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Topical Review

Neurotransmitter Release at Fast Synapses

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Introduction

Study of the mechanism underlying release of neurotransmitter in fast synapses began as early as 1921 (Loewi, 1921). One would hope that after so many years of study, a review of the subject might provide a comprehensive picture. Unfortunately, at the present writing much remains unknown and, consequently, we cannot conclude with a clear understanding of this mechanism.

Why is the release of neurotransmitter in fast synapses so difficult to unravel? A partial answer may be found on a poster announcing a recent conference held in Gif sur Yvette (France) on exactly this subject (*see* Fig. 1).

The present review attempts to pinpoint steps that might differ in the process of exocytosis between fast synapses and slow synapses or other slow releasing systems. This approach, we believe, may enhance understanding of the key steps in the release of neurotransmitter in fast synapses.

Duration of Release in Fast and Slow Systems

We begin our comparative analysis by schematizing the minimal number of macrosteps that must be involved in exocytosis and assign to each a time constant. The stimulus (different stimuli in different systems) triggers the release machinery, readying it for exocytosis. This step is termed TRIGGER, and the time constant associated with it is denoted τ_G. The next step involves fusion of the vesicle to the cell membrane and formation of the fusion pore. This step is termed FUSION and the time constant associated with it is denoted τ_F. After fusion, the content of the vesicle is emptied into its surroundings—in fast synapses, the synaptic cleft. This step is termed DISCHARGE and the time constant associated with it is denoted τ_D. The sum $\tau_G + \tau_F + \tau_D$, describes the time constant for release of a single vesicle and is denoted τ_S. The experimental correlate of this sum (τ_S) is the measured MINIMAL DELAY, that is the time elapsing from the onset of the stimulus until the first vesicle discharges its content.

In fast synapses, where release is monitored by the postsynaptic current, the *measured* minimal delay faithfully reflects the *theoretical* minimal delay, that is τ_S. This is not the case for systems where exocytosis is monitored by other means, which usually lack the temporal resolution needed to monitor release of a single vesicle. This technical limitation should be borne in mind in the course of the ensuing discussion.

The overall process of release, however, does not terminate with the discharge of a single vesicle's content; rather, the exocytosis of many vesicles is repeated until release eventually stops. We refer to the total time of such repetitions—the time elapsing from the onset of the stimulus until the rate of release returns to its resting level—as the DURATION of release. The corresponding time constant is denoted τ_T.

For fast synapses, such as neuromuscular junctions, where the stimulus is the action potential (depolarization), τ_T is in the range of a few milliseconds at or near room temperature. τ_T of this order has been observed, for example, in neuromuscular junctions of frogs (Katz & Miledi, 1965; Dudel, 1984*a, b*), crayfish (Parnas, Dudel & Parnas, 1986*a;* Parnas, Parnas & Dudel, 1986; Parnas, Hovav & Parnas, 1989; Arechiga et al., 1990;

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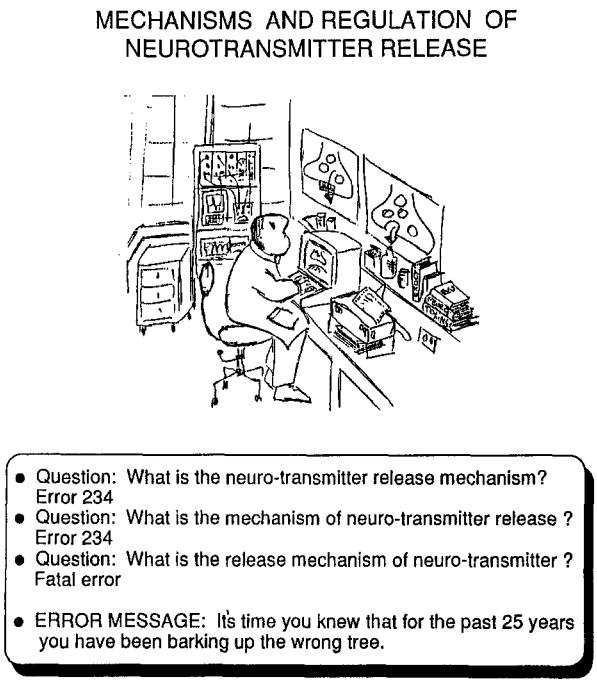

Fig. 1. Poster announcing the 17th Gif Lecture in Neurobiology, organized by L. Tauc, 1992. We are indebted to L. Tauc for permission to reproduce the poster.

Hochner, Parnas & Parnas, 1991), and mice (Datyner & Gage, 1980).

In slower systems by contrast, τ_T is in the range of seconds or even minutes. For example, Jankowski et al. (1992) reported τ_T for chromaffin cells to be 5–10 min at 23°C. They also found that τ_T was reduced when the extracellular Ca^{2+} concentration was reduced.

The same range of τ_T (7–12 min) was obtained for chromaffin cells by Bittner and Holtz (1992). In both instances, the stimulus for release was permeabilization of the chromaffin cells with digitonin. By contrast, when chromaffin cells were stimulated by depolarization to +10 mV for 25 msec (Chow, von Ruden & Neher, 1992), τ_T was in the range of seconds. The above suggests that τ_T depends strongly on the type of stimulus used.

In other slow systems, τ_T was equally long. For example, τ_T in the range of minutes was found in melanotrops (Okano, Monk & Fernandez, 1993), and in the range of seconds, for the same cells by Thomas, Surprenant and Almers (1990). In mast cells, another slow releasing system, τ_T was shown to be minutes (*reviewed in* Lindau & Gomperts, 1991). Finally, Kennedy et al. (1993) showed that in pancreatic β-cells, τ_T could reach 10 min when cells were stimulated by glucose, and 10–60 sec when stimulated by K^+.

What is the origin of the overwhelming difference in τ_T between fast and slow releasing systems? Does the slow τ_T reflect slow time constants of the various steps involved in the exocytosis of a single vesicle (granule)? Or, alternatively, is the slow τ_T a result of persisting release where many vesicles, each with a relatively fast τ_S, are not released synchronously?

To answer this question, let us examine the time constant of each of the macrosteps shown in Fig. 2.

Discharge Time Constant (τ_D) in Fast and Slow Systems

In fast synapses, the discharge (emptying of the vesicle's content) cannot be measured directly but can be inferred from the measured minimal delay. At room temperature (or below), the minimal delay was found to be as brief as 0.2 msec in the squid giant synapse (Llinas, Sugimori & Simon, 1982), around 0.5 msec (in the lobster, Parnas et al., 1989; and in the mouse, Datyner & Gage, 1980). As mentioned, the minimal delay corresponds to τ_S, and hence τ_D must be either close to τ_S (if τ_D reflects the rate limiting step) or much smaller. Khanin, Parnas and Segel (1994) demonstrated that in order to obtain the observed high concentration of neurotransmitter in the vicinity of the postsynaptic critical zone, the duration of the discharge in fast synapses must be in the range of 50–75 μsec, thus much shorter than the minimal delay (τ_S).

