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The 'Ca-voltage' hypothesis for neurotransmitter release

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The 'Ca-voltage' hypothesis for neurotransmitter release was reinvestigated by studying the kinetics of neurotransmitter release. These were independent of changes in intracellular or extracellular Ca^{2+} concentration. It is concluded that initiation and termination of release do not result from rapid entry and removal of Ca^{2+} although Ca^{2+} is essential for release. Quantal release of transmitter requires depolarization-dependent transformation of a membrane molecule from an inactive form T to a Ca^{2+}-binding form S. The depolarization-dependent $T \rightarrow S$ transformation initiates release in the presence of Ca^{2+}. The $S \rightarrow T$ transformation upon repolarization stops release even though the Ca^{2+} concentration at release sites is still high.

1. Introduction

Recent experiments at a variety of synapses suggest that the relation between Ca entry and transmitter release may be more complex than had previously been thought. Instead of increases in intracellular Ca close to the release sites being the only important variable, evidence is accumulating that a second mechanism, which depends on membrane potential, plays an important part. Thus, according to the 'Ca-voltage hypothesis' (fig. 1) for neurotransmitter release [1–4] two events must occur concomitantly. (i) Internal Ca^{2+} concentration must be elevated and (ii) the release machinery must become sensitive to Ca^{2+}. Normally these two events are produced by membrane depolarization. When the membrane is depolarized, Ca^{2+} channels open and Ca^{2+} flows into the nerve terminal. In parallel, a molecule or a complex in the membrane (T in fig. 1) undergoes a conformational change (S in fig. 1) and only then can it bind Ca^{2+} and start the chain of events leading to release. When the membrane is repolarized, the active molecules return to the inactive state, T, which cannot bind Ca^{2+} and release stops, even though the Ca^{2+} concentration may be higher than normal at the release sites.

The original 'Ca-hypothesis' [5] differs from this scheme in considering only the first of the two events. Namely, Ca^{2+} is not only necessary but sufficient on its own to evoke synchronized transmitter release. In the Ca-voltage hypothesis there are two limiting factors, Ca^{2+} and the S molecule (fig. 1). A clear illustration of the difference between both schemes is the explanation for termination of release after an action potential. According to the Ca hypothesis transmitter release stops because of rapid (within 2 ms) removal of Ca^{2+} from near the release sites. According to the Ca-voltage hypothesis release stops because of the conformational change (which can be very rapid) of the S molecule back to the T state upon repolarization.

In this article we analyse the steps that led us to consider membrane potential as an important factor in the process of release and compare experimental predictions of the two hypotheses.

Three groups of experiments indicated that

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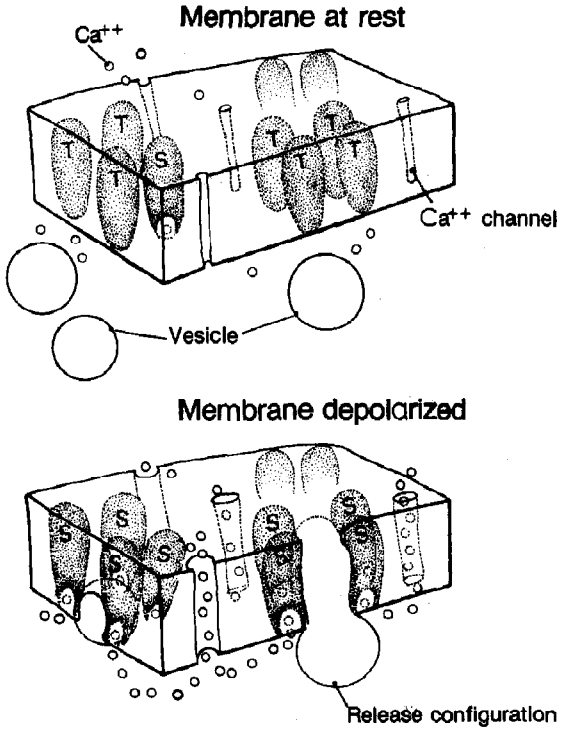

Fig. 1. Schematic representation of the Ca-voltage hypothesis. (Top) Membrane at rest. Ca^{2+} channels are closed and most of the particles are at the T state. T cannot bind Ca^{2+}. (Bottom) Upon depolarization Ca^{2+} channels open and Ca^{2+} flows in. In parallel the T particles change their conformation to become S which can bind Ca^{2+}. Four CaS complexes are required for the release of one quantum.

membrane depolarization plays a role in the release process in addition to its effect on membrane Ca^{2+} conductance.

