

Change of Transmitter Release Kinetics During Facilitation Revealed by Prolonged Test Pulses at the Inhibitor of the Crayfish Opener Muscle

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Vyshedskiy, Andrey and Jen-Wei Lin. Change of transmitter release kinetics during facilitation revealed by prolonged test pulses at the inhibitor of the crayfish opener muscle. *J. Neurophysiol.* 78: 1791–1799, 1997. A presynaptic voltage-control method was used to study synaptic facilitation at the inhibitory neuromuscular synapse of the crayfish opener muscle. The expression of the F_2 component of facilitation, monitored 150 ms after a conditioning stimulus, was examined by systematically changing the duration of the presynaptic test pulse. (Test pulses in all experiments were depolarized to 0 mV.) Control and facilitated inhibitory postsynaptic potentials (IPSPs) exhibited identical time courses when test pulse duration was brief (~ 2 ms). When the duration of the test pulse was increased beyond 2 ms, the transmitter release time course shifted to an earlier point in time during facilitation. Meanwhile, the increase in total transmitter release, measured from inhibitory postsynaptic conductance (IPSG) area (total release), became less pronounced with increasing duration of the test pulse. With a 20-ms test pulse, facilitation did not cause any detectable change in total release but the half-maximal point of the facilitated IPSG shifted by 3 ms (release shift). This change in release kinetics was not associated with a decrease in minimal synaptic delay. Furthermore, the relationship between total release and presynaptic pulse duration suggested that the transmitter release activated by a 20-ms pulse can be defined as a distinct component of continuous transmitter release (early component). The facilitation process accelerated the release kinetics of the early component but did not modify its total transmitter content. To test the hypothesis that the release shift is indeed mediated by the same mechanism that increases IPSP amplitude during facilitation, we investigated the correlation between the release shift and IPSP amplitude change. The two parameters were significantly correlated when the magnitude of facilitation was changed 1) during the decay of facilitation and 2) by varying the strength of the conditioning stimulus. The experimental approach reported here provides two new physiological parameters, release shift and total release, for the analysis of synaptic facilitation.

INTRODUCTION

Synaptic facilitation is a common form of synaptic plasticity within the CNS and at neuromuscular junctions. On the basis of kinetic and pharmacological characterizations, facilitation is classified under short-term synaptic plasticity, which includes the F_1 and F_2 components of facilitation, augmentation, and posttetanic potentiation (Magleby 1987). The decay time constants of the F_1 and F_2 components are in the range of tens and hundreds of milliseconds, respectively. In addition, the F_2 component can be selectively enhanced and prolonged by replacing extracellular calcium

with strontium (Zengel and Magleby 1980). These distinct properties have led to the speculation that the F_1 and F_2 components could be mediated by different molecular mechanisms (Magleby 1987).

Synaptic facilitation is typically activated by a conditioning stimulus, which can be one or a burst of action potentials, and then monitored by a single test action potential delivered at a defined time after the conditioning stimulus. It has been found that calcium influx associated with the conditioning stimulus is essential for the activation of facilitation (Katz and Miledi 1968). This observation, together with the fact that the amount of neurotransmitter release is related to the extracellular calcium concentration by a fourth-power relationship (Dodge and Rahamimoff 1967), has led to the residual calcium hypothesis for facilitation (Katz and Miledi 1968). Specifically, this hypothesis postulates that residual intracellular free calcium ions ($[Ca^{2+}]_i$) remaining after the conditioning stimulus participate in the secretion process such that the calcium influx associated with the test pulse becomes more effective at triggering transmitter release (Katz and Miledi 1968; Martin 1977). Advances obtained through several approaches to the study of intracellular calcium dynamics have suggested that the classical hypothesis requires further refinement to quantitatively account for facilitation and other components of short-term plasticity. Using the decay of calcium-activated potassium current to monitor the decay of $[Ca^{2+}]_i$, Blundon et al. (1993) showed that $[Ca^{2+}]_i$ decays faster than the decay time constant of F_1 facilitation. Direct monitoring of the presynaptic calcium transient by imaging methods has also suggested that the decay of F_2 facilitation was significantly slower than that of the calcium transients (Atluri and Regehr 1996). These observations are contrary to what the classical hypothesis would have predicted. Detailed modeling of intracellular free calcium diffusion has pointed to the conclusion that the classical model is quantitatively too simplistic to account for the impact of introducing an intracellular calcium buffer on F_2 facilitation (Winslow et al. 1994). Finally, imaging analysis of $[Ca^{2+}]_i$ has indicated that the classical model cannot account for augmentation and posttetanic potentiation quantitatively (Delaney and Tank 1994; Delaney et al. 1989). The difficulties encountered by the classical model in its attempt to account for different components of short-term plasticity can, however, be resolved by assuming that the affinities of the calcium binding sites of the secretion process are not equal (Bertram et al. 1996; Yamada and

