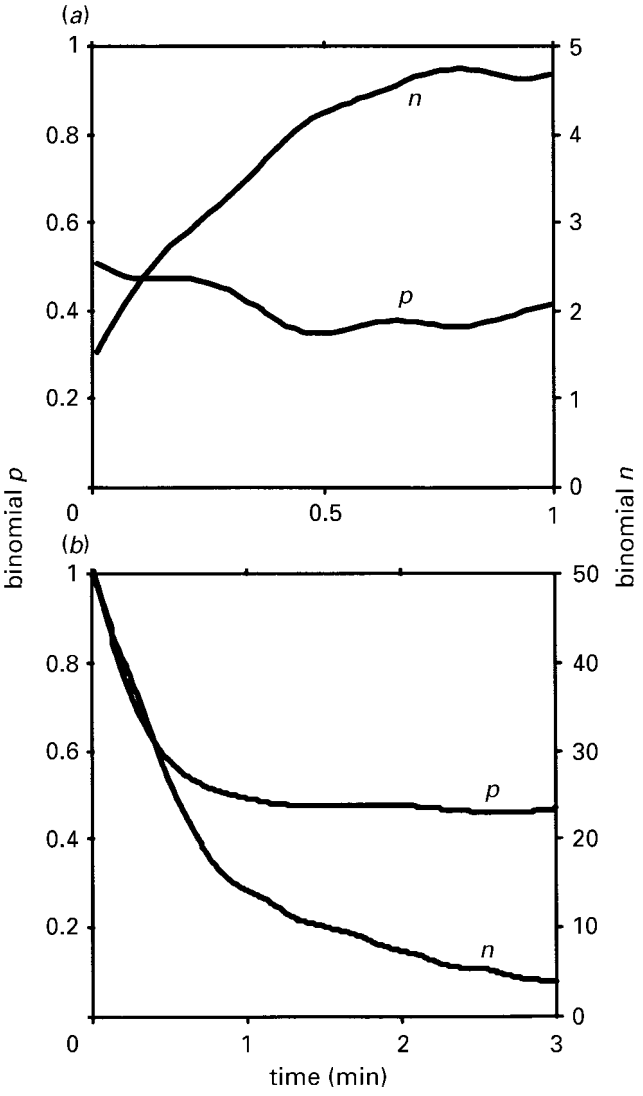

Figure 3. Binomial analysis of quantal content of EPPs during two trains of stimuli. Notice the gradual increase of binomial *n* from the initial very low value at reduced Ca^{2+} concentration (0.24 mM Ca^{2+} (12%), 25 Hz; (*a*)), and the decrease in both *n* and *p* during stimulation at 50 Hz in normal Ca^{2+} concentration (2 mM; 250 nM μ-conotoxin added; (*b*)).

Ca^{2+} concentration and the growing responses at lowered Ca^{2+} concentrations. Such changes reflect the dynamic balance between synaptic facilitation and potentiation processes on the one hand and synaptic depression or vesicle depletion on the other. Spontaneous quantal release can be measured by counting the mEPPs which occur between pairs of successive stimuli; during repetitive stimulation asynchronous release is also affected by facilitation and depression processes. However, asynchronous and evoked quantal release are known to be differentially regulated by Ca^{2+} and other divalent ions and dissimilarly affected by drugs and toxins. Figure 2 shows that repetitive stimulation also produces differential effects on evoked and asynchronous release: initial facilitation phenomena are markedly more evident in mEPP rate than in EPP size, whereas profound depression of evoked, but not asynchronous, release is apparent at late times during high-frequency stimulation in normal Ca^{2+}.

The results of binomial analysis under these experimental conditions are illustrated in figure 3. The results are consistent with a binomial model, as they point to a limited value for the parameter *n*, in agreement with most previously published observations under various experimental conditions. The decrease of the *n* parameter during the train in normal calcium is not unexpected and may reflect quantal depletion and/or fatigue.Instead, the apparent increase in *n*, rather than *p*, in low Ca^{2+}, is puzzling, as is the decrease in *p* which is observed in normal Ca^{2+} (although previous similar analyses also suggested a concurrent decline in both *n* and *p* during repetitive stimulation which produces synaptic depression; Mallart & Martin 1967).

The main problems arising with binomial analysis are related to variability among the *p*-values for different sites, which decreases response variance, and to

variability of n and p in time, which increase response variance. Although such phenomena prevent accurate and meaningful estimates of the parameters, relative changes in the estimates during an experiment or under different experimental conditions still yield relevant information on the underlying processes.

4. CORRELATION ANALYSIS

Under all conditions here examined the estimates of n at late times are consistently very low (< 10). It is worth pointing out that, although estimates of n and p may be inadequate, quantal analysis yields a solid piece of information: the variance in quantal content is too low for a Poisson system (characterized by a high number of possible events occurring with very low probability). Such low variance obviously is an important positive feature in terms of information processing at the synapse, and it might be observed that both a limited number of functional release sites and heterogeneous probabilities of release are advantageous features in terms of decreased output variability.

The clear indication of a limited number of release sites was exploited in the past to try and get relevant information on the process from the analysis of successive responses in a train of high frequency stimuli. A more robust approach would be the application of auto-regressive analysis to quantal contents during sustained stimulation. In particular, one could investigate the auto-correlation of EPP sizes (or quantal contents) in a reasonably stationary condition, i.e. the dependence of the size of each EPP on the size of the previous one, during prolonged stimulation. EPPs with a particularly high quantal content (due to random fluctuation) should be preferentially followed by undersized EPPs (due to larger momentary depletion). Systematic analyses by this approach should be able to validate the $n \times p$ model while yielding information on both parameters and on the time constant of the quantum reconstitution process.