(1) it was shown that release as a function of membrane depolarization continued to rise at strong depolarizations even though the Ca^{2+} entry was reduced because of the smaller driving force. Only at very large depolarization where entry of Ca^{2+} was totally suppressed was there no release. This result suggested that higher levels of the S molecule at the stronger depolarizations compensated for reduced entry of Ca^{2+} [1,4].

(2) Release was shown to stop abruptly after tetanic stimulation during which strong facilitation has occurred. At the same time Ca^{2+} remained in the terminal and a great deal of synchronized release could be produced by membrane depolarization. This depolarization caused little Ca^{2+} entry into the terminal [1,4].

(3) Release could be modulated by membrane hyperpolarization. In these experiments a hyperpolarizing pulse was given before or after a depolarizing test pulse. In both cases release was reduced by the hyperpolarization. The hyperpolarizing pre- or post-pulses were effective in reducing the release of the test pulse if applied up to 4 ms before or after the test pulse [4].

Together, these three groups of experiments showed that the quantal content of release depends on another factor in addition to Ca^{2+}, which is membrane potential dependent. This factor was produced by depolarization and disappeared on hyperpolarization. How can this factor be better characterized? How do its appearance and disappearance ($S \rightleftharpoons T$, fig. 1) influence the beginning and end of release? Since this putative factor cannot be directly identified we have assessed its role by measuring the kinetics of release under various experimental conditons.

2. Kinetics of release

Katz and Miledi [6] showed that under conditions of low quantal content and low temperature, depolarization of the terminal results in many failures. In addition, single quanta are released at different delays after the stimulus (fig. 2, inset) and the origin of this delay is presynaptic. Thus, a synaptic delay histogram (fig. 2) allows one to estimate the probability for a quantum to be released at any given time following the impulse. Histograms of the synaptic delays thus represent the kinetics of release. It can be seen that within a certain time after the impulse, the minimal delay, the probability for release is zero. Then, the probability for release increases sigmoidally to a peak which is reached a few milliseconds after the end of the pulse. Finally, the probability of release declines to very low values a few milliseconds later. Having a direct tool to measure the kinetics of release, we can address the first question: is the time course of release determined by that of increase and decrease in intracellular Ca^{2+} concentration after an impulse?

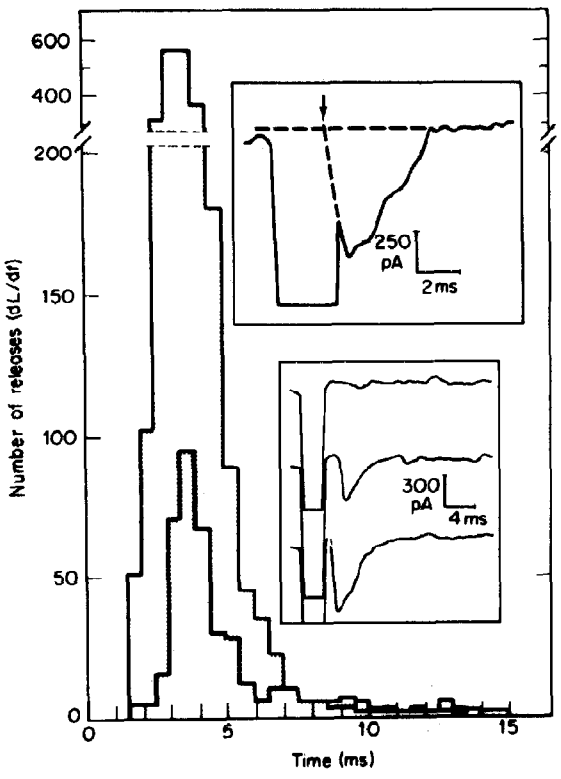

Fig. 2. Synaptic delay histograms obtained at low and high depolarizations. Lobster neuromuscular junction. (Inset) Samples of single-quantum recordings. Note variable delays (from ref. 16).