Zucker 1992). If one introduces an additional high-affinity calcium binding step to the secretion process (Yamada and Zucker 1992) or assigns appropriate calcium affinities to individual calcium binding steps (Bertram et al. 1996), one can account for the magnitude and time course of facilitation while retaining the essence of the classical model.

Despite the elegance of mathematical modeling and imaging studies, and despite the recognition that calcium ions are essential for the activation of short-term synaptic enhancement, experimental results that provide direct clues to molecular events that underlie an increase of vesicular secretion during facilitation are still missing. One physiological approach to addressing this problem has been to search for changes in the kinetics of transmitter release during facilitation in the expectation that any observed kinetic change may provide insights into the molecular mechanism of facilitation. Several rigorous studies performed with the use of action-potential-based protocols (Datyner and Gage 1980) and the macropatch approach (Parnas et al. 1989) have conclusively demonstrated that there is no change in the kinetics of transmitter release during F_1 facilitation. Here we reinvestigate the question of transmitter release kinetics with the use of prolonged presynaptic pulses to monitor facilitation. We demonstrate that an acceleration in the kinetics of transmitter release emerges as the duration of presynaptic depolarization is lengthened.

METHODS

Animals and preparations

Crayfish (*Procambarus clarkii*) were obtained from Carolina Biological, Burlington, NC. Animals were maintained at 23°C until use. All experiments were performed at 15°C unless otherwise indicated. The typical size of the animals was ~3.8 cm head to tail. The opener muscle of the first walking leg was used for all experiments. Details of the experimental setup have been described before (Vyshedskiy and Lin 1997b). Briefly, a presynaptic voltage electrode penetrated a secondary axon near a branch point where a tertiary branch emerged. Meanwhile, a presynaptic current electrode penetrated the main branch point of the inhibitor. The distance between the two presynaptic electrodes was between 100 and 150 μm . Two postsynaptic electrodes penetrated a muscle fiber near the presynaptic voltage electrode. The electrode placements around the primary branch point limited the postsynaptic recordings to central muscle fibers. A GeneClamp 500 (Axon Instruments) was used for presynaptic voltage control and two IE-201 intracellular amplifiers (Warner Instrument) were used to record from muscle fibers. Data were digitized with the use of ITC-16 Mac interface (Instrutech). Pulse Control (J. Herrington and R. J. Bookman, University of Miami, Miami, FL) was used for the control of data acquisition. Experimental data were typically filtered at 1.5–2 kHz and digitized at 10 kHz. All the data analysis was performed in Igor (Wavemetrics).

Dissection and the initial presynaptic electrode penetrations were conducted in a control saline composed of (in mM) 195 NaCl, 5.4 KCl, 13.5 CaCl_2 , 2.6 MgCl_2 , and 10 sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (NaHEPES), pH 7.4. After presynaptic electrode penetration, the control saline was replaced by an experimental solution composed of (in mM) 155 NaCl, 40 tetraethylammonium (TEA) chloride, 5.4 KCl, 10 CaCl_2 , 6.1 MgCl_2 , 10 NaHEPES, 1 4-aminopyridine, and 300 nM tetrodotoxin, pH 7.4. Addition of the other potassium channel blockers charybdotoxin (150 nM) or apamin (5 μM) did not cause any

detectable change in the recording characteristics of pre- or postsynaptic cells. The access of apamin was verified by its ability to uncover a long-lasting (~200-ms) afterdepolarization activated by a broadened presynaptic action potential in the presence of 40 mM TEA and 1 mM 4-aminopyridine. All chemicals were purchased from Sigma, except for charybdotoxin and apamin, which were ordered from Research Biochemicals International. Morphological examination of the terminal branches that innervated the recorded muscle fiber was performed after each experiment by sketching or photography (Vyshedskiy and Lin 1997b).