A problem arises if the parameters of the secretory process change with time. In this case, oversized and undersized EPPs will tend to cluster (positive correlation of EPP sizes), reflecting changes in momentary mean values. This idea was used to investigate whether slow fluctuations in transmitter release are sustained by calcium fluctuations in the nerve terminal (Meiri & Rahamimoff 1978). However, even though the mean EPP size may change with time, the departures of EPP sizes from the momentary mean value need not be correlated, unless the quantal content of an EPP directly influences subsequent output. [A memoriless (Poisson) system is characterized by the independence of its momentary output from previous history. Correlation of momentary output with previous history will generally indicate that the system holds some 'memory' of its output and is not Poisson. Here, a subtle question arises: if the parameters of a memoriless process change with time, correlation will appear in the sequence of its output values (high or low output values will tend to cluster in the periods when the output probability is high or low) in apparent contradiction with the independence of successive output values and the idea of 'lack of memory'. To discriminate statistical memory from time-varying features of the process, it is appropriate to study departures from momentary mean values: the output of a time-varying memoriless system can be predicted based on its time-varying parameters (input), and the departures from prediction (error) will remain random and uncorrelated; on the contrary, the output of a non-Poisson system depends not only on its parameters (input) but also on its own previous output (history and error), and correlation is expected to be present in statistical fluctuations as well.]

If an almost steady state can be reached, the number of quanta recovered between stimuli will balance the expected number of quanta lost ($n \times p$). Whenever an EPP with quantal content greater than expected occurs ($m > \nu \times p$), the number of quanta available for next EPP should decrease by the number of quanta released in excess (ε) and quantal content should be transiently depressed (by a factor close to $\varepsilon \times p$, neglecting rapid recycling). Such an effect of random fluctuations of quantal content on subsequent responses should be detectable if n is small (and thereby significantly affected by small changes in quantal release) and should fade away with the time-constant of quantum reconstitution. In other words, the autocorrelation of the departures of quantal content from its momentary mean value should pinpoint the memory of the system.

More precisely, let n_j be the number of activatable sites at stimulus j, and p_j the corresponding probability of release. The expected quantal content is $\mathrm{E}[m_j] = n_j \times p_j$. For a constant p and in the absence of recycling, we would have $n_{j+1} = n_0 - \mathrm{SUM}[m_1, m_2, \ldots m_j]$, and $\mathrm{E}[m_{j+1}] = (n_0 - \mathrm{SUM}[m_1, m_2, \ldots m_j]) \times p$. Thus, $-p$ would be the slope of quantal content versus cumulative previous release (Elmqvist & Quastel 1965). If r_j quanta are refilled between stimuli j and $j+1$, the available sites at stimulus $j+1$ will be $n_{j+1} = n_j m_j + r_j$. Thus, the next expected quantal content will be $\mathrm{E}[m_{j+1}] = (n_j - m_j + r_j) \times p_{j+1}$. If the stimulation proceeds until a steady state is reached, p will become constant and the average values of m ($\boldsymbol{m}$) and of r ($\boldsymbol{r}$) should become equal (release equals refilling).

Thus, at steady state, $\mathrm{E}[m_{j+1} - \boldsymbol{m}] = (n_j - m_j + r_j) \cdot p - \boldsymbol{m} = \mathrm{E}[m_j - \boldsymbol{m}] - (m_j - r_j) \times p$ and $\mathrm{E}[(m_{j+1} - \boldsymbol{m}) - (1 - p) \cdot (m_j - \boldsymbol{m})] = (r_j - \boldsymbol{r}) \times p$. If the departures of r from its mean value are random, this equation defines a first-order auto-regressive system, so that simple mathematical processing of the autocorrelation of fluctuations in quantal content at steady state should yield a value for p. In particular, the auto-correlation of quantal content should display the behaviour illustrated in figure 4*a*. However, if the recycling–refilling speed were highly correlated with quantal content (i.e. most of the quanta released in response to a stimulus are rapidly reconstituted before next stimulus), then no correlation should be detectable.

Actually, one may ask why such an approach has not been previously applied at all. The answer is probably a simple one: negative results never get published. In fact, no dependence of EPP quantal content on the size of previous EPPs could be detected at the mouse EDL muscle during repetitive stimulation, at frequencies from 10 to 100 Hz and under conditions of depressed as well as normal quantal content. Figure 4*b* illustrates the autocorrelation of EPP sizes at steady state during trains of stimuli in normal Ca^{2+} (250 nM μ-Conotoxin) at 10 or 50 Hz, and in reduced Ca^{2+}