3. The time course of release is insensitive to changes in extracellular or intracellular Ca^{2+} concentration

The Ca-hypothesis, in its simplest form, can be represented by

$$4Ca + X \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} Ca^4X \xrightarrow{k_3} L \text{ [1,7]} \qquad (1)$$

From this relation the change in the rate of release dL/dt with respect to time can be evaluated. This is shown in fig. 3 (continuous line). In order to obtain a minimal delay and for the peak to appear after the end of the pulse, the reactions which are dominated by k_3 must be slower than those of k_2 (see values of k_2, k_{-2} and k_3 in legend to fig. 3). For the kinetics of release to terminate in about 10 ms, one must assume that the Ca^{2+} is removed rapidly. This is shown in fig. 3 (left, continuous line), where the Ca^{2+} concentration builds up and declines in about 4 ms at this low temperature. The corresponding dL/dt curve shows all the features of the experimental delay histogram. It has a minimal delay, the peak of the probability of release is at about 3.5 ms and release terminates at about 10 ms. It is clear that the simple Ca-hypothesis can account for the initiation and termination of release after a single impulse. However, a more critical test is the effect of increasing

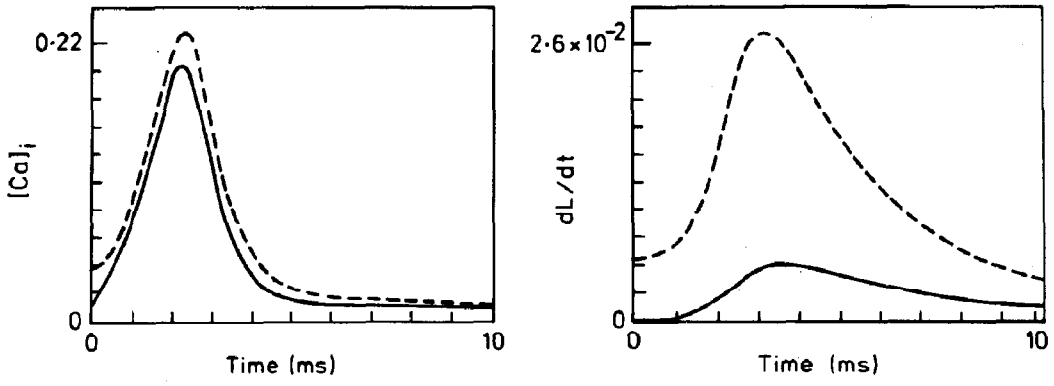

Fig. 3. Ca^{2+} concentration profiles for a test pulse (left) and corresponding delay histograms (right) for two levels of resting Ca^{2+} concentration; pulse duration, 2 ms. (———) Resting Ca^{2+} level 0.2 μM; (— — —) resting Ca^{2+} level 0.5 μM. Note the change in minimal delay; $k_2 = 1\ ms^{-1}\ \mu M^{-1}$, $k_{-2} = 0.1\ ms^{-1}$, $k_3 = 10\ ms^{-1}$ (adapted from ref. 16).

the intracellular Ca^{2+} concentration on the delay histogram produced by the same test pulse. This is demonstrated in fig. 3 (broken lines). In this case, the only modification is the initial value of the intracellular Ca^{2+} concentration, which is now 2.5-times greater than the control. The rest of the parameters were maintained as before including the rapid removal of Ca^{2+} from below the release sites. The profile of the change in Ca^{2+} concentration is almost unaltered. There is, however, a clear change in the corresponding delay histogram (fig. 3, right), as there is no minimal delay. The expected shortening of the delay is clear even though the slowest rate constant is still k_3. In other words, if intracellular Ca^{2+} concentration is higher, release is expected to start with a shorter delay. This can also be seen in fig. 4 for another set of experimental conditions. However, this prediction has never been substantiated experimentally. When the intracellular Ca^{2+} concentration was raised by repetitive simulation and large facilitation was obtained, the minimal delay was exactly the same as in the control. Application of a Ca^{2+} ionophore produced a large increase in quantal content without any effect on the minimal delay (Atwood, Parnas, Parnas and Wojtowicz, manuscript in preparation).