Experimental protocols

Only the F_2 component of facilitation is analyzed in this report. This was achieved by delivering the test pulse 150 ms after the end of a conditioning stimulus. This delay is sufficiently long to ensure that the F_1 component of facilitation, which has an averaged decay time constant of 19 ms (Vyshedskiy and Lin 1997a), has decayed to an insignificant level. All the recordings reported here, except those in Fig. 6, were obtained with the use of a protocol in which F_2 facilitation was activated by eight subthreshold conditioning pulses. (See Fig. 1B for example.) It has been demonstrated that this conditioning stimulus can activate a near-maximal level of F_2 facilitation without triggering significant transmitter release. In addition, this protocol does not activate augmentation or posttetanic potentiation (Vyshedskiy and Lin 1997a). This protocol eliminates possible complications caused by transmitter release triggered by the conditioning stimulus, such as transmitter depletion or postsynaptic receptor sensitization or desensitization. Accumulation of F_2 facilitation was minimized by lowering the repeat rate of experimental protocols to ≤ 0.1 Hz. To minimize data variation between different preparations, the level of test pulse depolarization was stepped to 0 mV in all the experiments reported here unless indicated otherwise in figure legends.

As in previous studies, the preparations used for data analysis were selected on the basis of their presynaptic space constants estimated from depolarization-release coupling curves (Vyshedskiy and Lin 1997a,b). Only those preparations with a presynaptic space constant ≥ 8 times than their actual length were selected for statistical analysis.

Measurement of postsynaptic responses

The use of chloride-containing electrodes in postsynaptic recordings increased intramuscular chloride concentration, which resulted in depolarizing inhibitory postsynaptic potentials (IPSPs). Because of the relatively small chloride driving force (ΔE_{Cl}) in the crayfish muscle fibers (10 ~ 15 mV) (Vyshedskiy and Lin 1997b), IPSP amplitude may not linearly reflect the quantity of transmitter release (Martin 1955). Meanwhile, signal-to-noise ratio considerations prohibited us from performing simultaneous pre- and postsynaptic voltage clamp. We therefore only performed postsynaptic current clamp and used the two postsynaptic electrodes to routinely measure the chloride equilibrium potential, the input resistance (R_m), and the time constant of the muscle fiber under investigation (τ_m). Knowledge of the ΔE_{Cl} , i.e., the difference between resting membrane potential and chloride equilibrium potential, allows one to correct for the nonlinear summation of IPSP peak amplitudes (Martin 1955). In addition, we were also interested in extracting information on the kinetics of transmitter release, which would dictate the time course of inhibitory postsynaptic conductance (IPSG). It is possible to reconstruct IPSG from IPSP if one has information on the R_m , τ_m , and ΔE_{Cl} of the muscle fiber under investigation (Vyshedskiy and Lin 1997b). The conversion can be derived from a circuit model of the muscle fiber membrane (Vyshedskiy and Lin 1997b) and requires two assumptions: 1) that the muscle fibers are isopotential and 2) that IPSPs do not activate voltage-sensitive currents. The isopotential assumption is