In at least one slow system, chromaffin cells, the duration of discharge was directly measured by using a carbon-fiber electrode as an electrochemical detector of the secreted catecholamines (Whightman et al., 1991; Chow et al., 1992). Using this method, they found the discharge from a single chromaffin granule to have a duration of 40–60 msec. Any attempt to evaluate τ_D from these measurements must take into account the time required for the released catecholamines to diffuse toward the electrode. This time will, of course, depend on the distance between the electrode and the point of release. Under the conditions reported in Whightman et al. (1991), the time will take about 10 msec using a diffusion coefficient of $D = 10^{-6}cm^2/sec$. If the diffusion time is subtracted from the measured time of 40–60 msec, we are left with τ_D of 30–50 msec in this slow system. This τ_D is much longer than in fast synapses, where, as mentioned, it is below 0.1 msec.

Two conclusions are relevant at this point: (i) The process of discharge is probably different in fast and slow systems (this will be further discussed below); and (ii) Even the slower τ_D in slow releasing systems cannot by itself account for τ_T in such systems.

Fusion Time Constant (τ_F) in Fast and Slow Systems

The other macrostep which must be considered in an attempt to elucidate the origin of τ_T is fusion and fusion pore formation. A full discussion of fusion is beyond the scope of the present review. However, several excellent

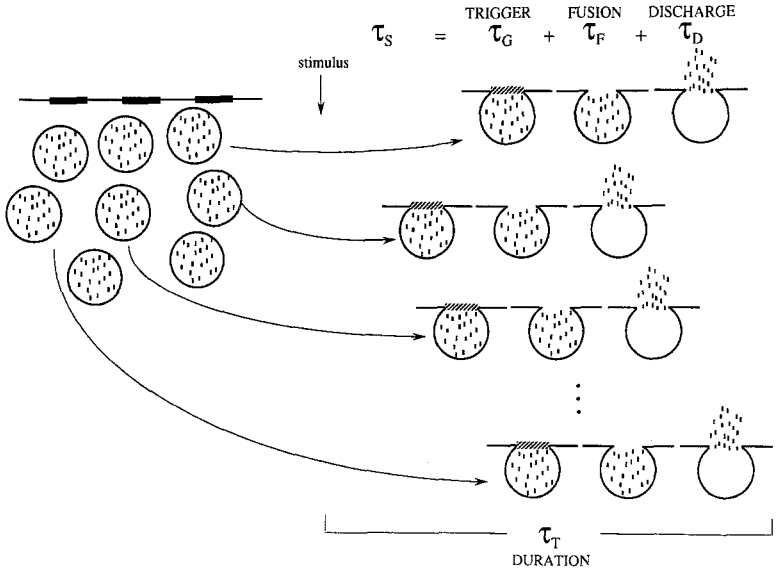

Fig. 2. Schematic presentation of the macrosteps involved in exocytosis, together with the time constants assigned to each step. Heavy line denotes resting state of release machinery. Broken line denotes release machinery in readied state after arrival of stimulus.

reviews exist on this subject (*see* Monck & Fernandez, 1992; and Zimmerberg, Vogel & Chernomordik, 1993). Here we focus on the time constant of fusion and fusion pore formation (τ_F).

This time constant cannot be measured directly but can be estimated based on the considerations outlined below. It is generally assumed that the mechanism of fusion and fusion pore formation is similar in all systems undergoing exocytosis (Monck & Fernandez, 1992; Zimmerberg et al., 1993).

We may therefore evaluate τ_F based on exocytosis in fast synapses. τ_F cannot be longer than the minimal delay which, as we recall, is of the order of 0.5 msec at room temperature. To evaluate τ_F, we first recall that τ_D (time constant of discharge) in fast synapses is less than 0.1 msec (*see above*). Next we note that based on experimental measurements of the time course of neurotransmitter release, Lustig, Parnas and Segel (1989, 1990) calculated the time constant of the lumped steps preceding the rate limiting step to be fast—less than 0.1 msec. Lustig et al. (1989, 1990) further argued that this fast time constant corresponds to a lumped step involving Ca^{2+}. Given that the Ca^{2+}-dependent steps precede the rate limiting step, it follows that in the context of Fig. 2, it is fusion and fusion pore formation which are the rate limiting steps in fast synapses. As such, its time constant must be close to the minimal delay, that is about 0.5 msec. In view of the assumed similarity of fusion in slow and fast systems, we may conclude that the time constant of fusion and fusion pore formation in slow systems is also about 0.5 msec.

Release Triggering Time Constant (τ_G) in Fast and Slow Systems

In fast synapses, the time constant for triggering the release machinery corresponds to the steps involving Ca^{2+} (and depolarization, *see below*) that precede fusion. This time constant is, as mentioned, around 0.1 msec (Lustig et al., 1989, 1990).

In slow systems, τ_G can be estimated from the time elapsing between onset of the stimulus and the first measurable change in capacitance. This obviously holds only for systems where changes in capacitance and conductance can be measured. In melanotrops, τ_G reaches 20 sec (Okano et al., 1993). In mast cells, the corresponding time constant is in the range of tens of seconds, depending on the experimental conditions (*reviewed in* Lindau & Gomperts, 1991).

For chromaffin cells, this time constant was shown to be a few seconds (Jankowski et al., 1992). However, under conditions where better time resolution was achieved (by using other than capacitance methods), a time constant of 10–15 msec was obtained (Chow et al., 1992). This overwhelming difference obviously casts doubt on the validity of the often held assumption that the time required to trigger release is very slow (seconds) in slow systems.

In summary, according to the findings cited, the time constant for triggering release is about 0.1 msec in fast systems but can be as long as a few seconds (but see above comments) in slow releasing systems. We may conclude, therefore, that the triggering mechanism is likely to differ as well in the two systems.

We shall next discuss possible factors that may account for the differences in τ_D and τ_G in fast and slow systems.

Possible Differences in the Mechanism of DISCHARGE between Fast and Slow Systems

In a series of experiments, Uvnäs and Aborg demonstrated that mast cell granules (Uvnäs et al., 1985), bovine chromaffin granules, and nerve granule enriched

preparations from various sources (Uvnäs & Aborg, 1984*a*) discharge their secretory products (biogenic amines and catecholamines) through an exchange to inorganic cations (e.g., Na^+, K^+). Present technology does not permit direct study of the mechanism underlying discharge from small synaptic vesicles. Nevertheless, Uvnäs and Aborg (1984*b*) proposed that ion exchange is a common mechanism of discharge for both slow and fast systems, though in fast systems only a fraction of the vesicle's content is discharged. R. Khanin, H. Parnas and L. Segel (*in preparation*) developed a complete mathematical model for discharge based on the idea of ion exchange. These authors showed that with sufficiently fast flow of ions into the vesicle, for instance through a fusion pore or vesicle membrane channels, an ion exchange can also account for a complete discharge of the vesicle's content in a brief time of about 0.1 msec, as required in fast synapses.