The Ca-hypothesis also predicts that the duration of release should depend on the amount of Ca^{2+} that enters during the depolarizing pulse. At higher extracellular Ca^{2+} concentrations more Ca^{2+} will enter during the impulse and it will take longer to lower the Ca^{2+} concentration to the level at which release stops [8]. Several experimental results show that this is not the case. Datyner and Gage [9] showed that the delay histogram is insensitive to changes in $[Ca^{2+}]_o$ even though the quantal content was greatly affected by these changes. In the presence of higher $[Mg^{2+}]_o$ which blocks Ca^{2+} entry the delay histogram was the same as in the low Mg^{2+} concentration [10]. In another set of experiments it was found that the kinetics of release of subsequent impulses in a train is the same despite the large facilitation [2,9,11].

From these results we conclude that the *time course of release is not determined by the time course of changes in intracellular Ca^{2+} after an impulse, in facilitation or during a train.*

It remains to be shown that a change in membrane potential does affect the kinetics of release. However, in order to do so, we must first determine where in the chain of events leading to release membrane potential exerts its effect.

4. What is the step at which the membrane potential acts?

Inspection of the Ca-hypothesis as given in eq. 1 shows that membrane depolarization could affect the release process by changing one or more of the rate constants. For example, membrane depolarization may increase k_2 or k_3 or decrease k_{-2}. In doing so, the depolarization-dependent factor would determine the kinetics of release independently of changes in $[Ca^{2+}]_i$.

Fig. 4 shows computer simulation of delay histograms, (dL/dt vs. time) as obtained from eq. 1 extended to include a dependence of k_2 (A) or k_3 (B) on membrane potential. During the depolarizing pulse the value of k_2 or k_3 is high and declines abruptly upon repolarization. The corresponding synaptic delay histograms show a common feature. The peak of the histogram is at the end of the depolarizing pulse. The same conclusion holds true if depolarization increases one or more of the other rate constants of reactions which take place after the entry of Ca^{2+}.

The experimental results do not support this prediction. On the contrary, synaptic delay histograms obtained at different depolarizations showed that the peak of the histogram was always significantly after the end of the pulse. We conclude that the effect of membrane depolarization does not affect a step, in the chain of events leading to release, which occurs after the entry of Ca^{2+}.

The remaining possibility is that membrane depolarization affects a stage in the release process which occurs in *parallel* with the entry of Ca^{2+}, as given in the following scheme:

$$\begin{array}{lcl} & [Ca^{2+}]_o & T \\ g(Ca) & \downarrow \quad k_1(d) & \downarrow\uparrow k_{-1}(h) \\ [Ca^{2+}]_i + & S & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CaS \xrightarrow{k_3} L \end{array} \qquad (2)$$

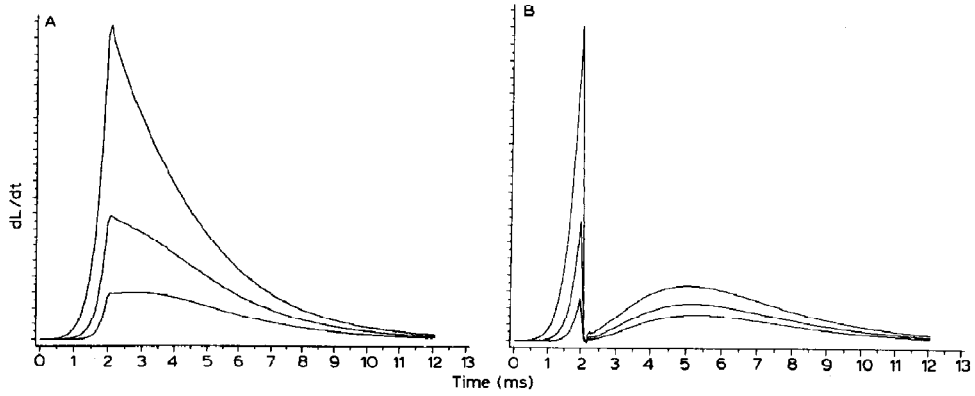

Fig. 4. Computer simulations of synaptic delay histograms. (A) k_2 increases with depolarization, (B) k_3 increases with depolarization. Pulse duration, 2 ms. k_2 at rest 0.1 μM^{-1} ms^{-1}. During the depolarizing pulse $k_2 = 1$ μM^{-1} ms^{-1}, k_3 at rest was 0.1 ms^{-1} and during the pulse 10 ms^{-1}. k_{-2} in both cases was 0.1 ms^{-1} and independent of membrane potential. The delay histogram simulations were performed for three levels of resting intracellular Ca^{2+} concentration: the lowest histogram at 0.01 μM, the middle line at 0.05 μM and the top line at 0.1 μM. Note shortening of the minimal delay as intracellular Ca^{2+} concentration is increased. The changes in Ca^{2+} concentration during and after the pulse were calculated as given in ref. 16.