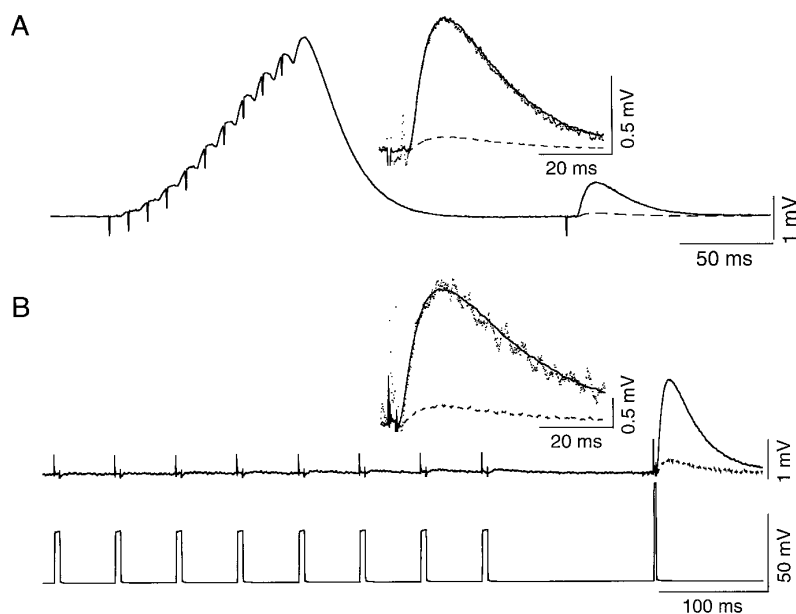


FIG. 1. Time course of inhibitory postsynaptic potential (IPSP) is not changed during facilitation if the duration of test pulses is brief. *A*: control IPSPs (IPSP_{cnt}) and facilitated IPSPs ($\text{IPSP}_{\text{test}}$) mediated by action potentials exhibit an identical time course. Facilitation is activated by a burst of 10 action potentials and the F_2 component is monitored by a test action potential delivered 150 ms after the conditioning train. There is a 7-fold increase in transmitter release during facilitation. *A, inset*: comparison between IPSP_{cnt} and $\text{IPSP}_{\text{test}}$ on an expanded scale. Time course of $\text{IPSP}_{\text{test}}$ (—) is exactly the same as that of IPSP_{cnt} (---) when scaled IPSP_{cnt} (···) is compared with $\text{IPSP}_{\text{test}}$. This experiment was performed in control saline and action potentials were activated by a suction electrode. Chloride driving force (ΔE_{Cl}) = 16 mV. *B*: time course of $\text{IPSP}_{\text{test}}$ is not changed when facilitation is probed by controlled presynaptic pulses with a waveform similar to that of an action potential. A burst of 8 subthreshold conditioning pulses is used to activate facilitation. Paired transients associated with individual conditioning pulses are artifacts. Facilitation is probed by a 2-ms pulse depolarized to 0 mV and the facilitated IPSP amplitude is 7.5 times larger than that of IPSP_{cnt} after the nonlinear summation is corrected for. *B, inset*: time course of IPSP_{cnt} (---; ··· after scaling) is identical to that of $\text{IPSP}_{\text{test}}$ (—). ΔE_{Cl} = 14.7 mV, input resistance (R_m) = 0.9 M Ω , time constant of muscle fiber (τ_m) = 18.5 ms. Traces in *A* and *B* are averages of 50 trials.

valid because it has been shown that muscle fibers in small crayfish are nearly isopotential (Wojtowicz and Atwood 1986). This is even more true under our experimental conditions, where a high concentration of potassium channel blockers further increases R_m (Vyshedskiy and Lin 1997b). Voltage-sensitive currents are not likely to be activated by IPSPs because their amplitudes are typically small (<10 mV) and the resting membrane potential of muscle fibers is high (−70 to −80 mV) (Araque and Buno 1995). The validity of converting IPSP to IPSPG has been demonstrated previously by comparing the time course of an IPSC calculated from IPSPG with that of an IPSC measured with the use of a two-electrode voltage clamp (Vyshedskiy and Lin 1997b). In this report, changes in transmitter release kinetics are inferred from the IPSPG time course. When nonlinear summation is not an issue, as in the measurement of synaptic delay or in the cases in which IPSP amplitude was <15% of ΔE_{Cl} , we directly compare the IPSP before and after facilitation (McLachlan and Martin 1981). IPSP traces displayed in the figures have not been corrected. The effect of nonlinear summation has been corrected for with respect to IPSP amplitude plotted in all the graphs in this report. ΔE_{Cl} , R_m , and τ_m of individual experiments are listed in the figure legends.

