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Neurotransmitter release and its facilitation in crayfish

VII. Another voltage dependent process beside Ca entry controls the time course of phasic release

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Abstract. Quantal synaptic currents were recorded at nerve terminations on the opener muscle of crayfish using a macropatch-clamp electrode, and the release was elicited by depolarizing current pulses applied to the terminal through the same electrode. After 2 ms depolarization pulses at low temperature, release started with about 2 ms delay after the onset of depolarization, and the maximum rate of release occurred at about 4 ms delay. Large variations in Ca inflow during the pulses were concluded from the facilitation of test EPSCs. The time course of release proved to be remarkably invariant in spite of large changes in release. If a conditioning train of depolarization pulses preceded the test pulse, release due to the test pulse was facilitated up to 60-fold, but the shapes of distributions of quantal delays were practically not affected by this facilitation. Facilitation by the conditioning trains must have raised the $[Ca]_i$ level at the onset of the test pulse. The invariance of the time course of release with respect to the level of $[Ca]_i$ cannot be explained by theories in which $[Ca]_i$ alone controls the time course of release.

The time courses of reactions controlling release were explored by mathematical analysis and simulation. A reaction scheme in which the activation of "release sites" directly by depolarization had rate limiting control on the release reactions, in which rise of $[Ca]_i$ only was a promoting cofactor, and in which a cooperative reaction involving the complex of release sites and Ca_i, (SCa_i) was one of the final steps eliciting release, was able to predict the delayed onset of release and the substantial latency between the end of the depolarization pulse and the maximum of the rate of release. Reaction schemes in which the direct effect of depolarization on release occurred at one or more steps following the entry of Ca could be excluded generally by showing conflict with the experimental findings.

Key words: Synaptic transmitter release – Time course of quantal release – Model of control of release by depolarization

Introduction

There is good evidence that inflow of Ca during depolarization of the nerve terminal activates phasic neurotransmitter release (Jenkinson 1957; Katz and Miledi 1968; Dodge and Rahamimoff 1967). Such inflow of Ca has been demonstrated directly in the giant synapse of the squid (Llinás and Nicholson 1975; Llinás et al. 1981a, b; Charlton et al. 1982; Miledi and Parker 1981). These findings supported the "Calcium hypothesis" stating that neurotransmitter release starts when intracellular Ca concentration $[Ca]_i$ reaches a certain threshold, and phasic release is terminated when $[Ca]_i$ returns to a subthreshold level (Katz and Miledi 1968; Llinás et al. 1981a, b; Zucker and Stockbridge 1983; Stockbridge and Moore 1984). However, as measured by Ca indicators, the elevation of $[Ca]_i$ lasted much longer than phasic neurotransmitter release (Zucker et al. 1980; Miledi and Parker 1981) which at low temperatures terminates within 10 ms after the depolarization of the nerve terminal (Katz and Miledi 1965, 1967; Barrett and Stevens 1972; Datyner and Gage 1980; I. Parnas et al. 1984; Dudel 1984a). Attempts to explain this discrepancy assumed rapid diffusion of Ca from near the release sites (Zucker and Stockbridge 1983; Simon et al. 1984; Stockbridge and Moore 1984) not detectable by the Ca indicators.

Another difficulty is encountered when the time course of single phasic release is compared with that of facilitation, the latter being explained by the residual calcium theory (Katz and Miledi 1968). While at room temperature phasic release terminates in about 2–3 ms, facilitation may last for hundreds of milliseconds. To circumvent this difficulty, it was proposed that Ca entering through the membrane is bound largely to intracellular protein which results in very steep concentration gradients changing rapidly with diffusion. Such models can explain the approximate time course of release in single pulses, and that of twin pulse facilitation (Zucker and Stockbridge 1983; Stockbridge and Moore 1984). It would be of interest to compare these predicted time courses of release with precise measurements. In another attempt to explain the short period of release in face of long-lasting elevations of $[Ca]_i$, Simon and Llinás (1984) assumed "peak" diffusion profiles at the single Ca channels. These authors attribute facilitation not to residual Ca, but to another unknown mechanism.

In our opinion the evidence for residual Ca as the basis of facilitation (Katz and Miledi 1968; Rahamimoff 1968) is rather convincing, and we have shown for the crayfish neuromuscular junction that this concept can describe changes in amplitude and time course of facilitation for a wide range of variation of $[Ca]_o$ as well as of other ions (H. Parnas et al. 1982a; I. Parnas et al. 1982b). However, we have given evidence that release is controlled critically by an additional factor, namely depolarization of the terminal (Dudel et al. 1983; Dudel 1983b; H. Parnas and Segel 1984).

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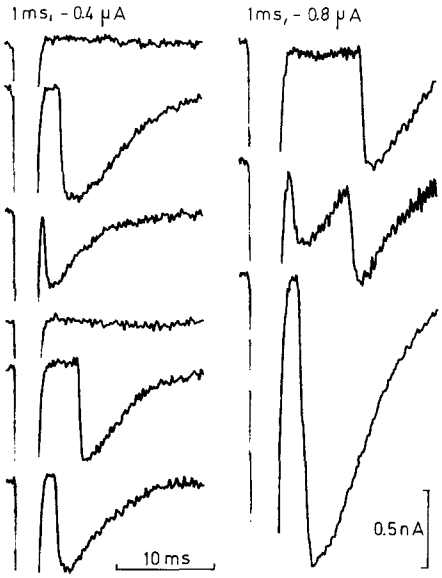


Fig. 1. Samples of EPSCs elicited by depolarizing current pulses through the recording electrode. The pulses had 1 ms duration and -0.4 μA or -0.8 μA amplitude in the *left* or *right hand columns*, respectively. At -0.4 μA, two failures are shown, while at -0.8 μA the *second* and *third traces* represent double releases. 0 °C. Most of the noise in the traces is generated by the FM-tape

We concluded that after a depolarization pulse release terminated largely due to repolarization, in spite of a slowly decaying, relatively high $[Ca]_i$.

The time course of release can be determined accurately, though laboriously, by measuring the distribution of delays of quantal releases after a depolarization (Katz and Miledi 1965, 1967b; Barrett and Stevens 1972; Datyner and Gage 1980; I. Parnas et al. 1984; Dudel 1984a). The different current hypotheses for the control of release predict variations in the time course of release on changing $[Ca]_i$ (Parnas and Segel 1984). We therefore have studied this time course of release for different amplitudes of depolarization and consequently Ca inflow, and also for different initial Ca levels.