Where, parallel to the increase in membrane Ca^{2+} conductance, $g(Ca)$, and the flow of Ca^{2+} into the terminal, a molecule T changes its conformation to become S with a rate constant $k_1(d)$ which increases with depolarization. S is transformed back to T with a rate constant, $k_{-1}(h)$, which increased with hyperpolarization.

Indeed, the solution to this scheme predicts the experimentally observed features of the synaptic delay histograms (see fig. 7).

5. Cooperativity in the process of release

The 'cooperativity' or 'apparent cooperativity' in the release process and its dependence on a variety of experimental conditions can also be used to test predictions of the Ca- and Ca-voltage hypotheses.

The quantal content of release was shown to have a sigmoidal dependence on extracellular Ca^{2+} concentration [7,12–14]. This sigmoidal relationship was interpreted to reflect a cooperative dependence of release on $[Ca^{2+}]_o$. The value of the cooperativity was estimated from the slope of log $m/\log[Ca^{2+}]_o$, where m is the quantal content. The cooperativity was found to be 4 [7]. It was shown, later, that with this method there is a possibility that the measured slope will underestimate the true cooperativity especially in cases where the change in Ca^{2+} concentration due to a depolarizing pulse is not large relative to the resting level of intercellular Ca^{2+} concentration [13–15]. In addition, this method of determining the cooperativity cannot distinguish between the possibility that four Ca^{2+} combine with one molecule of S or that four complexes of CaS are required for the release of one quantum.

In order to overcome these difficulties, we [16] have suggested a new way to determine the cooperativity of the release process, using the synaptic delay histogram. The slope of log number of quanta/log time at the beginning of the synaptic delay histogram estimates the cooperativity (fig. 5a–d). The cooperativity was found to be 4 (fig. 5 right and ref. 16). This applies to both the Ca-hypothesis given in eq. 1 and the Ca-voltage hypothesis model as given in eq. 2. Therefore, we now use the slope of $\log(dL/dt)/\log t$ and its behaviour under a variety of experimental conditions to distinguish between the Ca-hypothesis and the Ca-voltage hypothesis.

Fig. 6 shows the predictions for the Ca-hypothesis and the Ca-voltage hypothesis under three conditions. In the left panel we study the effect of pulse duration, in the middle panel the effect of