If we assume, then, that ion exchange underlies discharge in both slow and fast releasing systems, why is there a difference of two orders of magnitude in τ_D between the two? Recall that τ_D in fast synapses cannot exceed 0.1 msec, but lasts tens of milliseconds in slow systems.

Khanin et al. (1994) showed that for fusion pores of equal diameter, (and this is assumed to be the case for various exocytotic systems, *see* Monck & Fernandez, 1992; Zimmerberg et al., 1993), the duration of discharge will be directly proportional to the volume of the vesicle. Such a linear proportionality between the vesicle's volume and τ_D is expected also if ion exchange underlies discharge.

It is possible, therefore, that the difference in τ_D can be fully accounted for by variations in the diameter of the vesicles in the various systems, and variations in the diameter of vesicles have indeed been reported. For example, the mean diameter of synaptic vesicles in the frog neuromuscular junction is ~50 nm (Heuser & Reese, 1973) while the mean diameter of chromaffin granules for bovine adrenal medullary cells is 160 nm (Jankowski et al., 1992), and the mean diameter of the giant granule of the beige mast cell is 2.5 μm (Alvarez de Toledo, Fernandez-Chacon & Fernandez, 1993). The diameter of other vesicles and granules lie between the last two extreme values. If the mechanism of discharge in all these systems were identical, we would expect, based on the rule shown by Khanin et al. (1994), τ_D in chromaffin cells to be about 30 times longer than in neuromuscular junctions. But the observed τ_D in chromaffin cells is hundreds of times longer than that in neuromuscular junctions (*see above*). This discrepancy suggests that even if in both systems discharge is governed by ion exchange, nontrivial differences in the execution of the ion exchange in the two systems must exist.

Such differences could emerge if the content of the vesicle is stored differently in large and small vesicles. Indeed, synaptic vesicles contain all their neurotransmitter in an essentially dissolved fluid phase (Stadler & Fuldner, 1980). By contrast, in large dense-core secretory granules, a large fraction of the secretory compounds is bound to the protein complex (Trifaro & Poisner, 1982).

To appreciate the effect of the bound compounds on τ_D, we note a recent report showing that the main portion of discharge in slow systems is always preceded by a very small initial discharge (Chow et al., 1992; Alvarez de Toledo et al., 1993). This initial discharge lasts 8 msec in chromaffin granules (Chow et al., 1992) and up to hundreds of milliseconds in beige mouse mast cells (Alvarez de Toledo et al., 1993). The duration of the initial discharge indicates that a long interval is required for unbinding of secretory products that are to be eventually discharged.

R. Khanin, H. Parnas and L. Segel (*in preparation*) formulated a unified ion exchange theory that describes discharge by means of electrodiffusion of the vesicular content in fast and slow systems alike. These authors further showed that if the neurotransmitter is stored in an unbound state, the discharge is fast, as fast as 0.1 msec. If, by contrast, the transmitter is bound, then discharge may last tens of milliseconds.

Possible Differences in the Triggering Mechanism between Fast and Slow Systems

It is more difficult to reach decisive conclusions regarding possible differences in the triggering mechanism in fast and slow systems. This is because the reported time constants depend strongly on the experimental procedure used. In one example cited above, for the same type chromaffin cells, the triggering time ranged from 10–15 msec (Chow et al., 1992) to tens of seconds (Jankowski et al., 1992). The two procedures differed in the type of stimulus used: depolarization by Chow et al. (1992) and permeabilization with digitonin by Jankowski et al. (1992). The type of stimulus seems to influence the time constant of release in general and the triggering step in particular. This conclusion is substantiated by additional findings. For example, Okano et al. (1993) showed that the presence of ATP significantly affected the duration of release in melanotrops. Some inherent differences in the triggering mechanism might therefore exist between slow and fast systems, but their exact nature is still not clear. Whatever such mechanisms might be, the differences in their intrinsic time constants must be much smaller than those exhibited by the experimental measurements of the minimal delay.

Below, we discuss what the mechanism might be for triggering the release machinery in fast releasing systems.

Relationship between τ_S and τ_T

So far, we have seen that the steps that differ between fast and slow releasing systems are probably discharge

Table Relationship between τ_S and τ_T in fast and slow releasing systems

System	τ_S	τ_T	τ_T/τ_S	References
Fast				
Neuromuscular junction, lobster				
19°C	0.6 msec	3 msec	5	Parnas et al., 1989
9°C	2 msec	8 msec	4	
Neuromuscular junction, frog				
10°C	1.3 msec	5 msec	4	Dudel, 1984*a,b*
0°C	2 msec	10 msec	5	
Neuromuscular junction, mouse				
15°C	1.6 msec	5 msec	3	Datyner & Gage, 1980
Slow				
Bovine adrenal medullary cells	3 sec	5–10 min	100–200	Jankowski et al., 1992
	10–15 msec	3–5 sec	~300	Chow et al., 1992
Melanotrops	20 sec	5 min	15	Okano et al., 1993
Pancreatic β-cells (stimulus glucose)	~5 sec	3–10 min	~40–120	Kennedy et al., 1993
Pancreatic B-cells (stimulus K^+)	1 sec	10–60 sec	10–60	Kennedy et al., 1993

and triggering of the release machinery. It does not necessarily follow, however, that entirely different mechanisms underlie these steps in the two systems. Rather, as shown above for discharge (R. Khanin, H. Parnas and L. Segel, *in preparation*), differences in the details of a basically common mechanism can result in very different time constants. As a result of such putative differences, the time constant of all the steps that together lead to exocytosis of a single vesicle (τ_S) is in the range of 0.5 msec in fast systems and is typically in the range of seconds (but note earlier reservations) in slow systems. We further indicated that in both systems, the time constant for a single vesicle (τ_S) is small in comparison to the duration of the entire release process (τ_T). To assess the magnitude of such differences and draw conclusions on yet another step in which the two systems differ, we provide the Table.

In the Table, we list examples of both fast and slow systems where both τ_S and τ_T could be either measured or safely inferred. We confine the selected examples, particularly for slow systems, to findings where the measured minimal delay was of the same order of magnitude as most other reported cases. This is to avoid considering cases where the measured minimal delay reflected not only τ_S but also conditions specific to a particular experiment.

The Table depicts significant differences between the two systems. While the typical ratio τ_T/τ_S in fast systems is about 5, in slow systems it ranges from 10 to 200.