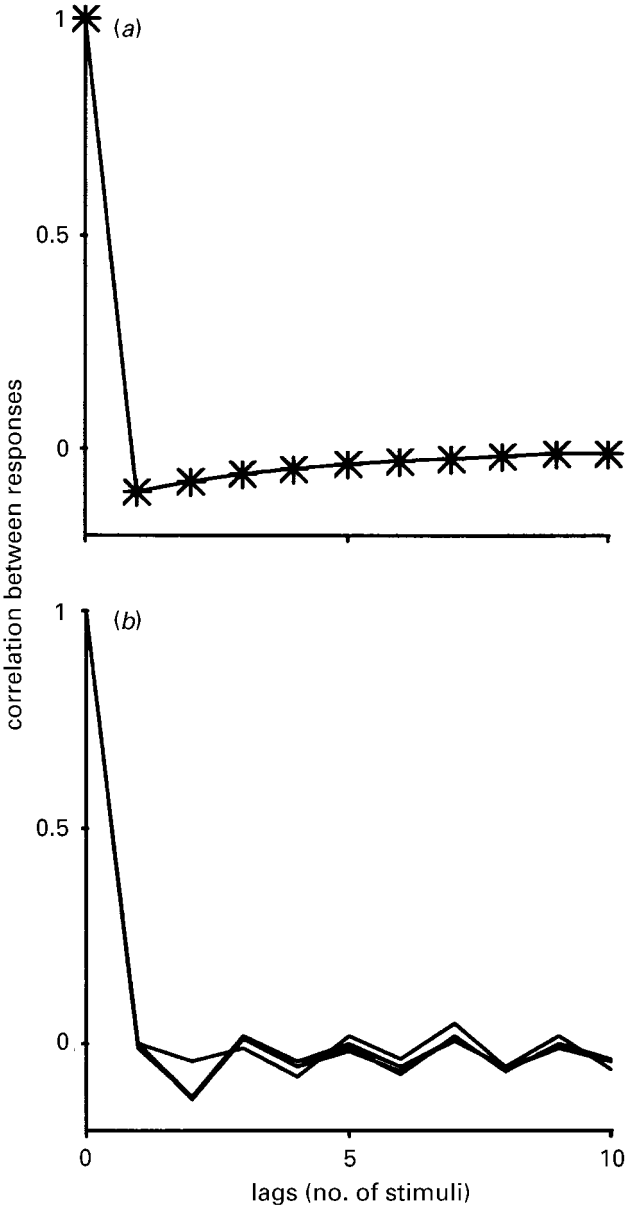

Figure 4. Expected and observed autocorrelation of EPP size fluctuations during repetitive stimulation; (*a*) illustrates the expected behaviour for low *n* and transient depression following oversized EPPs (see text); (*b*) shows the autocorrelation computed at late times during trains of stimuli at 10 and 50 Hz in 2 mM Ca^{2+} and 250 nM μ-conotoxin, at 25 and 50 Hz in 0.3 mM Ca^{2+} (15%) and at 100 Hz in 0.24 mM Ca^{2+} (12%). No consistent autocorrelation is observed on the fast time-scale considered here, and in particular no undershoot is observed for a lag of 1, as would instead be expected for a small pool of quanta subject to momentary depletion.

concentration (0.3 mM, 25 or 50 Hz; 0.24 mM, 100 Hz). No significant correlation could be detected under any of these conditions, although inconsistent fluctuations on a longer time-scale were often apparent.

If momentary depletion occurred after each stimulus, and its extent were related to recent quantal output, negative correlation should arise between EPP size and mEPP frequency between successive stimuli. Again, as shown in figure 5, no significant autocorrelation could be detected for either EPPs or mEPPs and no cross-

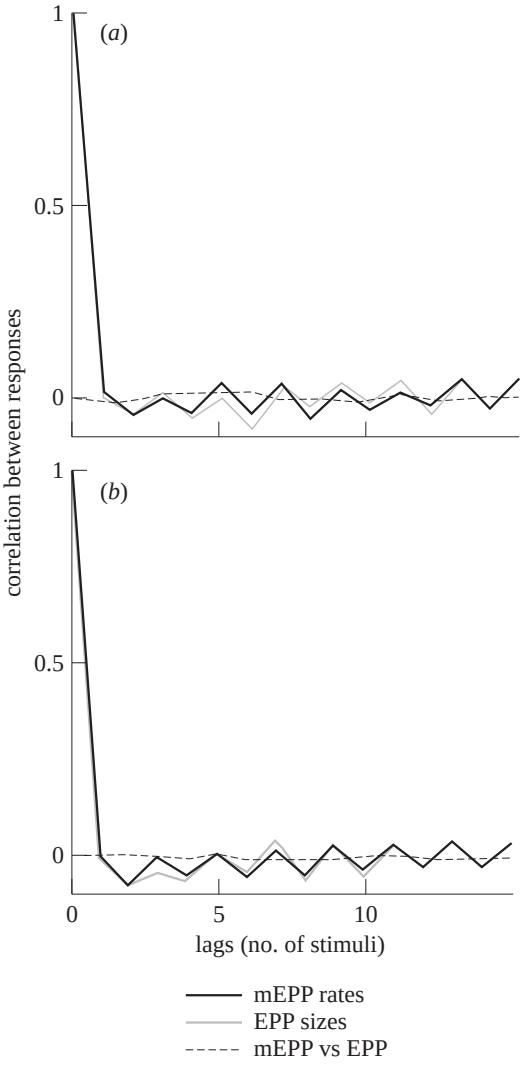

Figure 5. Autocorrelation of EPP size and mEPP rate of occurrence (between EPPs), and cross-correlation of mEPP rate versus EPP size at late times during trains of stimuli in 2 mM Ca^{2+} and 250 nM μ-conotoxin. No significant correlation can be detected, either at 10 Hz (*a*) or 50 Hz (*b*).

correlation was apparent between EPPs and mEPPs. These results are difficult to reconcile with a binomial model, considering in particular that estimates of *n* at steady state are quite small (<10) for the repetitive stimulation protocols examined here.