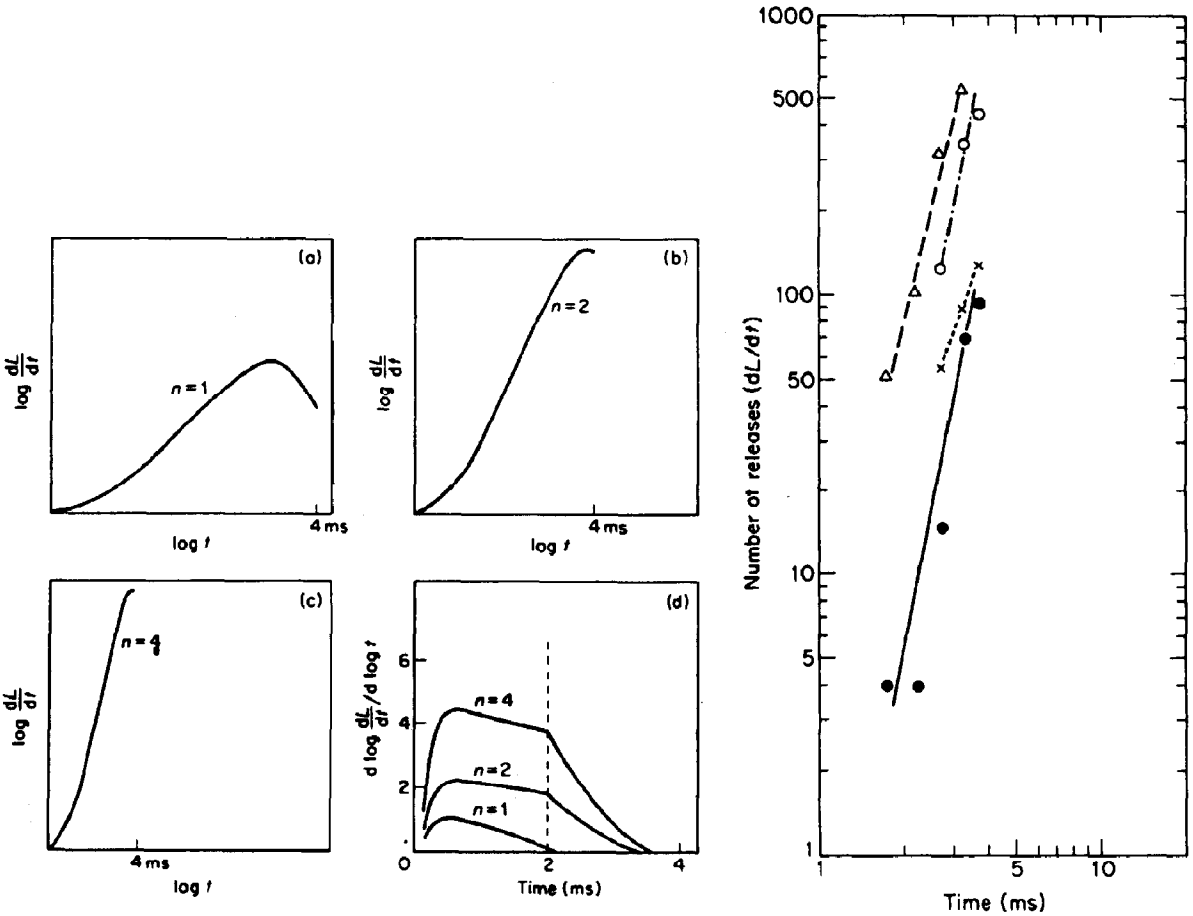

Fig. 5. (Left) Computer simulation of the initial part of a synaptic delay histogram when the cooperativity was assumed to be 1 in a, 2 in b and 4 in c. d shows the slopes for different times after the pulse. The maximal slope is 1, 2 and 4, respectively. (Right) Experimental results for the slope of $\log(dL/dt)/\log t$, obtained from the lobster neuromuscular junction; the value of the slope is 4. For more details see ref. 16.

pulse amplitude and in the right panel the effect of increasing the resting level of intracellular Ca^{2+} concentration. For these three conditions the Ca-hypothesis predicts that the maximal slope of $\log(dL/dt)/\log t$ should increase with longer pulses, increase with stronger depolarization and decrease when the resting $[Ca^{2+}]_i$ level is increased. The Ca-voltage hypothesis, on the other hand, predicts that the slope should be independent of pulse duration, decrease with increasing depolarization and be practically independent of the resting level of intracellular $[Ca^{2+}]_i$ concentration.

The experimental results contradict the predictions of the Ca-hypothesis and agree with those of the Ca-voltage hypothesis. The slope is independent of the pulse duration, [16]. The slope was unaffected by Ca^{2+} ionophore (Atwood, Wojtowicz, Parnas and Parnas, manuscript in preparation) or by repetitive stimulation [2] even though both these conditions increased the release to a test impulse. Finally, the slope became *smaller* and not larger at stronger depolarizations [16]. These sets of predictions and experimental results serve as support for the Ca-voltage hypothesis.