RESULTS

Changes in transmitter release kinetics during facilitation

The kinetics of neurotransmitter release can be inferred from the time course of postsynaptic potential. A comparison of the IPSP time course before and after facilitation should reveal whether there has been a change in transmitter release kinetics. Similar to the results observed in previous studies (Datyner and Gage 1980; Parnas et al. 1989), the facilitated IPSP ($\text{IPSP}_{\text{test}}$) mediated by an action potential of the crayfish inhibitor has a time course identical to that of the control IPSP (IPSP_{cnt}). Figure 1*A* illustrates an example where facilitation is activated by a burst of 10 action potentials and monitored 150 ms after the burst. When IPSP_{cnt} (---) is scaled to the same height as $\text{IPSP}_{\text{test}}$ (—), it is clear that there is no detectable difference in the time course of the two IPSPs (Fig. 1*A, inset*; ··· is scaled IPSP_{cnt}). The same

phenomenon can be recreated with the use of the presynaptic voltage-control method. Figure 1*B* shows an example where eight subthreshold conditioning pulses are used to activate facilitation and a 2-ms pulse, depolarized to 0 mV, is used to monitor facilitation 150 ms later. The test pulse waveform approximates that of a presynaptic action potential at 15°C. A comparison of the IPSP_{cnt} and $\text{IPSP}_{\text{test}}$ time courses reveals no significant difference in their shapes (Fig. 1*B, inset*). Similar results were obtained in nine additional preparations. Therefore the presynaptic voltage-control technique is able to recreate the same characteristics of facilitation as are revealed with action potentials.

A change in the kinetics of facilitated transmitter release emerges if the duration of the test pulse is lengthened. Figure 2*A* shows pre- and postsynaptic recordings of control and facilitated responses mediated by a 20-ms test pulse. The waveform of the control, presynaptic test pulse (---) is

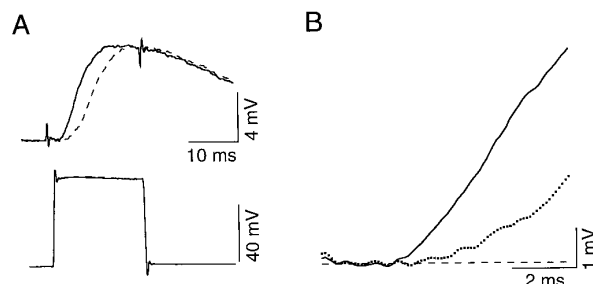


FIG. 2. Prolonged presynaptic depolarization reveals a change in the kinetics of transmitter release during facilitation. *A*: change in the transmitter release time course becomes apparent if a presynaptic pulse with a duration of 20 ms is used to probe facilitation. Peak amplitude of facilitated IPSP (—) is similar to that of IPSP_{cnt} (---). However, the $\text{IPSP}_{\text{test}}$ time course moves to the left. *A, bottom*: presynaptic recordings. Line styles of pre- and postsynaptic recordings are matched. *B*: acceleration in release kinetics does not modify the minimal synaptic delay. Initial rising phases of IPSP_{cnt} (···) and $\text{IPSP}_{\text{test}}$ (—), shown in *A*, are compared on an expanded time scale. Given the presence of background noise, it is difficult to determine whether the moment at which the 2 IPSPs deviate from the baseline (---) differs. ΔE_{Cl} = 11.6 mV, R_m = 0.78 M Ω , τ_m = 8.3 ms

indistinguishable from that of the test pulse delivered after conditioning stimulus (—; Fig. 2A, bottom). The amplitude of $IPSP_{test}$ (—) is not significantly different from that of $IPSP_{cnt}$ (- - -). The $IPSP_{test}$ time course, however, is shifted to the left. This shift represents an acceleration in the kinetics of transmitter release during facilitation. The leftward shift is not achieved by simply moving the $IPSP_{cnt}$ trace in a parallel manner, because the minimal synaptic delay does not seem to be affected by the facilitation process. Figure 2B shows the initial rising phase of $IPSP_{cnt}$ and $IPSP_{test}$ on an expanded scale. $IPSP_{test}$ exhibits a faster initial rate of rise than does $IPSP_{cnt}$. Given the signal-to-noise ratio of the recordings, it is difficult to conclusively determine whether there is a difference in the point in time at which the two IPSPs take off. This was a consistent finding in 12 preparations.