Methods

Opener muscles were dissected from the first walking leg of large crayfish (*Astacus leptodactylus*). They were held in a chamber and superfused with modified v. Harreveld solution containing in mM: 205 Na^+, 232 Cl^-, 5.4 K^+, 13.5 Ca^{2+}, 10 tris-maleate buffer, pH 7.6. Although most terminals are inexcitable (Dudel 1983a; Dudel et al. 1984), $2 \cdot 10^{-7}$ M tetrodotoxin was added to prevent local excitations. The solution was cooled to 0° to 4° C. Synaptic currents were recorded by means of the macro-patch-clamp electrode used by us previously (Dudel 1983a; Dudel et al. 1983). Through the same electrode depolarizing pulses could be applied to the terminal.

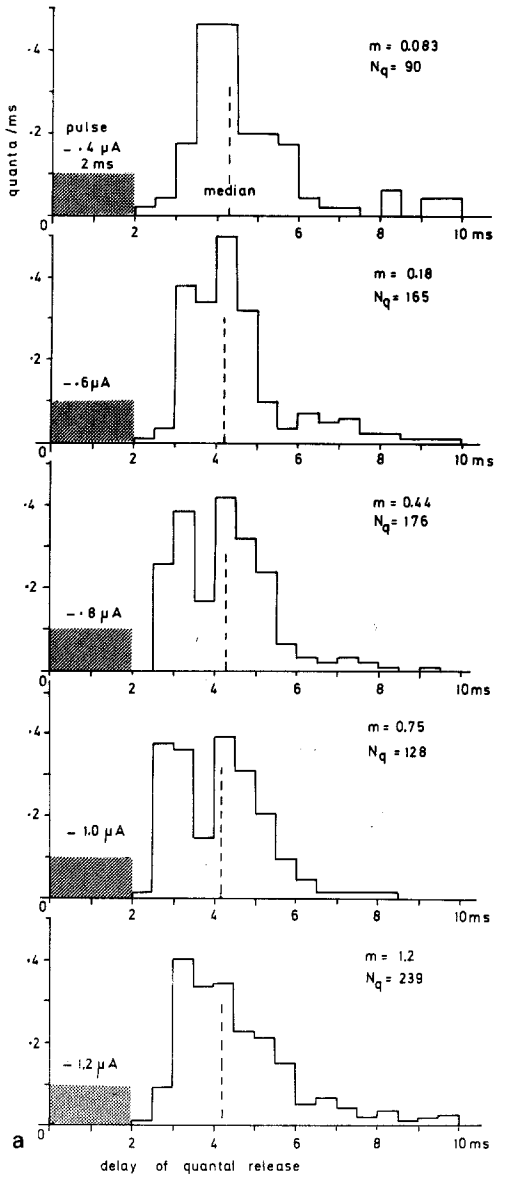
It has been argued that this method of recording and stimulation of release applies unknown depolarizations to a region of the terminal which possibly changes with variations of amplitude and duration of the applied current. As discussed in detail by Katz and Miledi (1967b, Fig. 1) current pulses applied to an electrode above the terminal shift the *extracellular* potential. The amplitude of this potential shift is proportional to the sealing resistance of the electrode tip with respect to the bathing medium. In the present experiments, in order to avoid damage to the terminal, this sealing resistance was held at the low level of 50–200 kΩ, typically about 100 kΩ. The current pulses used in this study to trigger release were between -0.3 and -1.7 μA, which thus should represent depolarizations in the range of 20–200 mV. Recorded releases were stable often for many hours: more than 50,000 pulses could be applied to a terminal with well reproducible results. The range of depolarizations concluded here also agrees well with the amount of release produced by an action potential recorded by the same electrode (Dudel 1983a). The shift of the extracellular potential during a current pulse should be proportional to the current amplitude within less then 0.1 ms, and should be limited sharply to the region inside the seal (Katz and Miledi 1967b). [Experimental evidence for the short time constant of the system is given in the succeeding paper (I. Parnas et al. 1986) and in Dudel 1984b.] The equipotential region below the tip with about 80 μm inner diameter was relatively large compared to the size of the terminal. As expected, quantal synaptic currents (EPSCs) elicited by release below the electrode were negative and rose sharply (Fig. 1). If release was triggered in the axon outside the electrode by stimulating the axon, damped positive EPSCs were observed. Such positive EPSCs were never seen (more then 10^6 observations) on eliciting release by depolarization through the recording electrode, even for the largest current pulses. We therefore can discount an appreciable effect of a larger depolarized stretch of the terminal on increasing current strength.

As seen in Fig. 1, quantal releases occur with variable delays. At the low temperature of 0° C, multiple releases can be discerned easily (Johnson and Wernig 1971) and their delays can be measured separately. The input amplifier is unbalanced during the current pulse and for about 0.5 ms afterwards, if optimal compensation is employed (Dudel 1983a). The start of EPSCs cannot be observed during this artefact, however, EPSCs last for more then 10 ms and their tails would have been observed. Actually there is enough latency of release after the depolarization (Katz and Miledi 1965) that at least the peaks of the first EPSCs could be resolved after the pulse artefact, and their start could be estimated with at least 0.5 ms accuracy. During the experiment, EPSCs elicited by pulses were displayed and counted directly. In parallel the current records were stored on FM tape. Samples of such stored records are given in Fig. 1. For the evaluation of synaptic delays, records synchronized to the stimulus were displayed on a digital oscilloscope. The delays of the starts of the single quantal EPSCs (including also the later ones in multiple releases) were evaluated by placing a cursor with a resolution of 0.1 ms, and fed into a PDP 11/23+ computer which established the respective distributions of delays. The bin width for the distributions was 0.5 ms or less.