In fast systems, both the minimal delay (τ_S) and the duration of release (τ_T) can be evaluated with a fair degree of precision from the measured synaptic delay histograms (Katz & Miledi, 1965). Thus, the measured ratio is presumably a true indicator of the intrinsic relationship between τ_S and τ_T. In slow systems by contrast, we recall that the measured minimal delay is strongly dependent upon experimental conditions and the type of stimulus used. Attempts to assess the true τ_S in slow systems must therefore rely on cases where the minimal delay was short; for example, *see* Chow et al. (1992) for chromaffin cells. The duration of release can be measured fairly accurately in slow systems as well. Consequently, given the numbers for τ_T (in the Table) and keeping in mind that, for most cases, the real τ_S is shorter than reported in the Table, the typical ratio of τ_T/τ_S in slow systems will be of several tens or even hundreds.

The results presented in the Table and the foregoing discussion imply that the single most dramatic difference between slow and fast releasing systems resides in the temporal distribution of the release process. In other words, release terminates very soon after it begins in fast systems. All the vesicles involved (sometimes hundreds following a single impulse) empty their contents almost simultaneously over a very brief time that does not much exceed the time needed for a single vesicle to undergo exocytosis. In slow systems by contrast, once started the process of release persists for a long period much exceeding the time needed for exocytosis of a single vesicle. We conclude that one major difference between fast and slow systems is in the mechanism of TERMINATION of release. It is this mechanism which accounts for the brief τ_T in fast synapses and the long τ_T in slow systems.

The natural question at this point is what could be the mechanism of termination in the two systems. The answer is not known, and a discussion of every possible candidate is beyond the scope of this review. We shall confine ourselves to fast synapses and, in these, to suggestions which have been evaluated, at least partially, in a quantitative way.

Common to most suggestions is the notion that it is Ca^{2+} (whether kinetics of Ca^{2+} channels and/or spatiotemporal distribution of Ca^{2+} near the release sites)

which is responsible for both triggering and terminating release in fast synapses. Since Ca^{2+} is also assumed to be the trigger, and in fact the only needed factor for release, in many slow systems (in the chromaffin cell, Burgoyne, 1991; Neher & Zucker, 1993; in pancreatic cells, Maruyama et al., 1993), it is difficult to see how τ_T can differ so much in the two systems. In spite of this difficulty, which seems unsolvable, we shall outline suggested answers to the question of termination release in fast synapses.

Ca^{2+} Channels in Fast and Slow Systems

It is commonly believed that the kinetics of the Ca^{2+} channels involved in release determines the kinetics of the release itself. That is to say that release begins as a result of the opening of Ca^{2+} channels and the consequent influx of Ca^{2+} (Fogelson & Zucker, 1985; Simon & Llinas, 1985; Augustine, Charlton & Smith, 1987).

Since Ca^{2+} is considered both necessary and sufficient to evoke release in both fast and many slow systems (*see* above references for fast and slow systems), differences in triggering or termination of release (or both) could be explained if the Ca^{2+} channels involved would differ in their kinetics in the two systems. This, however, is not the case. The same type of Ca^{2+} channels was shown to be involved or implicated in release in both fast and slow systems. Both N-type and P-type channels were shown to be involved in release from nerve terminals (Nowycky, Fox & Tsien, 1985) and in chromaffin cells, a Ca^{2+}-requiring, slow system (Fenwick, Marty & Neher, 1982; Artajelo, Adams & Fox, 1994).

We must conclude that the very different τ_T in the two systems is not caused by variations in the Ca^{2+} channels involved. Moreover, in view of the identity in Ca^{2+} channels in the two systems it is also unlikely that the differences in the time constants of triggering (τ_G), can be attributed to differences in Ca^{2+} accumulations near the release sites. The basis for the last conclusion can be explained as follows. In fast synapses, a release site is believed to be situated at a distance of 50 nm from a Ca^{2+} channel (*see,* for example, Fogelson & Zucker, 1985). Simple calculations show that roughly 1 μsec is needed for Ca^{2+} to diffuse across this distance. Diffusion of Ca^{2+} is therefore not the rate limiting step even in fast synapses (*see also* Parnas et al., 1989). In slow systems, it is often suggested that the structure of the release zone is not as precise as in fast synapses. In particular, a much larger distance could exist between the Ca^{2+} channel and the release site (*see,* for example, Verhage et al., 1991). Following the same calculations, we find that in order for diffusion of Ca^{2+} to account for the time constant of triggering in slow systems (tens of milliseconds or even seconds, *see above*), the distance between the Ca^{2+} channel and the release site must be of the order of several micrometers rather than the nanometers suggested for fast systems. From a structural point of view, this cannot be the case. Furthermore, the expected Ca^{2+} concentration at such distant sites will hardly exceed the resting level (*see* Aharon, Parnas & Parnas, 1994). One must therefore conclude that also in the Ca^{2+}-dependent slow systems a reasonable proximity between the Ca^{2+} channel and the release site must exist.

In conclusion, the identity of the Ca^{2+} channels in the two systems, together with the calculations mentioned above, suggest that even in fast systems, it is not the closing of Ca^{2+} channels that is responsible for termination of release. Moreover, it seems that the rapid triggering of release in fast systems might not be governed by the opening of channels and influx of Ca^{2+}.

Such a conclusion is strengthened by the finding that evoked release could be obtained in the neuromuscular junction of crayfish in the absence of Ca^{2+} influx, where intracellular Ca^{2+} was provided by light-dependent release of Ca^{2+} from intracellularly injected caged-Ca^{2+} compound (nitr-5, Hochner, Parnas & Parnas, 1989).

Ca^{2+} Domains

As already implied, the most common hypothesis for describing neurotransmitter release in fast synapses is the "Ca hypothesis," according to which Ca^{2+} is both necessary and sufficient to evoke release of neurotransmitter. According to this hypothesis, release starts upon arrival of the action potential at the nerve terminal due to the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, and release stops due to rapid removal of Ca^{2+} from the vicinity of the release sites. We have seen that triggering and termination of release in fast systems are not likely to be controlled by the kinetics of Ca^{2+} channels. Is it yet possible that Ca^{2+} controls these mechanisms in some other way?

Numerous experimental results cast doubt on the validity of the Ca hypothesis. These were discussed in a previous review (Parnas, Parnas & Segel, 1990). Here we wish to mention but one group of findings. An unavoidable prediction of the Ca hypothesis, resulting from its basic assumption of Ca^{2+} being both necessary and sufficient to trigger release, is that the time course of release will vary significantly with experimental conditions that alter the amount of Ca^{2+} that enters, or with the kinetics of the ion's intracellular spatio-temporal distribution.

Experimental results, in contrast, show that the time course of evoked release is completely insensitive to experimental manipulations that alter the intracellular Ca^{2+} concentration (level and temporal distribution). For example, procedures such as changing extracellular Ca^{2+} (Andreu & Barrett, 1980; Datyner & Gage, 1980); injec-

tion of a fast buffer, nitr-5 (Hochner et al., 1991); and application of a Ca^{2+} ionophore (Parnas & Parnas, 1989), all failed to affect the time course of neurotransmitter release, but strongly affected the amount of transmitter released (i.e., the quantal content).