5. IS A BINOMIAL MODEL (AND A STATISTICAL MODEL IN GENERAL) OF ANY USEFULNESS?

Even though statistical and kinetic analysis of quantal content are unable to yield a consistent dynamic model of the release machinery, the conceptual framework of binomial statistics is helpful in considering the features of information processing at the synapse. An optimally designed synapse, in terms of consistency of information processing, should display both low variability under stationary conditions and no memory of past output (and especially of its random fluctuations) under repetitive stimulation. If one were to choose a statistical system for quantal release such that error (and variability) is kept to a minimum, a limited number of release sites with high

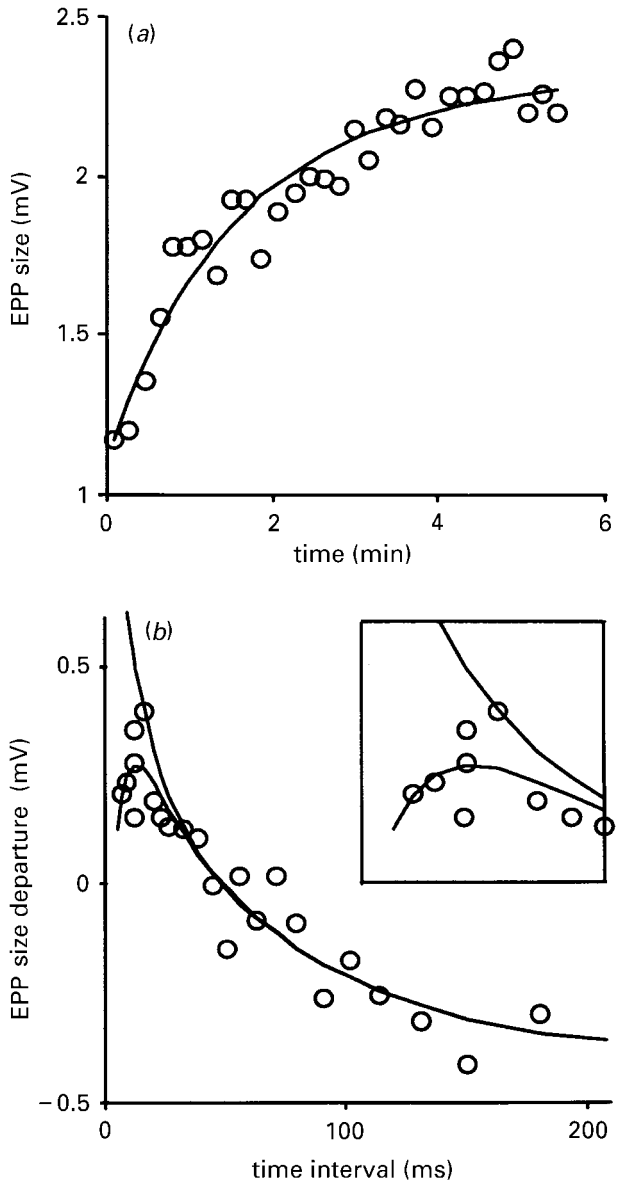

Figure 6. Analysis of EPP sizes during stimulation at random time intervals. Mean stimulation frequency, 15 Hz; 0.4 mM Ca (20%); (*a*) shows successive averages of groups of 166 EPPs and a smooth analytical fit to the overall time-course (two-exponential rise with time-constants of 75 and 175 s, respectively); (*b*) displays the departures of EPP sizes from the momentary average. Each point represents 100 EPPs, grouped according to the time interval which precedes the corresponding stimulus. The smooth decaying indicates facilitation which decreases as the interval gets longer. It is well fit by a two-exponential decay with time-constants of 68 and 9.8 ms, respectively. For short intervals, the points fall below the fit, indicating an initial depression, which vanishes with a 6.5 ms time-constant (also see inset).

probability of releasing a quantum would fit best, and quantal contents would obey binomial statistics with finite n, as observed distributions at rest usually do. On the other hand, such a system would be affected by correlation between successive events (occasional over-sized responses would significantly depress subsequent release), whereas an unlimited number of release sites with very low release probability (a Poisson system) would be best to grant the observed independence.

Thus, simple models may be inadequate to describe the synaptic release machinery, which is endowed with a particularly favourable behaviour: it is characterized by low variability (high reliability) under basal conditions; it can be modulated by activity over a wide range of efficiency levels; and it is sufficiently memoriless not to be affected by random fluctuations in its own previous output (low correlation and again high reliability).

An intriguing perspective which has not been investigated in detail regards the possibility that release may actually produce a momentary depression of activated release sites, but refilling of the active sites (or of a readily releasable pool of vesicles) might be so fast that the momentary depletion which follows a release event is rapidly recovered from before the next stimulus arrives, so that no correlation between successive responses can be observed.

6. RECOVERY BETWEEN STIMULI

Alternative hypotheses can be put forward to explain the lack of correlation between successive EPPs at steady state: release sites might be loaded with more than one releasable quantum although they release only one at a time; or the number of quanta and/or release sites might be so high that the number of quanta released with any single EPP cannot noticeably affect the release machinery (although this is in contradiction with the low values for n consistently reported in the literature).

To investigate these possibilities, neuromuscular junctions were subjected to 'random interval' stimulation, i.e. repetitive stimulation at variable time intervals, with mean stimulation rates of 10–20 Hz. The analysis of a representative experiment is illustrated in figure 6. Here, the average interval between stimuli was 67 ms (15 Hz) and extracellular Ca^{2+} concentration was 0.4 mM. The average EPP sizes (groups of 166 successive EPPs) are plotted in figure 6*a* as a function of time, to illustrate the overall time-course of release. In figure 6*b*, the departures of EPP sizes from the momentary average were computed and the values were grouped according to the time interval between the corresponding stimulus and the previous one. The decaying facilitation is apparent from the smooth curve (two-exponential decay with time-constants about 10 and 70 ms). However, at short time intervals the data depart from the exponential decay fit to the data, suggesting an initial depression which fades away with a time-constant of about 6 ms.

This suggests that a momentary depletion of releasable quanta (or silencing of release sites) does occur after each episode of evoked release, although recovery is so rapid (within 10–20 ms) that no 'memory' of recent output is detected for stimulation rates up to 50 Hz.