Results

Time course of release after a depolarization pulse

Distributions of delays of quantal releases from the beginning of a 2 ms depolarization pulse are given in Fig. 2. The amplitude of the pulses was varied in the range -0.4 to -1.2 μA, increasing the average rate of release of quanta,



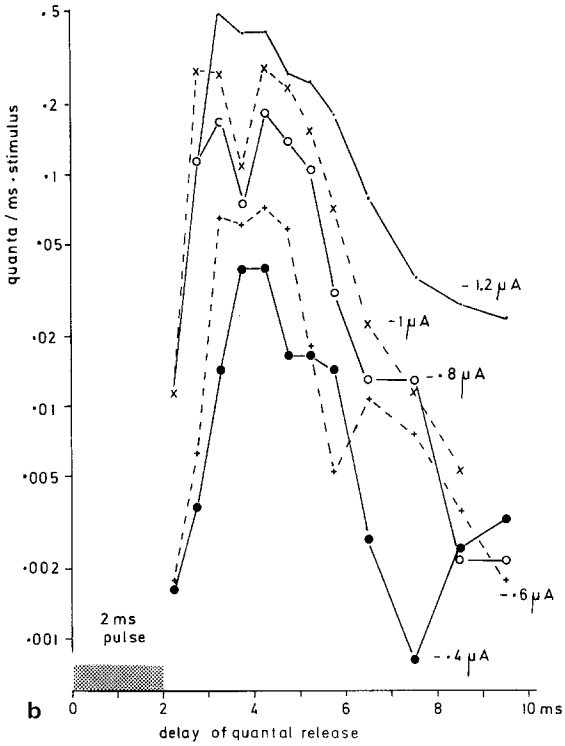


Fig. 2. A Distributions of the delays of quantal releases after the start of a 2 ms depolarization pulse, for pulse amplitudes of −0.4 to −1.2 μA, as indicated in each plot. In addition, the average number of quanta observed per stimulus, *m*, and the number of quanta, N_q, entered into the respective distribution are indicated. The rate of release in the ordinate (quanta/ms) gives the probability that the delay of one quantum is in the respective time interval of the distribution, i.e. the distributions are scaled to the same area 1 ms. Repetition rate of pulses 0.45/s, temperature 4° C. The *broken lines* indicate the median values of the distributions. **B** Distributions of delays of quantal releases of same experiment as in Fig. 2A, but the *ordinate* represents (semilogarithmically) the absolute probability of occurrence, per stimulus, of a quantum at the respective time interval (quanta (ms)$^{-1}$ (stimulus)$^{-1}$). The distributions are generated from that of Fig. 2A by multiplying the ordinate values by the respective average number of releases per stimulus, *m*. The resulting values are inserted at the middle of the respective time intervals

m, from 0.08 to 1.2. In Fig. 2A the amplitude of the distribution is on a linear scale and gives the probability of an observed delay to occur within the respective 1 ms interval. Consequently the area of all distributions is 1 ms which allows a good comparison of the time courses of release at amplitudes varying by a factor of 15. An alternative plot of the same data in Fig. 2B uses a logarithmic amplitude scale, and the absolute probability per stimulus of a quantum occurring within a specific time interval is presented. The distributions show clearly that the time course of release is quite independent of the amplitude of depolarization. Release starts with a delay of 2 ms, with still a very low rate of release between 2 and 2.5 ms, and the maximum of release is between 3 and 4.5 ms after the onset of depolarization. The rates of release decline steeply about 5 ms after the onset of depolarization with no obvious differences in the time constants. Ten milliseconds after the beginning of the pulses, release has reached a relatively low level in all plots.

To show the consistency of the results, the effects of different amplitudes of depolarization are presented for another terminal in Fig. 3. Six different depolarizations were given, the quantum content of the EPSCs rising from m = 0.08 at −0.3 μA pulse amplitude to m = 1.8 at −1.4 μA. As in Fig. 2, the time courses of releases show remarkably small differences (see also Fig. 5, left hand part). The first releases in the experiment of Fig. 3 appear between 2 and 2.5 ms after the beginning of the pulse, i.e. directly after the end of the 2 ms depolarization. However, there is a tendency for relatively more early releases with increasing depolarization. The release rate between 2 and 3 ms delay is 0.03, 0.07, 0.04, 0.15, 0.09 quanta/ms, respectively, in order of rising depolarizations. For 2 ms pulses, the first releases usually appear directly after the end of the pulse, but in some experiments already during the last 0.5 ms of the depolarization pulse (see Fig. 5).

The peaks of the distributions in Fig. 3 are between 4 and 5 ms, and they have the tendency to become broader with increasing amplitude of release. The exact position of the peak is difficult to determine. A good measure of the center of the distribution is the median value. These medians