The transition from no change in release kinetics but a large change in IPSP amplitude to no change in amplitude but a significant change in kinetics is illustrated in Fig. 3. Superimposed $IPSP_{cnt}$ (- - -) and $IPSP_{test}$ (—) activated by 2-, 5-, and 20-ms test pulses are shown in Fig. 3, A1–A3, respectively. The increase in $IPSP_{test}$ amplitude becomes less pronounced as the test pulse duration increases. As with the result shown in Fig. 1B, $IPSP_{cnt}$ mediated by a 2-ms pulse has the same time course as does $IPSP_{test}$; compare the scaled $IPSP_{cnt}$ (dotted trace) to $IPSP_{test}$ (—) in Fig. 3B1. To obtain a quantitative comparison of transmitter release kinetics, the effects of nonlinear summation on the IPSP time course have to be corrected for. This is achieved by comparing IPSP time courses. Figure 3, B2 and B3,

illustrates $IPSG_{cnt}$ (- - -) and $IPSG_{test}$ (—) converted from IPSPs in Fig. 3, A2 and A3, respectively. In Fig. 3B2, the $IPSG_{cnt}$ mediated by a 5-ms test pulse is scaled (dotted trace) to the same height as $IPSG_{test}$ to illustrate that $IPSG_{test}$ exhibits characteristics intermediate between those mediated by 2- and 20-ms test pulses. Specifically, the $IPSG_{test}$ mediated by a 5-ms test pulse exhibits an increase in IPSP amplitude, but the increase is proportionally less than that mediated by the 2-ms pulse (Fig. 3B1). Meanwhile, the shift in the $IPSG_{test}$ time course is not as pronounced as that mediated by the 20-ms pulse (Fig. 3B3).

Quantitative analysis of the changed release kinetics was achieved by measuring the difference in time, at the half-maximal point, between $IPSG_{cnt}$ and $IPSG_{test}$ (Fig. 3B3, □; release shift). A second parameter that was analyzed quantitatively is the normalized change in total transmitter release calculated from the IPSP area (total release). The total release measured during facilitation was then normalized by control total release to provide an estimate of normalized facilitation (F_n). Figure 3C summarizes the transition of the release shift and F_n , measured from the synapse shown in Fig. 3, A and B, as test pulse duration increases. The relative increase in total release declines as the test pulse duration increases. Meanwhile, the release shift increases and reaches a plateau as the test pulse duration increases. To illustrate the transition statistically, the release shift and F_n averaged from 12 preparations are summarized in Fig. 3D. Therefore the facilitation process accelerates the kinetics of vesicular release without changing the total amount of transmitter secretion activated by a 20-ms pulse.