To further investigate this point, an autoregressive deconvolution procedure (Fesce 1990; Sacchi *et al.* 1998) was applied to the time-course of the averaged EPPs during repetitive stimulation. Figure 7 illustrates the results of this approach for a train of stimuli at 50 Hz in normal extracellular Ca^{2+} concentration: the average EPP time-courses are shown for the first 150 EPPs in the train and for groups of 1000 EPPs at 2 and 5 min after the onset of stimulation (figure 7*a*). The analytical shape of the mEPPs (the difference between two exponentials,

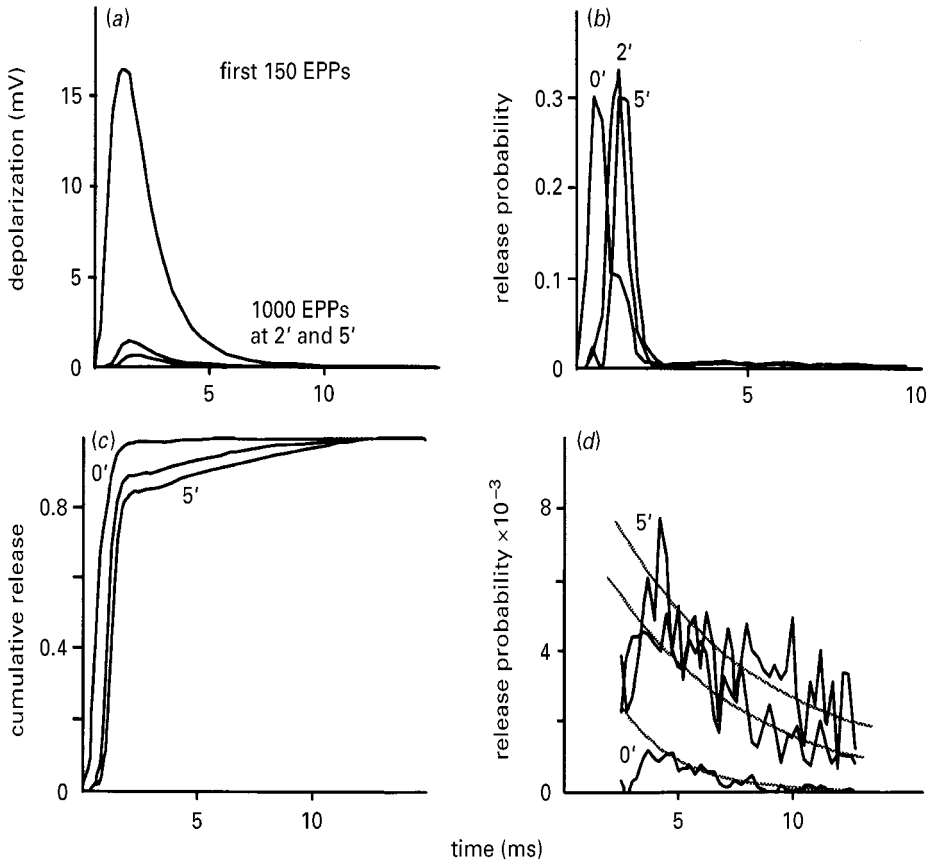

Figure 7. Deconvolution analysis of the EPP waveform during a 50 Hz train of stimuli delivered in 2 mM Ca^{2+} and 250 nM μ-conotoxin. (*a*) Average waveform for the first 150 EPPs and for groups of 1000 EPPs at 2 and 5 min after the onset of the stimulation. (*b*) Release probability curves obtained by deconvolving the average mEPP waveform (the difference between two exponentials with 0.6 ms rise time-constant and 4 ms decay time-constant) into the averaged EPP. Notice the shoulder in the deconvolution for the first group (0 min), produced by flattening of the peak, due to non-linear summation, for the first oversized EPPs. (*c*) Cumulative release curves obtained by integrating the release probability curves. Notice momentary flattening and increasing slow component for later times. (*d*) Tails of release probability curves redisplayed at higher gain (the first 3 ms have been cancelled). Notice the initial depression and the exponential decays, after 4–5 ms.

fit to averaged mEPPs isolated from the same recording) was deconvolved into the average EPP waveform to yield an estimate of the probability of release as a function of time after the delivery of the stimulus (figure 7*b*). Deconvolution reveals a peaky pulse of synchronous release (for the initial oversized EPPs non-linear summation is relevant and smoothes the peak somewhat, giving rise to an artefactual shoulder on the decay phase). The pulse of synchronous release is delayed at late times, but is over by 2 ms after the stimulus, and is followed by a tiny tail of release. The relative relevance of the tail is appreciated from the cumulative release curves (figure 7*c*) which are obtained by integrating the probability of release over time. The tail becomes more prominent at late times, in agreement with the less marked depression observed for asynchronous release (figure 2). The cumulative release curves display a momentary flattening (particularly apparent for late EPPs) which precedes the slow asymptotically rising phase. This indicates temporary silencing of release. Inspection of the curves of release probability at high amplification (figure 7*d*) confirms this impression: the probability of release decays exponentially at times greater than 5 ms, but backward extrapolation of the decay highlights the temporary silencing that follows synchronous release, from which the synapse rapidly recovers.