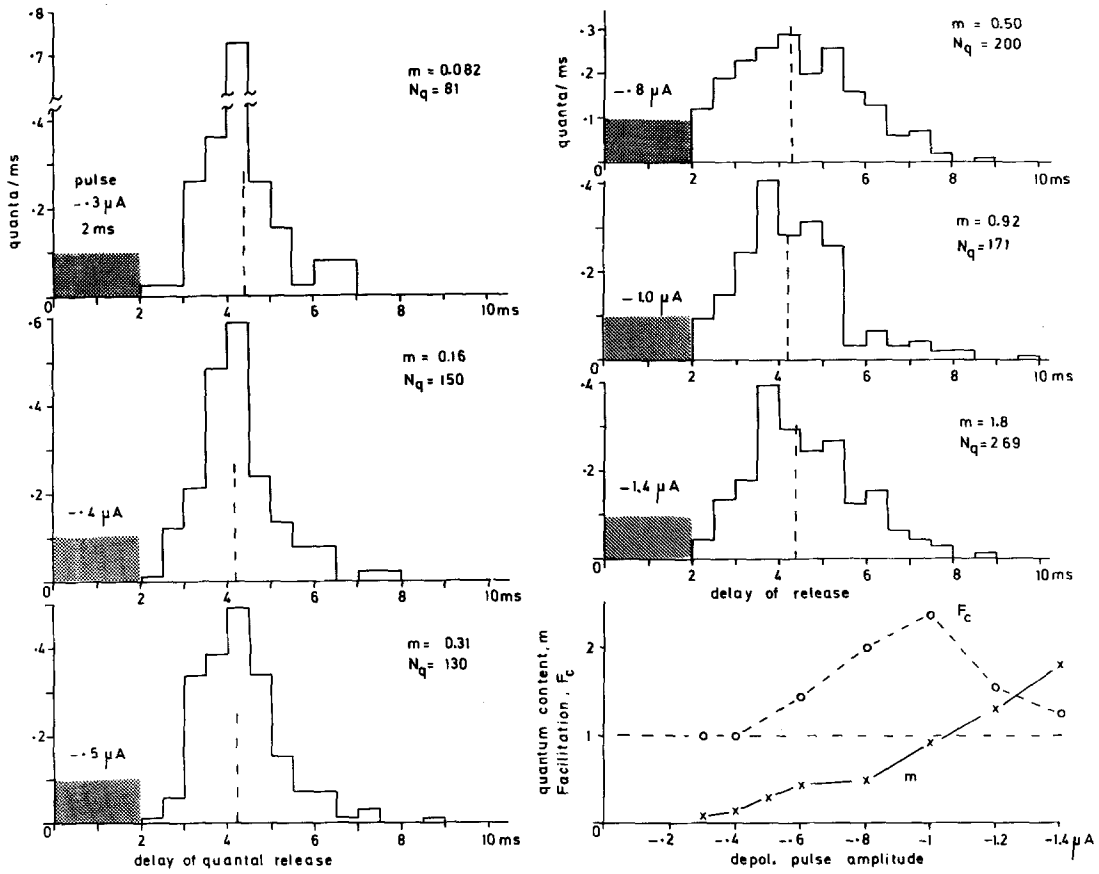


Fig. 3. Time courses of quantal release after a 2 ms depolarization pulse. The amplitudes of the pulses rise from −0.3 to −1.4 μA as indicated in the respective plots, which also contain the average number of quanta per stimulus, *m*, and the number of delays of quantal releases, N_q, contained in the respective distributions. The *ordinates* or the distributions of delays give the probability for one quantum to occur in a particular range of delays in quanta/ms. In the last graph, the dependence of the quantum content, *m*, and of the facilitation F_c on the amplitude of the releasing pulses are plotted. F_c, as defined in the text, indicates the amount of Ca inflow during the depolarization pulse. Superfusion of v. Harreveld solution containing $2 \cdot 10^{-7}$ M tetrodotoxin, at 3° C

are represented by broken lines in the different plots, and they do not seem to be affected at all by the amplitude of the depolarization. In Fig. 3, the medians are at 4.3, 4.2, 4.2, 4.3, 4.2, 4.4 ms in the order of rising depolarization amplitude, and similarly in Fig. 2 the median values lay between 4.2 and 4.3 ms. The constancy of these values shows, how little the time scale of the distributions is affected by differences in the amplitude of the releasing pulse and of the consequent Ca inflow.

With regard to Ca inflow, Fig. 3 gives some additional information in the plot of *m* and F_c at different depolarization amplitudes. F_c is the facilitation of a test EPSC which is elicited by a depolarization pulse of constant amplitude. This test EPSC is placed 10 ms after the respective depolarization pulses of −0.3 to −1.4 μA amplitude, and is facilitated by these prepulses. As seen in all experiments of this type, F_c is about one after low prepulses, rises steeply to a maximum for larger prepulses, but then declines for even larger prepulses, although release *m* increases further. As argued in Dudel et al. (1983), F_c represents the amount of Ca entering during the prepulse, and the maximum of F_c occurs at the depolarization which causes the maximum Ca inflow, i.e. a depolarization to about 0 mV. The implications of the potential dependence of F_c in the context of the measured time courses of release will be treated in the discussion.

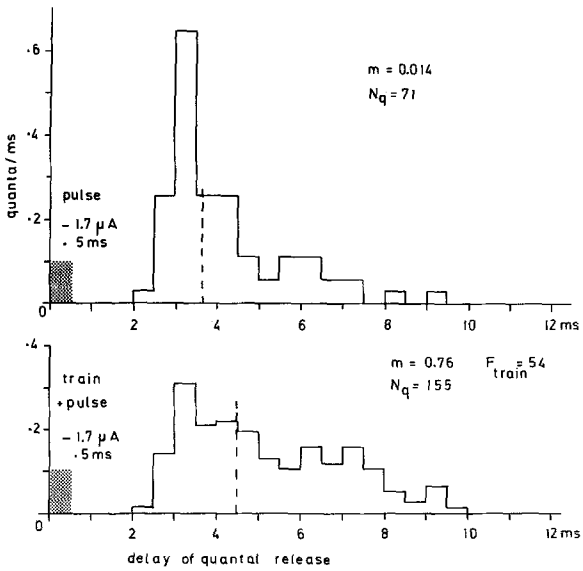


Fig. 4. Distributions of delays of quantal releases after the beginning of a −1.7 μA, 0.5 ms test pulse. The representation is like in Fig. 2A. In the *lower* graph, the test pulse was preceded by 60 ms by a conditioning train of 5 depolarization pulses (−1 μA, 2 ms) at 200 Hz which itself caused large release and Ca entry. Release due to the test pulse was facilitated by the conditioning train by a factor $F_{train} = 54$. Temperature 1.5° C