In view of this discrepancy, in an attempt to keep the Ca hypothesis viable, the supporters of the Ca hypothesis had to invoke the complex notion of Ca^{2+} domains. According to this modification of the Ca hypothesis, Ca^{2+} builds up to very high concentrations for a very brief time in the vicinity of the Ca^{2+} channel and the nearby release site. It is this high concentration of Ca^{2+} which is needed to trigger release, and release terminates shortly thereafter due to an abrupt decline in the local Ca^{2+} concentration. At present, it is impossible to verify this assertion experimentally. However, testing whether inclusion of Ca^{2+} domains can cure the erroneous behavior of the Ca hypothesis is possible by studying the existing mathematical models of the Ca hypothesis including Ca^{2+} domains (Fogelson & Zucker, 1985; Simon & Llinas, 1985; Yamada & Zucker, 1992). It is noteworthy that in the Yamada-Zucker version of the Ca hypothesis, termination of release is already attributed to an inactivation of the release machinery and not to the fast decline in Ca^{2+} concentration at the Ca^{2+} domains. By including inactivation in addition to Ca^{2+} domains, these authors acknowledge one of the major difficulties in the Ca hypothesis.

The question, then, is whether Ca^{2+} domains, even if they do exist, can account for the tremendous difference in τ_T in the two systems and for the finding that the time course of release is not affected by variations in the concentration of Ca^{2+}.

A priori, it is very difficult to see how Ca^{2+} entering from similar types of channels (*see above*) into fairly similar geometrical configurations (i.e., nerve terminals or slow releasing systems) can distribute in such different ways as to result in τ_T of a few milliseconds in fast synapses and seconds to minutes in slow systems. In spite of this obvious problem, we shall briefly consider whether including Ca^{2+} domains in the Ca hypothesis achieves the desired purpose of predicting a time course of release independent of the spatio-temporal distribution of intracellular Ca^{2+}. In this connection, Parnas et al. (1989) and Aharon et al. (1994) showed that even with inclusion of Ca^{2+} domains, the extended Ca hypothesis retains its basic problem: the predicted time course of release is very sensitive to changes in intracellular Ca^{2+} concentration.

We must conclude that the spatio-temporal distributions of intracellular Ca^{2+} in general and formation of Ca^{2+} domains in particular cannot account for termination of release, certainly not in fast synapses.

Two additional factors related to Ca^{2+} domains must be considered. It has often been suggested by advocates of the Ca^{2+} domain hypothesis that the high Ca^{2+} concentrations at the release sites are achieved by an overlap of Ca^{2+} entering from many channels (Simon & Llinas, 1985; Smith & Augustine, 1988). If the required high concentration can indeed be obtained only by influx through many channels, one would expect that opening of only one channel would not be sufficient to evoke release. However, in very sophisticated experiments, Stanley (1993) showed that Ca^{2+} entering through a single open channel evokes release in an adjacent site. Thus, the concentration obtained by influx through a single Ca^{2+} channel is sufficient to sustain evoked release.

The other aspect deserving discussion concerns the structure of the release zone. It is well known that the release zone exhibits a very specialized structure. The question is, to what extent does the highly complex structure contribute to the fast release in fast synapses. The answer is that it is irrelevant. As shown above, in order for Ca^{2+} to accumulate near the release site, a close proximity between the Ca^{2+} channel and the release site must exist. This proximity, however, does not guarantee **high** concentrations. It only ensures some level of Ca^{2+} at the release sites. Aharon et al. (1994) showed that the concentrations of Ca^{2+} at a release site situated 50 nm away from a Ca^{2+} channel reaches only a few micromolars. This concentration is much lower than assumed to be necessary by the proponents of the Ca^{2+} domain hypothesis (*see* references above). It should be emphasized, however, that even these relatively low concentrations of Ca^{2+} would not be obtained had the Ca^{2+} channel and the release site not been in close proximity to each other. The Ca^{2+} channel is the source for Ca^{2+}; below the channel mouth the concentration after a brief depolarizing pulse may reach very high concentrations in the millimolar range. It is this high concentration below the channel mouth which guarantees the much lower—but sufficient for release—Ca^{2+} concentration (few micromolars) at the relevant sites.

Of special importance in this regard is the recent imaginative work of Dan and Poo (1992). These authors formed an artificial system that released acetylcholine. They loaded myocytes with acetylcholine and obtained evoked release upon membrane depolarization. The time resolution of the measurements in the myocytes could not detect changes in the millisecond range. Nevertheless, several important and revealing differences exist between the time course of evoked release in the artificial system of myocytes and the time course of evoked release in conventional fast synapses.

In the myocytes, evoked release occurred shortly after a brief depolarizing pulse (+80 mV, 5 msec, 1 Hz). The minimal delay was at most a few milliseconds, and the rate of rise of release decreased as extracellular Ca^{2+} increased. It will be recalled that in regular fast synapses the minimal delay is independent of extracellular Ca^{2+} concentrations and that the rate of rise of release is not

altered when Ca^{2+} concentration is varied (Andreu & Barrett, 1980; Datyner & Gage, 1980).

Another important difference concerns the duration of release. In the myocytes, evoked release lasted about 100–150 msec while in fast synapses, it lasts only a few milliseconds (*see* the Table). Moreover, the duration of release in the myocytes was somewhat longer at a higher extracellular Ca^{2+} concentration. In fast synapses, as will be recalled, the duration of release is independent of the extracellular Ca^{2+} concentration (Andreu & Barrett, 1980; Datyner & Gage, 1980). Finally, in the myocytes the time of peak release is dependent as well on extracellular Ca^{2+}. The time of peak release became shorter as extracellular Ca^{2+} increased. In contrast, in fast synapses the time of peak release is independent of extracellular Ca^{2+} concentration (*see* above references).

Interesting and suggestive is the observation that the duration of evoked release in the myocytes exhibits a time constant similar to that of TWIN-PULSE FACILITATION in fast synapses (Rahamimoff, 1968; H. Parnas, Dudel & I. Parnas, 1982; I. Parnas, Dudel & H. Parnas, 1982). Facilitation, as is well documented, is governed by residual Ca^{2+} (Katz & Miledi, 1968) and as such reflects the time course of removal of the Ca^{2+} that has entered. In fast synapses, facilitation lasts orders of magnitude longer than the duration of evoked release.

Taken together, the results in the myocytes suggest that here, but not in fast synapses, evoked release indeed follows the Ca hypothesis. That is to say that it is controlled by the kinetics of intracellular Ca^{2+}. In particular, release is probably triggered in the myocytes by the influx of Ca^{2+} and its accumulation, and release terminates as a result of removal of Ca^{2+}. Indeed, in findings identical to the experimental results of Dan and Poo, Parnas et al. (1989) showed that if evoked release is governed by Ca^{2+}, as assumed by the Ca hypothesis, release will begin after a shorter minimal delay, will reach its peak sooner, and will last longer as the extracellular concentration of Ca^{2+} increases. It should be noted that in this work of Parnas et al. (1989), the Ca hypothesis was modeled following Fogelson and Zucker (1985) with Ca^{2+} domains included.