The results of random-interval stimulation and deconvolution of the EPP waveform indicate that temporary silencing of release follows synchronous secretion induced by the action potential during sustained stimulation. Paired pulse experiments at single hippocampal synapses at rest (Stevens & Wang 1995) revealed a rapidly waning depression (about 5 ms silencing followed by recovery with a time-constant of about 5 ms) at synapses where a quantum had just been released. From those experiments it could not be concluded whether such a depression would also be present (and if so, to what extent) at actively secreting synapses. The data given here indicate that momentary depression after evoked release also occurs at intensely stimulated synapses, and suggest that the recovery time may even become shorter under these conditions (although the data come from a different preparation).

7. MOMENTARY SILENCING AND THE BINOMIAL MODEL. WHAT IS *n*? WHAT IS *p*?

Fast recovery from momentary depletion of releasable quanta (or active sites) following evoked release fully explains the lack of correlation in EPP size fluctuations during repetitive stimulation, even though the number of active sites (or releasable vesicles) is very limited, as suggested by binomial analysis. Thus, it appears that

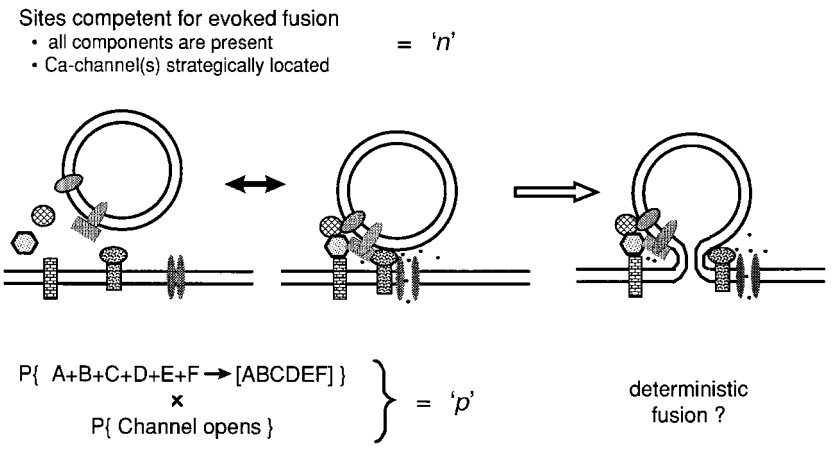

Figure 8. A reinterpretation of the *n* and *p* binomial parameters. It is suggested that the stochastic aspects of release concern the assembly of a functional fusion complex and the opening of calcium channel(s) at a competent site, whereas the action potential simply induces exocytosis at all competent sites loaded with an adequately assembled fusion complex.

kinetic and statistical data can be reconciled with a binomial model for quantal release.

Our increasing awareness of the complexity of the synaptic release machinery makes the $n \times p$ model for quantal release look like a wildly oversimplified view. However, the binomial model is far more general than it may appear at a first glance. For example, if *n* vesicle docking sites, competent for evoked release, were present in the presynaptic membrane and the fusion of a vesicle required the proper location of the vesicle, the correct formation of a multimolecular protein complex (fusion or SNARE complex) and the opening of one (or more) appropriately located calcium channel(s), then the compound probability of occurrence of all of these required conditions (which could be computed, in principle) can be called *p*, and the system will again be described by binomial statistics with parameters *n* and *p* (figure 8).

The most popular interpretation of the binomial model is based on the idea that in response to the presynaptic spike, a defined maximum number (*n*) of secretory events could occur, each with a fixed probability of occurrence, *p*. The binomial parameter *n* is generally thought of as the number of 'readily releasable quanta' or, equivalently, sites in a terminal which are ready to release a quantum, i.e. they are loaded with a vesicle which is docked to the presynaptic membrane through an adequately assembled and primed multimolecular fusion complex ('cocked' or docked-competent for fusion); *p* would then be the probability of each 'readily releasable quantum' being released. Implicit in this interpretation is the idea that the stochastic aspects in quantal release are confined to the last step (release of a releasable quantum) rather than to preceding events (assembly of a releasable quantum). In other words, this view implicitly suggests that the assembly of a vesicle fusion complex competent for evoked release need not be considered as a reversible (stochastic) process, whereas the fusion events are a possible, stochastic consequence of calcium influx during the action potential.

The interpretation proposed in figure 8 is somewhat reversed: most of the stochastic aspects in this model move here to early steps in the vesicle mobilization and preparation, and the assembly of a 'releasable quantum' is more properly considered as an equilibrium (reversible and stochastic) process. The fusion event might add further stochastic variability, but as regards evoked release it might even be a deterministic consequence of calcium inflow during the action potential. Indeed, paired pulse depression at single hippocampal synapses (Stevens & Wang 1995) as well as random-interval trains and deconvolution data reported above, consistently point not only to transient depression, but to actual silencing of release for a short period (a few milliseconds) which follows nerve-evoked secretion. This strongly suggests that whatever can be secreted in response to the action potential is actually released, i.e. the vesicle armed for firing cannot help releasing its content when Ca^{2+} floods the release site.

It is interesting to examine how such a revised binomial view of the release machinery could explain some intriguing aspects of transmitter release. Let then *p* represent, according to figure 8, the combined probability of all elements of the fusion machine correctly assembling and calcium channels opening to let calcium ions in; *n* would then represent the number of release sites competent for synchronous evoked release, i.e. where all necessary elements are present and a fusion complex can be formed close enough to one or more calcium channels for the action potential to trigger exocytosis. Notice also that the apparent value of *n* (measured from quantal content variance) would be decreased by heterogeneity of *p* among the sites.

The parallel changes that are often observed in *n* and *p* during stimulation are not easily explained by the traditional interpretation of the binomial model. According to the view proposed here, however, disassembly of protein complexes and partial depletion of vesicles and/or SNARE-complex components would directly produce a decrease in *p*; some release sites might even be silenced, thereby producing a concomitant decrease in *n*. Opposite changes would be produced by enhanced fusion-complex formation and/or vesicle mobility.