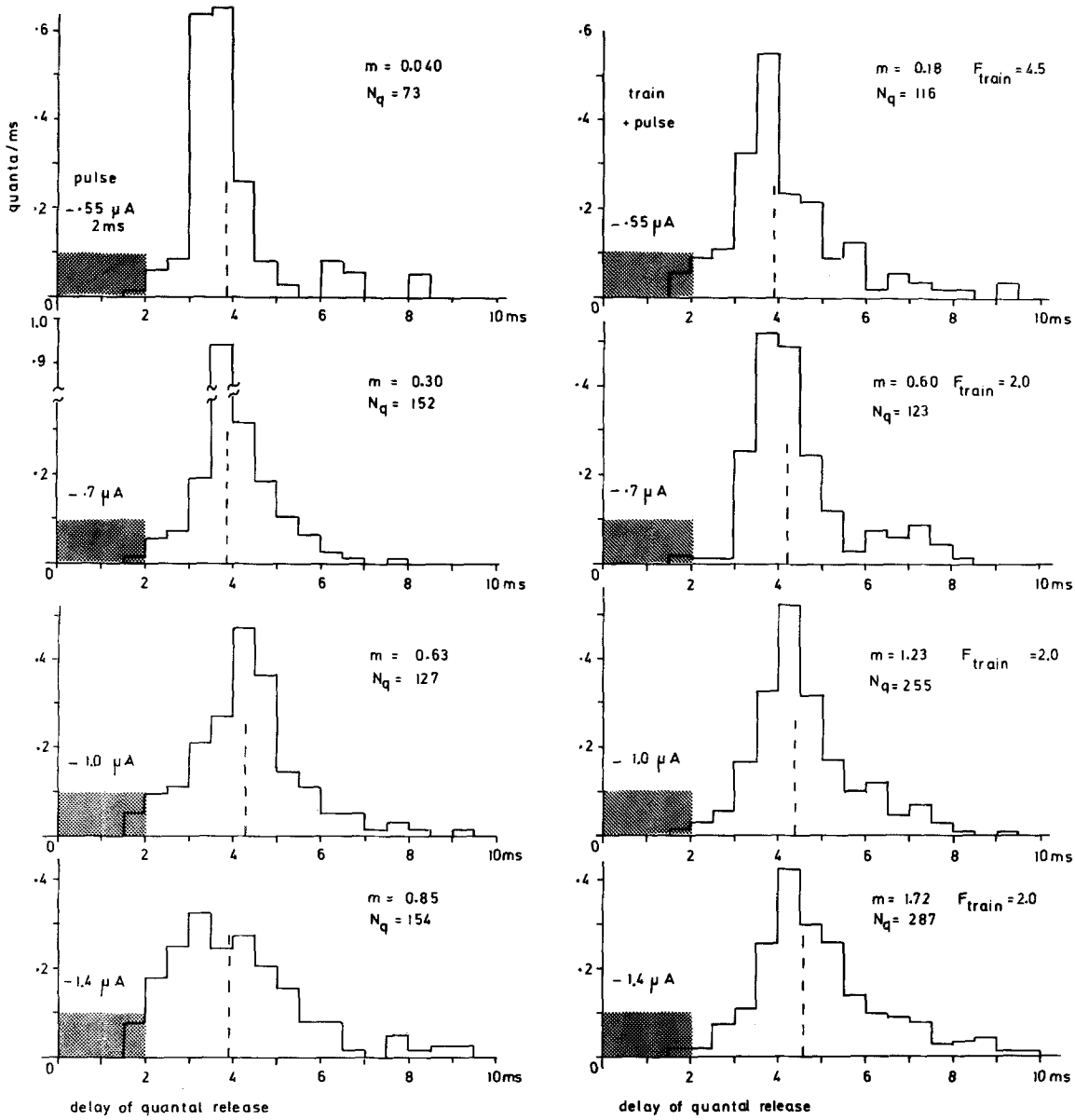


Fig. 5. Distributions of quantal delays after the beginning of 2 ms depolarization pulses. Ordinate quanta/ms, representation like in Fig. 2A. The pulse amplitude rises from top to bottom from −0.55 μA to −1.4 μA. In the *left hand row* of plots, only the test pulses were applied, while in the *right hand row* of plots, the respective test pulses were preceded by 50 ms by conditioning trains of pulses (3 pulses, −1.2 μA, 2 ms, at 100 Hz). F_{train} gives the respective facilitation by the preceding train. Temperature 4° C

Time courses of release at different amplitudes of depolarization have been measured in 23 terminals. All the features discussed in Figs. 2 and 3 were seen in all these experiments. The time courses were almost invariable in the respective terminals, if the duration of the depolarization pulses was constant.

Effects of facilitation on the time course of release

If release is controlled by $[Ca]_i$ only, shifts in the level of $[Ca]_i$ present before a further Ca inflow during a depolarization pulse should modify the time course of release considerably. A high initial Ca concentration can be achieved by a train of prepulses which precede the test pulse, as done in the experiments of Figs. 4 and 5. In the experiment of Fig. 4, a 0.5 ms test pulse was adjusted in amplitude to result in the very low average release rate $m = 0.014$. If this test pulse was preceded by a train of conditioning pulses, release was facilitated by a factor of 54. If this facilitation is due to residual Ca, and if release is proportional to $[Ca]_i^4$ (an assumption which presents a lower limit of the change in $[Ca]_i$; I. Parnas et al. 1982b), the facilitation factor of 54 represents a change in $[Ca]_i$ by the $\sqrt[4]{54} \approx 3$. In spite of these large shifts in $[Ca]_i$, the minimum delay for release of quanta is the same in Fig. 4 before and after facilitation. Also the rest of the time course of release is little affected by the facilitation. The median value of the distribution of delays rises from 3.7 to 4.4 ms, but in view of the low number of quantal delays, $N_q = 71$, obtained out of 5,000 stimuli in the non-facilitated test EPSCs this rise may not be signifi-

cant. The increased median value was not generally observed in facilitated EPSCs (see Fig. 5).

Figure 4 gave an example of a very strong facilitation of a very small EPSC. Figure 5 shows the results of an experiment in which the facilitating train was shorter, and in which the test EPSCs varied in the average rate of release from 0.05 to 0.9 due to different amplitudes of the test pulses. Consequently, the resulting facilitations went down from 4.5 to 2. The time courses of release seem to be totally unaffected by facilitation: all minimum delays were 1.5 ms and the changes in median value of the delays were from 3.8 to 3.9, 3.9 to 4.2, 4.3 to 4.4 and 3.9 to 4.6 ms, respectively. The absence of an effect of even very large facilitations on the time course of release was seen in all 13 experiments of this type.

Models that can describe the experimental results

The observation that start, maximum and termination of release after a depolarization pulse are practically not affected by large variations of Ca inflow during the pulse or even of the level of $[Ca]_i$ present before the pulse, appear to exclude $[Ca]_i$ as the only factor that controls release. We shall argue this conclusion with respect to the relevant theories of release in the discussion. It remains to formulate a scheme of reactions which can cover the results obtained. The essential features at low temperature are: (1) release starts with about 2 ms delay after the beginning of a 2 ms depolarization pulse, or clearly after the end of a shorter depolarization pulse. (2) The maximum of release is reached 4–5 ms after the beginning of a 2 ms depolarization pulse, i.e. always after the end of the pulse. (3) Release declines to a low level within about 10 ms. (4) Changes in the amplitudes of the depolarization pulse and in the level of $[Ca]_i$ present before this pulse do not appreciably change the time course of release described in Eqs. (1)–(3).