The experimental results summarized so far clearly indicate that the time course of evoked release in fast terminals is not governed by Ca^{2+}. In particular, evoked release does not seem to terminate as a result of the closing of Ca^{2+} channels, or the removal of Ca^{2+} from the vicinity of the release sites, be it by diffusion or any other mechanism. Some of the experimental results discussed earlier (use of caged-Ca^{2+} and a Ca^{2+}-ionophore) further suggest that triggering of evoked release is not directly linked to Ca^{2+} influx. Ca^{2+} is necessary but insufficient to evoke release of neurotransmitter.

What, then, controls the time course of evoked release in fast synapses? A possible answer to this is discussed in the next section.

The Ca-Voltage Hypothesis for Release of Neurotransmitter in Fast Synapses

The shortcomings of the classical and extended Ca hypotheses led to the development of the Ca-voltage hypothesis for neurotransmitter release. According to this hypothesis, depolarization—the natural stimulus for release in nerve terminals—induces at least two processes. It opens voltage-dependent Ca^{2+} channels, through which Ca^{2+} flows in. Concurrently and independently, depolarization also activates the release machinery, readying it for release (more details concerning the Ca-voltage hypothesis are provided below). It is the depolarization-induced activation of the release machinery which triggers release and release is terminated when the release machinery is deactivated upon membrane repolarization (Dudel, Parnas & Parnas, 1983; H. Parnas, et al., 1986*a;* I. Parnas et al., 1986; Lustig et al., 1989; 1990; and *see review* Parnas et al., 1990). The role of Ca^{2+} in the framework of the Ca-voltage hypothesis is discussed below.

Several factors prompted development of the Ca-voltage hypothesis. Most notable among these are: (i) The time course of evoked release was found to be independent of the level and kinetics of intracellular Ca^{2+} concentration (*see* above references). By contrast, the Ca hypothesis, in any of its versions, unavoidably predicts that the time course of release must faithfully reflect the temporal distribution of intracellular Ca^{2+} near the release sites (Parnas et al., 1989; Aharon et al., 1994). (ii) Furthermore, it was shown that evoked release could be obtained in the absence of influx of Ca^{2+} provided that the terminal was depolarized and intracellular Ca^{2+} was raised by other means such as photolysis of caged-Ca^{2+} (Hochner et al., 1989). Also, when intracellular Ca^{2+} was raised by means of a Ca^{2+} ionophore, evoked release commenced only upon membrane depolarization (Parnas & Parnas, 1989). The time course of release in the presence of the Ca^{2+} ionophore and following photolysis of caged-Ca^{2+} coincided precisely with that obtained when Ca^{2+} entered only via the voltage-dependent Ca^{2+} channels. (iii) The duration of facilitation and evoked release differ by orders of magnitude. Evoked release lasts but a few milliseconds (*see* above references), while facilitation in the same preparations lasts tens or hundreds of milliseconds (Rahamimoff, 1968; H. Parnas et al., 1982; I. Parnas et al., 1982). Twin pulse facilitation was attributed to residual Ca^{2+} (Katz & Miledi, 1968). It was shown that the duration of facilitation was prolonged as extracellular Ca^{2+} increased (Rahamimoff, 1968; H. Parnas et al., 1982). Furthermore, the duration of facilitation was prolonged following inhibition of the $Na^+ \rightleftarrows Ca^{2+}$ exchange (by reducing the extracellular Na^+ concentration, I. Parnas et al., 1982). In contrast, the duration of facilitation was shortened following injection of a fast Ca^{2+} buffer (Hochner et al., 1991). The experimen-

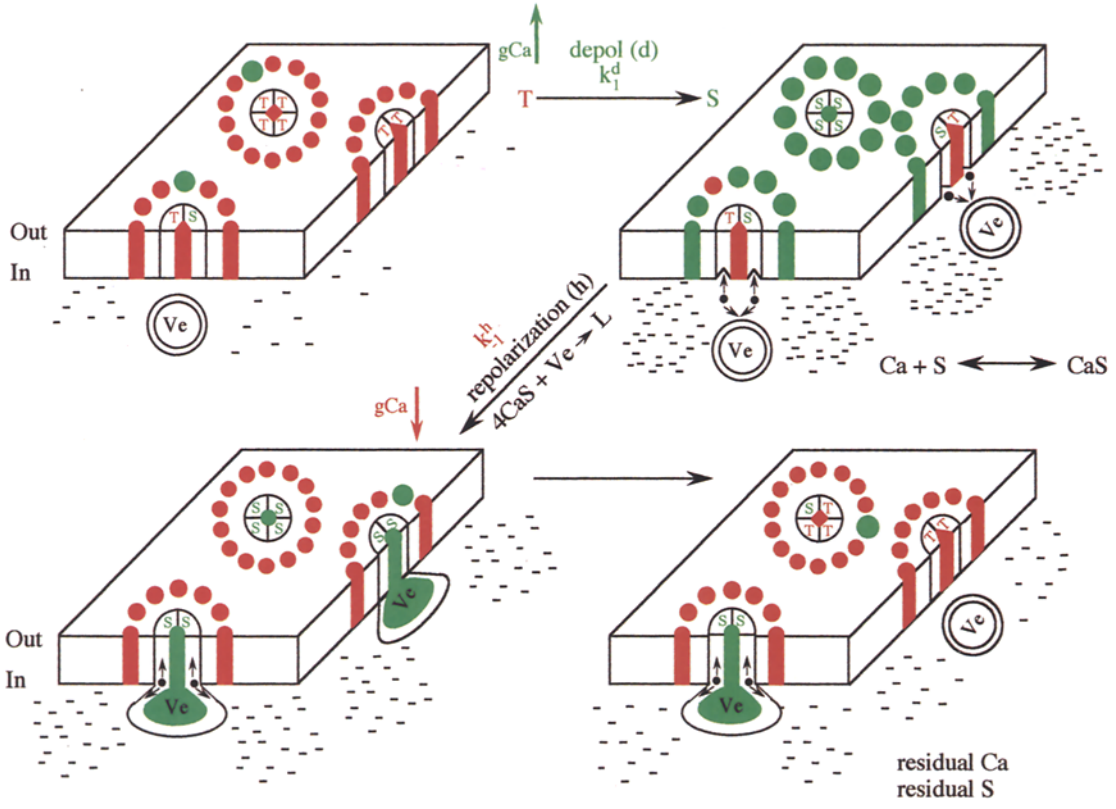

Fig. 3. Schematic presentation of the Ca-voltage hypothesis. (Upper left) Closed Ca^{2+} channels (red spots) and the release machinery in its inactive form (*T*). Note that even at resting membrane potentials, there exist few *S* and few open channels. (Upper right) Following depolarization, *T* transforms to *S* and Ca^{2+} channels open (green spots). *S* forms a complex together with Ca^{2+}, *CaS,* and four such complexes enable the fusion of one vesicle (*Ve*) to execute release (*L*). The actual exocytosis is seen in the lower left. Note that when release actually begins (lower left), Ca^{2+} channels are already closed. (Lower right) Gradual return to resting conditions; residual Ca^{2+} and residual *S* are observed. The requirement of a cluster of four *CaS* to enable vesicle fusion was deduced by H. Parnas et al. (1986*b*) from the steep rise in the rate of release.