The dependence of *n* on extracellular Ca^{2+} at rest also is puzzling, if *n* represents the number of readily releasable quanta. In principle, vesicle mobility or the assembly of fusion complexes might be influenced by extracellular Ca^{2+} concentrations, but there are no indications on how extracellular calcium might influence intracellular organization at rest. The view proposed here, where *n* represents the number of sites competent for synchronous release in response to an action potential, suggests an alternative interpretation. The organization of

the release sites might vary to some extent. In particular, if the number and precise localization of calcium channels recruited by the fusion complex were not fixed, then the number of sites that can be activated by the action potential (and the heterogeneity of p) would depend on the amount of calcium entering each channel. At low extracellular Ca^{2+} concentrations, it is very unlikely that a single channel may let in, during an action potential, enough Ca^{2+} to trigger release; thus, only those sites of release where a sufficient number of Ca^{2+} channels are favourably located might be capable of releasing a quantum of transmitter and contribute to n. This would account for the dependence of n on extracellular Ca^{2+} concentration. During stimulation at low extracellular [Ca^{2+}], as intracellular calcium rises and activation of kinases ensues, mobilization of vesicles and availability of other components might be enhanced, and more sites might be gradually recruited, thereby producing the observed increase in n.

In general, estimates of p do not increase during stimulation as one would expect if vesicle mobility and SNARE complex formation were enhanced by facilitation processes. However, the rate of fusion complex formation might be bounded by limited availability of some of its components (DeBello *et al*. 1995). An increased probability of asynchronous exocytosis might divert a significant proportion of vesicles along their way to competence for evoked release. Furthermore, studies of responses to paired pulses at single synapses indicate that the onset of facilitation reduces p heterogeneity (Stevens & Wang 1995). Thus, decreased variability might significantly contribute to the fall (or lack of increase) in estimates of p during repetitive stimulation.

8. SYNCHRONOUS AND ASYNCHRONOUS RELEASE

In contrast with evoked release, asynchronous quantal release is only partly dependent on Ca^{2+}, and it can be enhanced by several factors in addition to the facilitation and potentiation processes which also affect evoked release. Hypertonic solutions, black widow spider venom, ouabain and other drugs induce increased rates of quantal release (see, for example, Ginsborg & Jenkinson 1976; Ceccarelli & Hurlbut 1980), and their actions variably depend on the extracellular concentration of Ca^{2+} and other divalent ions. Even post-tetanic potentiation affects asynchronous and evoked release to different extents and can be sustained by divalent cations other than Ca^{2+} (Hurlbut *et al*. 1971). It is therefore likely that the synaptic vesicle can follow more than one path to fusion and that ionic requirements are different along the various paths. Given the high number of interactions that appear to be involved in the formation of a vesicle ready to fire in response to an action potential, it is likely that several intermediate conformations might lead to vesicle fusion in response to local stimuli such as changes in volume and ionic environment. Thus, the concept itself of 'readily releasable quanta' might be misleading, as different stimuli might exert specific actions on distinct intermediate complexes. For example, the dissimilar degree of depression of evoked and asynchronous release, observed during sustained stimulation (figure 2*b*), suggests that synchronization of release is somehow depressed or the transition of quanta from a 'readily but asynchronously releasable' condition to a 'synchronously releasable' condition is somehow impaired. It is very reasonable that a specific conformation might be needed for a vesicle fusion complex to be capable of producing instantaneous exocytosis (on the microsecond time-scale) in response to a transient increase of local calcium concentration to the 100 µM range (Llinás *et al*. 1992), and that an increased probability of asynchronous exocytosis might reduce the probability for a vesicle fusion complex to reach such competence for synchronous release.

We have suggested here that the action potential may not act as a stochastic activator of fusion on vesicles that are docked to the presynaptic membrane and primed for fusion, but rather act as a deterministic trigger on fusion complexes competent for synchronous release. If such a population of synchronously releasable quanta—which we may refer to as 'armed vesicles'—did exist, then it should be possible to specifically interfere with their formation, i.e. to produce desynchronization of evoked release. Knock-out studies on the proteins of the fusion complex—SNAREs (for review, see Wu & Bellen 1997)—and observations using clostridial toxins which cleave these same proteins (Montecucco & Schiavo 1995) yield relevant information on this aspect. Two qualitatively distinct effects are produced on synaptic response by different botulinum toxins that cleave SNAREs on the presynaptic membrane (BoTx A and C) rather than the vesicle SNARE synaptobrevin (tetanus toxin and BoTx B; Gansel *et al*. 1987; Molgo *et al*. 1989), and these results have been confirmed by knock-out experiments (Broadie *et al*. 1995). In particular, cleavage or knock-out of synaptobrevin does not abolish asynchronous release and a burst of miniatures can still be elicited by an action potential, but they are not synchronized to give rise to a typical EPSP. In contrast, most other treatments and experimental conditions may even depress more deeply spontaneous as well as evoked release—as is the case for BoTx A—but still the few (if any) quanta released by an action potential give rise to a synchronous response.

9. CONCLUSIONS

The data discussed here suggest that in interpreting the functional aspects of neurotransmitter release, the schema of a pool of readily releasable quanta which, in response to an action potential, have a certain probability of undergoing fusion, may be inadequate. The dynamics of binomial parameters during repetitive stimulation and the demonstration of transient silencing of release during a brief period following synchronous transmitter release evoked by the presynaptic action potential point to a different schema (figure 9), where the vesicle fusion complex assembly is a reversible process and can proceed, at specifically competent sites, to a final state of competence for synchronous release (indicated as armed vesicle in figure 9). Exocytotic fusion may occur—and be differentially regulated—at several stages in the process, so that several different pools of readily releasable quanta may exist, depending on the means used to stimulate release.