As we have concluded from other evidence, in addition to $[Ca]_i$ depolarization of the terminal as such controls release (Dudel et al 1983; Dudel 1984a; I. Parnas et al. 1984). To introduce this potential dependence into the kinetics of release, we assume that "release sites" S are produced by depolarization from an inactive precursor T:

$$T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S. \tag{1a}$$

in which the rate constants change from a resting level k_1^0 and k_{-1}^0, to k_1^D and k_{-1}^D respectively on depolarization, and back to k_1^0 and k_{-1}^0 on repolarization. These release sites react with intracellular Ca, as assumed in the control of release by $[Ca]_i$ alone:

$$S + Ca_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (SCa_i), \tag{1b}$$

and then n complexes (SCa_i) will combine with a "vesicle", V, to result in release, L:

$$n(SCa_i) + V \overset{k_3}{\rightarrow} L. \tag{1c}$$

The fate of the complex (SCa_i) after combination with a vesicle has not been included into this scheme; the complex might revert into the components S (or T) and Ca_i, or S might disintegrate. Regarding the low rates of release found in the terminals, it does not seem essential to include such reaction steps, and it is not possible, at present, to obtain evidence for one of the possibilities. The differential equations describing the model are:

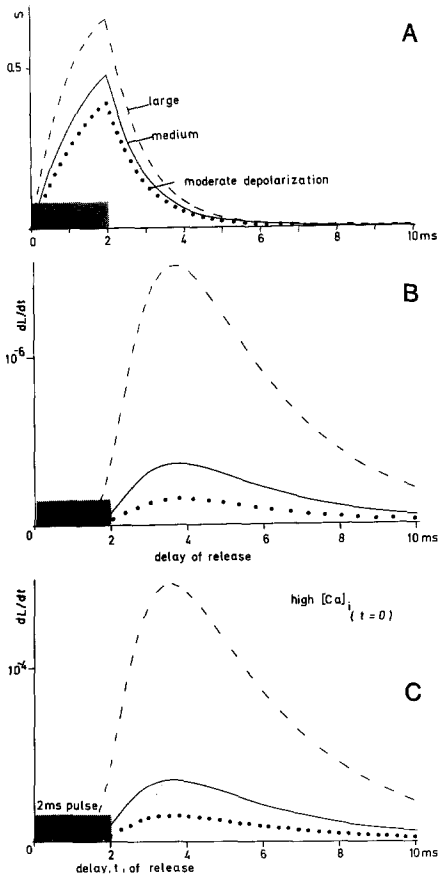


Fig. 6A–C. Simulation of the effects of a 2 ms depolarization pulse according to the reaction scheme of Eqs. (1) and (2). **A** Time course of number of activated release sites, S, with $k_1^D = 0.3$/ms for the moderate, $k_1^D = 0.4$/ms for the medium, and $k_1^D = 0.7$/ms for the large depolarizations. $k_{-1}^D = 0.1$/ms, $k_{-1}^0 = 1$/ms and $k_1^0 = 0$. This time course is insignificantly different for low and high $[Ca]_i$, which were assumed to have constant levels of $[Ca]_i = 0.05$ μM in *B* and $[Ca]_i = 0.2$ μM in **C. B, C** Time courses of the rates of release dL/dt for low and high $[Ca]_i$, with $k_2 = 1(\text{ms} \cdot \mu\text{M})^{-1}$, $k_{-2} = 0.1$/ms, $k_3 = 0.1$/ms and $n = 4$

$$dT/dt = k_{-1}S - k_1T, \tag{2a}$$

$$dS/dt = k_1T - k_{-1}S - k_2S[Ca]_i + k_{-2}(SCa_i), \tag{2b}$$

$$d(SCa_i)/dt = k_2S[Ca]_i - k_{-2}(SCa_i) - k_3(SCa_i)^n, \tag{2c}$$

and

$$dV/dt = -k_3(SCa_i)^n \cdot V, \tag{2d}$$

$$dL/dt = k_3(SCa_i)^n \cdot V. \tag{2e}$$

These equations have a lengthy solution. It shows that the maximum of dL/dt can be at a delay, t_{max}, longer than the duration of the pulse. t_{max} is determined by a number of parameters, the most important being k_3 and the rate of inactivation of release sites k_{-1}^0. Figures 6A and B give a simulation of the behaviour of the model of Eq. (1). For

simplicity it was assumed that $[Ca]_i$ stays constant; it will be shown below that the time course of release is not altered essentially by this simplification. Figure 6A shows the time course of appearance of activated release sites, S, for three different depolarizations, which are reflected in the respective rates k_1 (see legend). The resulting rates of release in Fig. 6B seem to agree largely with the features of the phasic release listed above: (1) release starts with a delay of 1–1.5 ms. This is almost as much latency as the experiments show; with the present model this latency can only be lengthened by increasing k_3, which also prolongs the total release curve, or by raising n above the value of four employed here. Increasing n to six does not change the latencies significantly, and much higher powers seem unrealistic. (2) The maximum of release in Fig. 6B is at almost 4 ms, i.e. considerably after the end of the pulse. (3) Release declines to a low level within about 10 ms. (4) Changes in the amplitude of the depolarization pulse do not appreciably affect the time course or release. There is a last characteristic of release which has to be covered by the model which is not contained in Fig. 6B: the time course of release should be insensitive to increases in the level of $[Ca]_i$ present before the depolarization pulse. The influence of an increase of pre-pulse $[Ca]_i$ is modelled in Fig. 6C: compared to Fig. 6B, Ca_i at $t = 0$ was raised from 0.05 μM to 0.2 μM. This results in an about hundredfold facilitation of release (see ordinate scales), however, the time course of release is unchanged, as concluded from the results of Figs. 4 and 5.

The model of Eqs. (1) and (2) seems to conform in all essential points to the experimental results. However, an approximation was made in the simulation of this model in Fig. 6B and C: the intracellular Ca concentration, $[Ca]_i$, was assumed to be constant. It should be examined whether phasic changes of $[Ca]_i$ which are likely to occur during and after a depolarization pulse, affect the simulation critically. Therefore, the simulation of the model of Eqs. (1) and (2) in Fig. 7 includes phasic changes in $[Ca]_i$. The time courses of $[Ca]_i$ for the different depolarizations are shown in Fig. 7B. They were modelled assuming potential dependent entry of Ca according to Hagiwara and Takahashi (1967), and subsequent removal according to Eq. (3) of I. Parnas et al. (1982a). The time courses of release (Fig. 7C) show a latency of the first measurable releases of 1.5–2 ms, a maximum near 4 ms and almost complete decay 10 ms after the onset of depolarization. The most obvious effect of the phasic changes in $[Ca]_i$ is a slightly later maximum of release at the large depolarization. This is due to the assumed relatively large late Ca entry during the tail Ca current. The fact that the latency of release is by no means longer for the large depolarization, as might be expected from the late Ca entry, demonstrates that the time course of release is essentially determined by the activation of sites, S, and very little by $[Ca]_i$ in this model. Actually, relatively more early releases are elicited by the large pulse as compared to release by lower pulses, which may correspond to the similar observation in Figs. 2 and 3. In summary, the reaction scheme of Eqs. (1) and (2) predicts very well the measured distributions of delays of quantal releases. This is true if $[Ca]_i$ is assumed to be constant, and also if large phasic changes of $[Ca]_i$ are added. Even if very sharp rises in $[Ca]_i$ and declines with time constants of about 5 ms (Stockbridge and Moore 1984) are assumed, the resulting time course of release is not appreciably different from that in Fig. 7C.