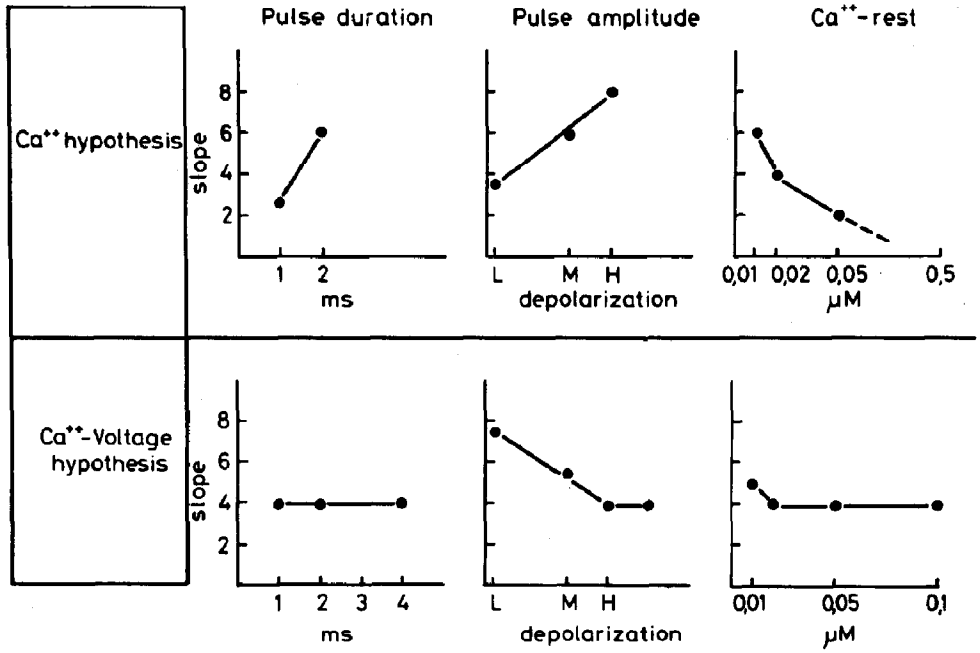

Fig. 6. Theoretical predictions for behaviour of the slope of synaptic delay histograms under different experimental conditions. (Top) Predictions for the Ca-hypothesis. (Bottom) Predictions for the Ca-voltage hypothesis.

6. Nature of the cooperativity

Two conclusions can be drawn with respect to the cooperativity. There is a cooperative dependence of m on the Ca^{2+} concentration [7,13,14] and there is a sigmoidal rise of the synaptic delay histogram, where the slope of $\log(dL/dt)/\log t$ is 4. The latter finding was interpreted as reflecting a cooperative dependence of release on the S molecule [16].

There are three possible ways in which Ca^{2+} and S can interact and show a cooperative dependence [16],

$$4S + Ca^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (S^4Ca) \xrightarrow{k_3} L \qquad (3a)$$

$$4Ca^{2+} + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (Ca^4S) \xrightarrow{k_3} L \qquad (3b)$$

$$Ca^{2+} + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (CaS) \qquad (3c)$$

$$4(CaS) \xrightarrow{k_3} L$$

In eq. 3a four S molecules bind one Ca^{2+}. This configuration will show the proper cooperativity for the delay histogram but not a cooperative dependence on extracellular Ca^{2+} concentration. It is therefore rejected.

The case where four Ca^{2+} bind to one S molecule will show a cooperative dependence with respect to extracellular Ca^{2+} concentration but a linear dependence in time for the synaptic delay histogram. Therefore, this possibility is also rejected.

Only the configuration given in eq. 3c, namely, one Ca^{2+} binds one S, and four complexes of (CaS) are involved in the release of one quantum, will show the correct cooperative dependence, both on $[Ca^{2+}]_o$ and on time in the synaptic delay histogram. The experimental results are thereby compatible with the predictions of the relation given in eq. 3c [16].

This analysis shows that measurements of a synaptic delay histogram (in addition to quantal content) elucidate not only the intrinsic cooperativity (and its value = 4) in the process of release

but also the step at which cooperative action takes place.

7. Simulation of release by the Ca-voltage hypothesis

Fig. 7 shows a simulation of the Ca-voltage hypothesis. The upper panel shows the change in S at three levels of depolarization. The middle panel, the change in $[Ca^{2+}]_i$ and the lower panel the expected synaptic delay histograms. There is a reasonable agreement between the predicted and observed minimal delay, time of peak release and termination of release. It should be noted that the decline in $[Ca^{2+}]_i$ is slow and termination of release is because of the S → T transformation. This slow decline in $[Ca^{2+}]_i$ is responsible for residual Ca^{2+} and therefore for facilitation. The dilemma of explaining termination of release with the same kinetics after a single impulse and after many impulses, even at high frequency, no longer exists.