tal results cited above convincingly show that the time course of facilitation is indeed determined by the level of residual Ca^{2+} concentration. In view of this, the large difference between the duration of facilitation and that of evoked release indicates that evoked release must be controlled by a mechanism other than the kinetics of intracellular Ca^{2+} (Hovav, Parnas & Parnas, 1992).

This is precisely the case according to the Ca-voltage hypothesis. The time course of facilitation is controlled by the residual concentration of intracellular Ca^{2+}. The time course of release, in contrast, is determined by the time constants of the voltage-dependent activation and deactivation of the release machinery.

Figure 3 shows a schematic representation of the Ca-voltage hypothesis together with the corresponding mathematical formulations. *T* stands for the inactive release machinery, which we postulate to be a membrane protein or bound complex of proteins. Upon depolarization, *T* undergoes conformational change (activation) to become *S*. The activated *S* state interacts with Ca^{2+} and induces release. After the action potential when the membrane repolarizes, *S* deactivates back to *T* with a rate constant that increases as a function of membrane hyperpolarization. It is this $S \rightarrow T$ transition that terminates release regardless of the high Ca^{2+} concentration which persists near the release site as is evident from measurements using Ca^{2+} indicators (Connor, Kretz & Shapiro, 1986).

In summary, according to the Ca-voltage hypothesis, release is triggered by the transition $T \rightarrow S$ that occurs due to depolarization. Note that the Ca-voltage hypothesis requires that the $T \rightarrow S$ transition be slower than the opening of Ca^{2+} channels that also take place upon membrane depolarization. Release terminates due to the back reaction of $S \rightarrow T$ on membrane repolarization. Thus, Ca^{2+} is necessary but only as a cofactor, and as such, it does not control the time course of release in fast synapses but does control, together with *S,* the quantal content of evoked release.

The Ca-voltage hypothesis has gained further support from the following experiment. A brief post-pulse hyperpolarization, administered immediately or shortly

after a brief depolarizing pulse, was found to reduce quantal content, and more significantly, to shorten the duration of evoked release compared with release obtained by administration of the same depolarizing pulse alone (Dudel, 1984*b*; I. Parnas et al., 1986; Arechiga et al., 1990). Recalling that the time course of release showed complete independence of the kinetics of intracellular Ca^{2+}, these results were interpreted by the above authors as the brief post-pulse hyperpolarization that accelerated the hyperpolarization-dependent transition $S \rightarrow T$ and hence accelerated termination of release.

Possible Role of Ca^{2+} in Fast Synapses

If in fast synapses, Ca^{2+} does not trigger release and its removal does not cause termination of release, what could be the role of Ca^{2+}?

Under conditions prior to the stimulus, the distance between the vesicle and the release site is about 20–30 Å. This distance is determined by a balance between attraction forces (van der Waals) and repulsion forces (electrostatic and hydration) (Rand & Parsegian, 1984). This distance exists although the vesicles are in the vicinity of the release sites. Obviously, the vesicular and plasma membranes cannot fuse until they are brought into intimate contact. Hydration being the main force that determines the distance of 20–30 Å between the membranes, intimate contact is possible only if the repulsive forces of hydration are abolished.

Rand and Parsegian (1986) calculated the energy needed to overcome the repulsive hydration forces to be 100 erg/cm^2. This is too high an energy barrier to be overcome spontaneously. Some energy producing events(s) must therefore precede the step of fusion. Rand and Parsegian (1984) suggested that the needed energy could come from the binding of Ca^{2+} ions to the phospholipids of the membrane. These authors calculated the energy derived from the formation of a Ca-phospholipid complex to be 50 erg/cm^2. If part of this energy is utilized for the apposition step, then only a few Ca^{2+} ions are necessary to bring the two membranes to the intimate proximity needed for fusion.

We could therefore postulate that the influxed Ca^{2+} makes vesicle and plasma membrane fusion possible by forming the necessary Ca-phospholipid complex. This, however, cannot be the entire mechanism since pure lipidic fusion is too slow (seconds). For Ca^{2+} to exert the role suggested (overcoming repulsive forces of hydration), the involvement of proteins is needed to reduce the energy barrier. Such Ca^{2+} binding proteins may be situated in the plasma membrane, in the vesicular membrane, or in both.

There are several vesicular and plasma membrane proteins, extensively studied and amply reviewed (*see*, for example, Sudhof & Jahn, 1991), which have been shown to be involved in exocytosis. In the present review, however, we shall not discuss the possible involvement of these proteins in overcoming the repulsive forces of hydration.

What Could be the Voltage-dependent Mechanism That Controls Release in Fast Systems?

From the perspective of general mechanisms, it is very efficient for a process that includes a cascade of events to be controlled by the product of that process. Indeed, feedback inhibition is a common mechanism of control in various cascade-type biochemical and physiological processes. In such cases, the product feeds back to the **first** key step in the cascade of events. Also concerning release of neurotransmitter, effect of the released neurotransmitter on its secretion, usually to block release, was shown. For example, acetylcholine was shown to feed back on its own release via a presynaptic muscarinic receptor (Michaelson et al., 1979; Kloog, Galron & Sokolovsky, 1986). Glutamate was shown to do the same in glutamatergic systems (Lovinger et al., 1993; Wu & Dun, 1993).

These findings, however, are not sufficient to imply that the transmitter *controls* its own release. Indeed, the interpretation of the above authors has been that high concentrations of transmitter *modulate* release.

For the transmitter to control release it must—as in any other feedback regulation—be involved with regulating the *first* event in the cascade of events leading to release of neurotransmitter.