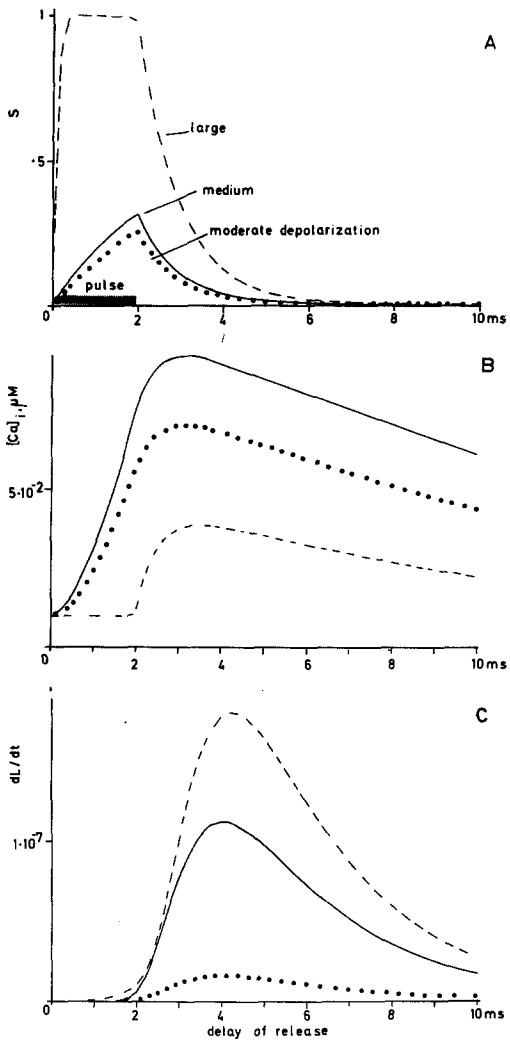


Fig. 7A–C. Simulation of release reactions according to Eqs. (1) and (2) (Fig. 6), but including variation in $[Ca]_i$ due to Ca inflow and removal (see text). Effects of moderate, medium and large depolarization pulses of 2 ms duration on S (**A**), $[Ca]_i$ (**B**), and the rate of release dL/dt (**C**), represented like in Fig. 6. Parameters: $k_1^D = 0.15$/ms for moderate, $k_1^D = 0.2$/ms for medium, $k_1^D = 10$/ms for large depolarization; $k_1^0 = 0$, $k_{-1}^D = 0.01$/ms, $k_{-1}^0 = 1$/ms. $k_2 = 1/(\text{ms} \cdot \mu\text{M})^{-1}$, $k_{-2} = 0.1$/ms; $k_3 = 10$/ms

In the model of Eq. (1) the cooperativity in the reactions leading to release is in the last step, in the reaction of n (SCa_i) with one V. n is assumed to be four, to cover the dependence of release approximately on $([Ca]_i)^4$ (H. Parnas et al. 1982). In an alternative formulation of the model, the cooperativity was assumed to be at one step earlier, as the combination of n Ca_i with one S. With these assumptions the time course of release can be very similar to that shown in Fig. 6, only the initial delay is missing: release starts from the beginning of the depolarization. Since this does not match the essential features of the results, the cooperativity was placed at the last step [Eq. (1c)].

Discussion

In our experimental studies we have tried to vary the amplitude and the time course of changes in $[Ca]_i$ after a

stimulus to the nerve terminal. This was done with the aim to obtain information which might allow a critical evaluation of existing theories of the control of synaptic release. Since there is little chance to measure the time course of $[Ca]_i$ at the release sites in the small motor terminals, indirect procedures to change $[Ca]_i$ in a predictable manner had to be employed. The first were variations of the amplitude of depolarization in a wide range. As discussed in Methods, the terminals were depolarized, usually for 2 ms, by 20 to 200 mV. If one accepts the residual-Ca theory of facilitation, the extent of facilitation F_c of an EPSC which is elicited by a constant depolarization pulse placed at a fixed interval after a preceding EPSC, will indicate the amount of Ca entering during the preceding EPSC. If the depolarization triggering the latter is varied, F_c and consequently Ca inflow are seen to increase up to a depolarization to about 0 mV, and to decline for larger depolarizations, as expected for an ionic current on approaching its equilibrium potential (Hagiwara and Takahashi 1967; Llinás et al. 1981a, b). The measurement of the depolarization dependence of F_c gives reliable and well reproducible results in motor terminals of crayfish (Fig. 3; Dudel et al. 1983), lobster (I. Parnas et al. 1984a) and frog (Dudel 1983, 1984b). It has been argued that the peak in F_c at medium large depolarizations might be due to change in the spatial distribution of the depolarization with increasing current strength. We have pointed out in the Methods that this seems unlikely. In addition, the typical depolarization dependence of F_c is found irrespective of the size of the electrode which should affect such suggested artefacts, and the potential dependence is the same if the duration of the pulse is varied (Dudel et al. 1983). This last observation also seems to exclude inactivation of Ca inflow due to a rise in $[Ca]_i$ (Chad et al. 1984; Eckert and Chad 1984) as the cause of the decline of F_c after large prepulses. It has to be accepted, therefore, that the changes of F_c measured for instance in Fig. 3 indicate a rise and fall of Ca entry within the range of depolarizations employed.