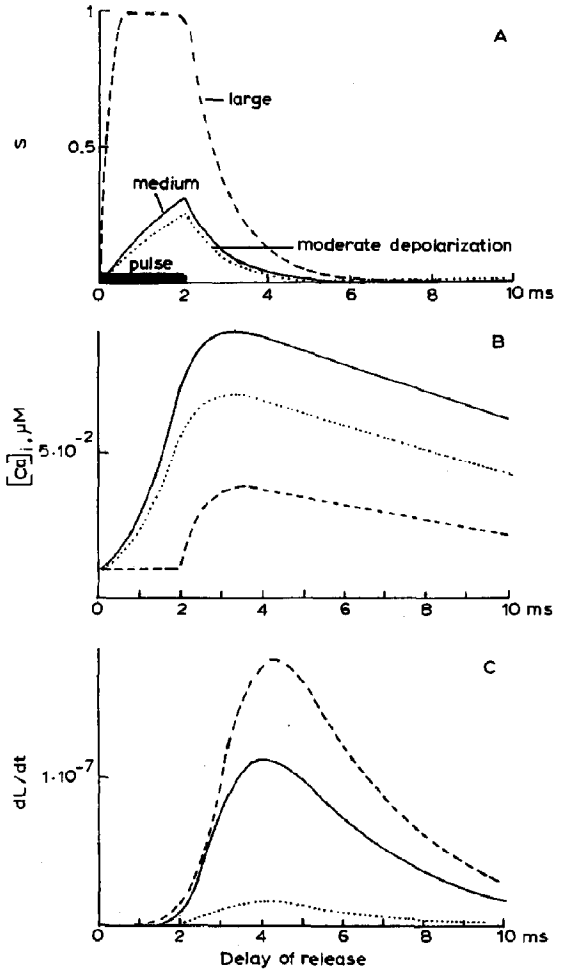

Fig. 7. Computer simulation of the Ca-voltage hypothesis. (A) Change of S in time for three levels of depolarization (B) Changes in intracellular Ca^{2+} concentration for the same three depolarizations. Note that Ca^{2+} removal is assumed to be relatively slow. (C) Synaptic delay histograms. Note that release stops even though intracellular Ca^{2+} concentration is still high. For further details see ref. 2.

8. Experimental manipulation of the synaptic delay histogram

We have demonstrated that the synaptic delay histogram is insensitive to changes in Ca^{2+} available for release, and concluded that the S⇌T transformation determines the kinetics of release. As the termination of release is mainly produced by the S → T transformation, it should be possible to accelerate the termination of release if a hyperpolarizing pulse is given immediately (or with a short delay) after a depolarizing test pulse.

Fig. 8 (upper panel) shows computer simulations of synaptic delay histograms of a test pulse alone and of the same test pulse followed by a hyperpolarizing post-pulse. The peak is shifted to an earlier time and the decline of the histogram is faster. Fig. 8 (lower panel) shows that the experimental results are consistent with this prediction. These results confirm that the kinetics of release is controlled more by the factor S which is sensitive to changes in membrane potential than by rapid changes in intracellular Ca^{2+} concentration.

In summary, we have used measurements of synaptic delay histograms to demonstrate that release is controlled in addition to Ca^{2+}, by another factor, S, which is depolarization dependent. These findings do not diminish the important role of Ca^{2+} in the release process. According to the Ca-voltage hypothesis, Ca^{2+} serves as an *essential cofactor* in the release process, but there is another important factor, the S molecule, which is required

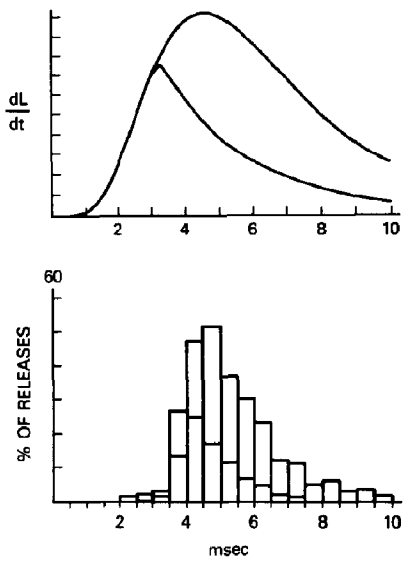

Fig. 8. Effect of a hyperpolarizing post-pulse on the synaptic delay histogram. (Top) Computer simulation for a 2 ms test pulse and for the same test pulse followed by a hyperpolarizing post-pulse at zero delay. Note that the area of the histogram is smaller (reduced quantal content). The peak moves to the left and the termination of release is accelerated. (Bottom) Experimental results for the same conditions.

as well. Release cannot occur unless the two factors are present, both in sufficient quantities. Release is modulated by changes not only in Ca^{2+} conductances and intracellular Ca^{2+} concentration but also in membrane potential.

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