Recently, H. Parnas et al. (1994) reported that glutamate, when externally applied, exhibits a voltage-dependent effect on the release of glutamate at the crayfish neuromuscular junction. Four important findings were reported: (i) At low depolarizations of the nerve terminal, glutamate blocked release. The magnitude of the block decreased as depolarization rose, and at high depolarizations glutamate even enhanced release. NMDA (*N*-methyl-D-aspartate) mimicked the voltage-dependent inhibitory effect of glutamate but did not possess the facilitative effect seen at high depolarizations; (ii) Glutamate exerted its inhibition at concentrations as low as 5×10^{-7} M – 10^{-7} M (I. Parnas, H. Parnas and J. Dudel, *in preparation*). (iii) When the NMDA antagonist APV (DL-2-amino-5-phosphor-valerianic acid) was added alone, release was enhanced in comparison to control, at all membrane potentials (H. Parnas et al., 1994); (iv) The voltage-dependent transition from inhibition (low depolarization) to recovery from inhibition or even facilitation (high depolarization) was extremely fast. To complete the picture, we mention that at low

depolarization it took seconds to minutes for inhibition to evolve, and several or even tens of minutes for it to recover after wash of glutamate (I. Parnas, H. Parnas and J. Dudel, *in preparation*).

Together, the above results are consistent with the idea that, under normal physiological conditions, the release machinery is kept (at resting potentials) in a blocked state by the low concentrations of glutamate present in the synaptic cleft. Upon depolarization (when the action potential arrives at the terminal), inhibition of the release machinery is rapidly relieved. The readied release machinery, together with the entered Ca^{2+}, evokes release of neurotransmitter. Upon membrane repolarization (end of action potential), the blocked state of the release machinery is reinstated and release terminates in spite of the presumably rather high concentration of Ca^{2+} that still exists near the release sites.

It is still too early to know whether this is a correct interpretation of the above experimental findings and, even if it is, whether this could be a general mechanism in other fast systems. Support for such a presumption, however, can be gained from the following results. It was shown that as in glutamatergic systems, in an acetylcholine-releasing system (neuromuscular junction of the frog), acetyl-β-methylcholine chloride (a muscarinic agonist of acetylcholine) blocked release at low concentrations of K^+ in the bathing solution (i.e., low depolarization) but enhanced release at high K^+ concentration (i.e., high depolarization) (Sarengor-Yashuv, 1982). Though the interpretation given by the above author to these findings differs from ours, this does not diminish the supportive significance of these observations to the hypothesis presented above.

Another finding consistent with the idea of a voltage-dependent relationship between neurotransmitter and the corresponding receptor is the following. Cohen-Armon and Sokolovsky (1991) reported that the muscarinic receptor shows low affinity and high affinity binding to acetylcholine; the transition between the two states depends directly on membrane potential.

Taken together, the results reported may suggest that a mechanism similar to that described here for a glutamatergic system exists in acetylcholine-releasing systems as well. Accordingly, for a cholinergic system, a presynaptic muscarinic autoreceptor regulates release in a voltage-dependent manner; it blocks release at resting and low depolarizations and the block is relieved at high depolarizations, thus readying the release machinery for release.

Summary

As stated at the beginning of this review, the mechanism of neurotransmitter release is not yet known. Keeping this in mind, we shall, nevertheless, attempt to speculate and outline a possible scenario of events as it emerges from the foregoing discussion.

At resting membrane potentials, the release machinery is in a blocked state produced by the constant presence in the synaptic cleft of neurotransmitter at low concentrations. At resting potentials, Ca^{2+} channels are closed, but this is probably not associated with the presence of low levels of neurotransmitter. Upon arrival of the action potential at the nerve terminal, (as suggested by the Ca-voltage hypothesis) two things happen independently: The release machinery is relieved of its block, being activated and readied to **trigger** release. Concurrently, Ca^{2+} enters the presynaptic terminal, and together with specific Ca^{2+} binding proteins, it abolishes the hydration repulsive forces without which the intimate contact between the vesicle and the plasmatic release machinery is not possible.

The biophysical meaning of triggering release is at present not known. There are several suggestions, the one most consistent with the arguments of this review being the mechanism discussed and modeled by Nanavati et al. (1992; *see also review:* Monck & Fernandez, 1992). According to that hypothesis, an activated scaffold of proteins forms a dimple in the plasma membrane upon stimulation. This dimple, which exhibits high tension—perhaps together with Ca^{2+}—overcomes the repulsive forces of hydration, permitting the two membranes to "jump" into intimate contact. As a result, a single hemifused bilayer is formed. In this hemifused bilayer, a lipidic fusion pore opens.

In the context of the lipidic fusion pore hypothesis, the role of the depolarization-dependent triggering could be to start those manipulations in the plasmatic membrane that result in increased lateral bilayer tension and formation of the dimple. Ca^{2+} could then, in view of reduced repulsive forces and increased attractive forces, be responsible for the intimate docking of the vesicle at the release site. Under such conditions, hemifusion could take place with the final formation of the lipidic fusion pore. Finally, once the fusion pore opens, discharge of the vesicular content takes place immediately and lasts for up to 50–70 μsec. To be so fast, discharge must occur by a mechanism other than diffusion, possibly by ion-exchange (R. Khanin, H. Parnas and L. Segel, *in preparation*).

Based on experimental measurements of the time course of release and the discussion provided in this review, the time constants associated with the various steps in release of a single vesicle in fast synapses are as follows: Lustig et al. (1990) calculated the time constant of the voltage-dependent step (i.e., triggering) to be less than 0.1 msec. Also based on measurements of the time course of release, the same authors calculated the time constant of the Ca^{2+} involved steps to be equally brief, that is less than 0.1 msec. The above two steps precede

the rate limiting step, probably fusion, which has a time constant of 0.5–1 msec. Finally, the time constant of the last event, discharge, is also very brief—less than 0.1 msec. It goes without saying that the various time constants dependent on temperature and the above values correspond to temperatures around 18°C.

In conclusion, we offer a few philosophical comments. The main argument of this review is that, at least in fast synapses, release of neurotransmitter is governed by two factors, both of which are generated upon arrival of the natural stimulus—the action potential. Safety measures of this kind are very much to be expected in the operation of a system so vital as the release of neurotransmitter. It is expected that the system be protected from actively responding to random fluctuations in the Ca^{2+} concentration. Such fluctuations are probable, given that Ca^{2+} is involved in almost every physiological process. Dependence on two factors ensures neurotransmitter will be released only in response to the proper stimulus.

The involvement of two factors, both necessary, provides yet another advantage. It enables modulation of the amount of release for periods of time much exceeding the duration of a single evoked release. The duration of a single evoked release must be kept brief for efficient neuronal communication. Therefore, it is important that one factor decisively terminate release, despite persisting elevations of the other needed factor. The other factor, however, could provide "memory" to the systems. Memory which differs appreciably in its duration from the duration of release following a single pulse is impossible to obtain if only one factor is necessary and sufficient to induce release. In the case of neurotransmitter release, then, $T \rightleftarrows S$ transitions determine the duration of release after a single pulse. By contrast, Ca^{2+} is responsible for the memory of the presynaptic nerve terminals. The Ca^{2+}-dependent memory of the presynaptic nerve terminal lasts as long as seconds under moderate activity of the presynaptic terminal (facilitation) and can last minutes under more intensive activity (post-tetanic potentiation).

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