The second parameter of release manipulated experimentally was $[Ca]_i$ at the start of the releasing depolarization. This was achieved by a conditioning train of depolarizations which was presumed to raise $[Ca]_i$. The maximum facilitations achieved by such conditioning trains in case of small, just threshold releasing pulses go up to 1,600-fold (Dudel et al. 1983); assuming, as a lower limit (I. Parnas et al. 1982), a dependence of release on $([Ca]_i)^4$, this means that $[Ca]_i$ before the releasing pulse was at $\sqrt[4]{1{,}600} - 1 = 5.3$ times the threshold value for release. At such high $[Ca]_i$ release should proceed continuously, if only $[Ca]_i$ controls release. It has been argued, therefore, that these large facilitations are not due to residual $[Ca]_i$, but to some change in excitability of the terminal after the conditioning train. This seems very improbable: (a) the large facilitations can be obtained irrespective of the presence of TTX; (b) some of the test pulses are so small that they would not even reach the threshold for excitation in excitable terminals (Dudel 1983a); (c) the amplitude of the test EPSC can be graded within a wide range by changing the depolarization pulse, and consequently facilitation of this EPSC varies also in a graded manner (Fig. 4; Dudel et al. 1983, Fig. 7). Changes in excitability of the terminal thus seem to be excluded as a source of the large facilitations. If one does not want to invoke another unknown mechanism, it appears that residual $[Ca]_i$ can reach very high levels in the crayfish terminals after conditioning trains.

The main result of the present experimental studies was that changes in Ca inflow due to variations in depolarization, as well as changes in the initial $[Ca]_i$ levels due to pretrains, do not appreciably affect the time course of release in spite of large changes in the amount of release. Similar results have been reported for the mouse by Datyner and Gage (1980), who changed the extracellular concentration of Ca and K and also pH, and did not obtain appreciable changes in the time course of release elicited by action potentials, although the amplitude of release was much altered. Also facilitation within a train of action potentials did not affect the time course of release. Dudel (1984a) also applied pulses of different amplitude to the motor terminal of frog and found no change in the time course of release.

If one tries to explain these findings with theories in which $[Ca]_i$ alone controls release, a number of difficulties become obvious, as already concluded by Datyner and Gage (1980). The initial delay of release is mostly disregarded by such theories (Stockbridge and Moore 1984); it can result only from a strong sigmoidity of the $[Ca]_i$ release relation. Increasing the Ca inflow in a larger depolarization, or a high initial level of $[Ca]_i$ should accelerate the sigmoid rise of release and shorten the latency of release – contrary to experimental results. Larger inflow of Ca should also prolong the time course of release (Parnas and Segel 1984). The same is true for the effect of a higher initial $[Ca]_i$ level. This is also demonstrated in the simulations of Stockbridge and Moore (1984). They constructed a time course of $[Ca]_i$ in a submembrane layer which is most favourable to account for the time course of release and for facilitation for several 100 ms later. They simulated a double-pulse facilitation by the factor 2. Even in this small facilitation, the time constant of decay of "release" was prolonged in the facilitated EPSCs by more than 50%, and the duration of release was more than doubled. A facilitation of the order of 100× (Fig. 6) would prolong the time course of release enormously in this system. Models in which $[Ca]_i$ alone controls release, even if they are as refined as those of Zucker and Stockbridge (1983), or Stockbridge and Moore (1984), thus cannot cover the details of facilitated release even for the second EPSC in a pair. Much more difficulty is expected for longer series of pulses, in which the slowing of the decay of release will become more and more prominent with the progressive rise of the level of bound Ca in the terminal.

It seems necessary, therefore, to have another voltage-dependent factor in addition to $[Ca]_i$ participating in the control of release. In our model [Eqs. (1) and (2)], depolarization generates release sites S in parallel to the entry of Ca, and this model thus can be classified as "parallel" for control of release by depolarization and $[Ca]_i$. One could think also of serial models as the one suggested by Llinás et al. (1981b), in which depolarization triggers entry of Ca, and in addition influences the reaction of Ca with release sites S, or another later step. The model can be formulated as in Eq. (1b) and (1c), with S potential independent, but k_2 and/or k_3 potential dependent. [If the cooperative reaction is shifted to the step (1b), the conclusions reached below will not be affected.] We assume that $[Ca]_i$ rises fast with depolarization and stays constant; as shown before this simplification does not affect the conclusions qualitatively. The influence of the potential-dependent rate constants k_2 and k_3 can be deduced from Eq. (2c) and (2e). In Eq. (2c) the last term is much smaller than the first two terms and can be neglected. At constant $[Ca]_i$, only k_2 changes, increasing on depolariza-

tion. Equation (2c) can be solved for steady states reached finally during a long depolarization or at the resting potential:

$$(S\mathrm{Ca_i}) = S \cdot [\mathrm{Ca}]_i \cdot (k_{-2}/k_2 + [\mathrm{Ca}]_i)^{-1}. \quad (3)$$

Equation (3) shows that $(S\mathrm{Ca_i})$ increases as long as k_2 increases, and the same is true for dL/dt if only k_2 is potential-dependent [Eq. (2e)]. The same is true also if k_3 is potential-dependent, because k_3 does not appear in Eq. (3) and is a factor in Eq. (2e) only. Thus, if k_2 or k_3 increase on depolarization, dL/dt will rise during depolarization, and if k_2 or k_3 drop to a lower value on repolarization, dL/dt will start to decline immediately. Therefore, the peak of dL/dt will be at the end of the depolarization pulse, or even before.

In a similar manner, any other steps following the entry of Ca and affecting release can be ruled out as being potential-dependent.

It seems, therefore, that only the model of Eqs. (1) and (2) can account for the known characteristics of phasic release and facilitation. This model is "parallel", depolarization causes independently the inflow of Ca and the activation of "release sites", S. This activation of release sites is the rate controlling step and the time course of S largely determines the time course of release. $[\mathrm{Ca}]_i$ is a powerful cofactor and has large effects on the amount of release. In this model the "release sites" and "vesicles" naturally are not physically identified, but only denote consecutive steps in the kinetics of the control of release. The model also cannot be unique, although control only by $[\mathrm{Ca}]_i$ or with following modulation by a potential dependent factor seem to be ruled out. Alternative models, however, would be required to generate the many details covered by our model: synaptic delay, maximum release considerably after the end of depolarization and consequent rapid decline of release, facilitation for up to seconds and very large facilitations without appreciable changes of time course of the facilitated release, and independence of the time course of release of the amount of entering Ca.

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