This schema suggests that the stochastic nature of quantal release arises from the dynamic equilibrium

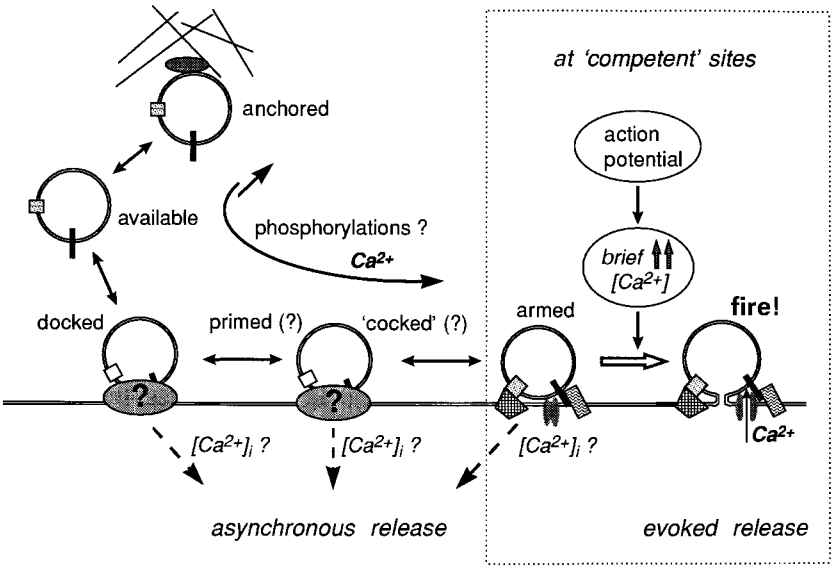

Figure 9. A model for asynchronous and synchronous quantal release. A series of reversible steps lead the vesicle through various conformational stages. Asynchronous release might occur from any of these stages and be differentially regulated by Ca^{2+} or other factors. Changes in Ca^{2+} concentration (even in the submicromolar range) and biochemical changes presumably affect the rates and probabilities of the molecular interactions and conformational changes involved. If a fusion complex forms at a release site competent for synchronous evoked fusion—adequately organized and endowed with calcium channel(s)—the vesicle is ready for synchronous release ('armed'); then, if it does not fuse spontaneously before a nerve pulse invades the terminal, it will be discharged in response to the high but rapidly vanishing increase in local Ca^{2+} level which accompanies the action potential.

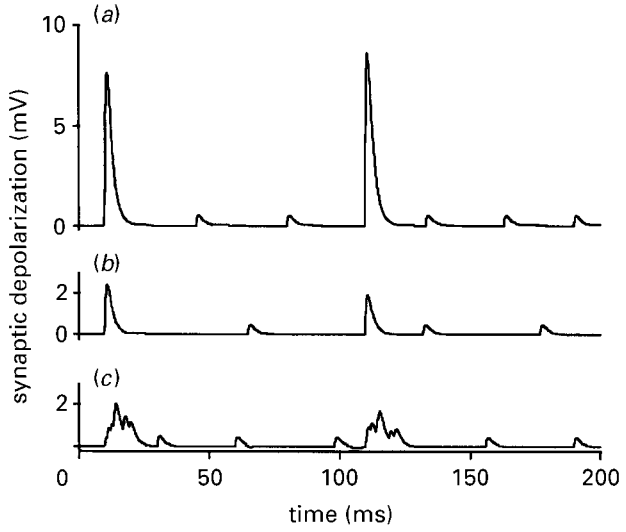

Figure 10. Schematic drawing illustrating the possible differential effects of treatments which inhibit quantal release. (*a*) Simulated evoked and spontaneous EPSPs under control conditions; (*b*) simulation of overall depression of release, with preserved synchronization of evoked release, as observed, for example, with BoTx A (Gansel *et al*. 1987) or injection of synthetic SNAP peptides (DeBello *et al*. 1995); (*c*) simulation of depression of quantal release with selective disturbance of synchronization, as observed, for example, with tetanus toxin or BoTx B (Gansel *et al*. 1987) or in synaptotagmin I knockout mice (Geppert *et al*. 1994).

among the various intermediate forms, whereas the final fusion step following the action potential can be considered as a deterministic, all or nothing, process, which occurs synchronously, with probability close to 1, at all sites where a competent fusion complex is exposed to a flash of extremely high local calcium concentration, thanks to the proper assembly of a multimolecular complex involving SNARE proteins and calcium channel(s). The model is consistent with the partly common and partly differential sensitivity of spontaneous and evoked release to ionic conditions and experimental situations. In particular, it can account for the sensitivity of both asynchronous and synchronous release to intracellular Ca^{2+} concentration in the micromolar or submicromolar range, which might sustain facilitation processes by favouring fusion-complex formation. However, the model suggests that only a specific conformation of the fusion complex (armed vesicles) can undergo fusion in response to the huge, but rapidly vanishing, increase in local Ca^{2+} concentration that follows the action potential, so that evoked release can be specifically affected by ionic and experimental conditions. This helps in interpreting the experimental observations, illustrated by the diagrams in figure 10, that synchronous quantal release may be preserved, albeit depressed, under conditions that massively inhibit quantal release, but is selectively impaired by inactivation of specific proteins or by substituting other divalent cations for Ca^{2+} (e.g. Katz & Miledi 1967), treatments which presumably interfere with the competence of the fusion complex for synchronous Ca^{2+}-evoked release.

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