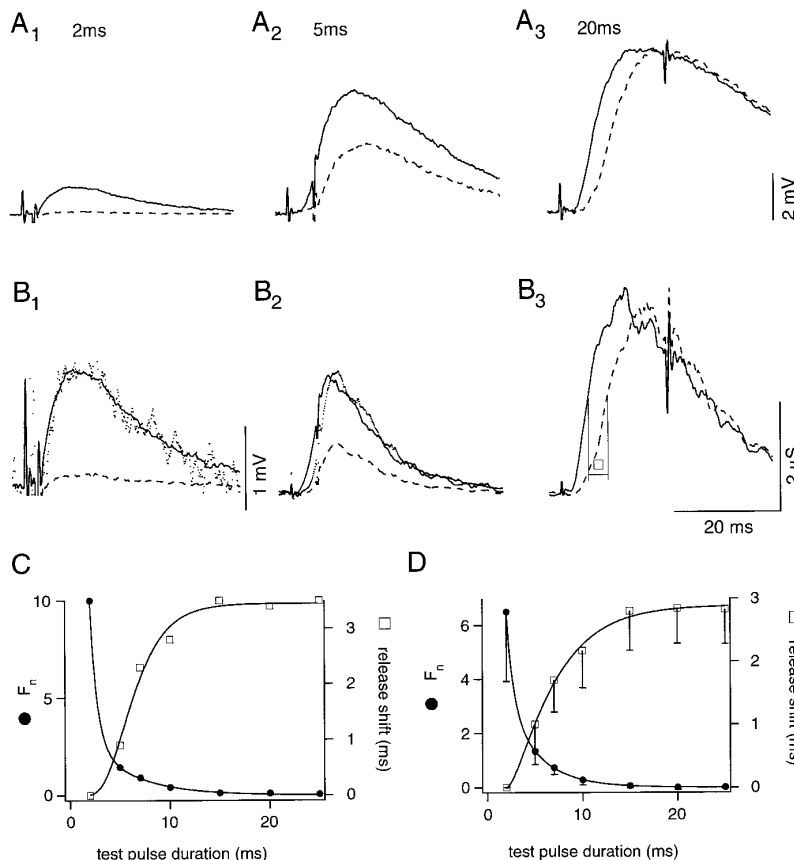


FIG. 3. An acceleration in transmitter release kinetics during facilitation becomes apparent as the duration of test pulses is increased. A1–A3: effect of test pulse duration on the time course of $IPSP_{cnt}$ (- - -) and $IPSP_{test}$ (—). Recordings were obtained from the same synapse used in Fig. 2. All the traces in A share the same vertical calibration. Time calibration in B is shared by traces in A. B1: IPSPs in A1 at a high magnification; dotted trace represents scaled $IPSP_{cnt}$. B2 and B3: inhibitory postsynaptic conductance (IPSPG) traces converted from the corresponding IPSPs in A. Dotted trace in B2: scaled $IPSG_{cnt}$. Quantitation of the change in kinetics is achieved by measuring the time difference at the half-maximal point between $IPSG_{cnt}$ and $IPSG_{test}$ (B3, □). IPSPs tested with a 2-ms pulse were not converted to IPSPG because the effect of nonlinear summation on small IPSPs is minimal. In addition, the conversion procedure introduces considerable noise, which renders IPSPGs derived from small signals unrecognizable. Therefore normalized facilitation (F_n) and release shift calculated for 2-ms points were obtained from IPSP rather than IPSPG traces (see following text). C: effects of test pulse duration on F_n and release kinetics. F_n estimates fractional changes in total transmitter release. It is calculated by integrating the IPSPG trace, and the fractional change of the area during facilitation is then calculated as $F_n = (IPSG_{test} \text{ area} / IPSPG_{cnt} \text{ area}) - 1$. F_n decreases with increasing test pulse duration, whereas the release shift becomes more apparent with test pulses of longer duration. D: averaged results illustrating the transition in F_n and the release shift as a function of test pulses duration. Means \pm SD are shown for all the data points. Data are pooled from 12 preparations and sample sizes are different for individual points. Number of samples averaged for 2, 5, 7, 10, 15, 20, and 25 ms: 10, 12, 8, 12, 12, 12, and 12, respectively.

Early component of transmitter release identified by a prolonged presynaptic depolarization

The observation that the total release mediated by 20-ms pulses is not modified by the facilitation process suggests that it may represent a distinct component during prolonged transmitter release. To identify the presence of multiple components of transmitter release during a continuous release, the duration of the presynaptic step was increased to 300 ms. Figure 4A shows the presynaptic recording and IPSP obtained from such an experiment. The initial transient component (---) rises rapidly and reaches its maximal level in ~ 10 ms (early component). The early component is distinctly different from the steady-state component (—), which persists for up to 300 ms in this preparation and up to 10 s in other preparations.

The early component was further characterized by changing the duration of presynaptic steps systematically. Recordings in Fig. 4B are superimposed IPSPs activated by 5-, 10-, and 20-ms pulses depolarized to 0 mV. The peak amplitude and total area of IPSP increase relatively little when the pulse duration is lengthened from 10 to 20 ms (compare — and $\cdot\cdot\cdot$). Furthermore, the IPSP mediated by the 20-ms pulse starts to decline before the end of the pulse. Both observations suggest that 20-ms steps have released most of the vesicles releasable by

the early component. The total IPSP area plotted against presynaptic pulse duration, calculated from the synapse shown in Fig. 4B1, follows a single-exponential curve with a time constant of 6.9 ms (Fig. 4B2). Averaged results obtained from 12 preparations, normalized by the IPSP area activated by a 20-ms pulse, also follow a single-exponential curve with a time constant of 9.4 ms (Fig. 4B3). The time constant of 9.4 ms suggests that a 20-ms pulse, depolarized to 0 mV, activates the release of almost 90% of the transmitter content of the early component. Therefore transmitter release activated by 20-ms pulses should correspond to the early component.

Correlation between release shift and facilitation as measured by IPSP amplitude increases

To provide supporting evidence for the hypothesis that the change in the kinetics of transmitter release is indeed a reflection of the synaptic facilitation process, we tried to correlate release shift, monitored by 20-ms test pulses, and IPSP amplitude change, monitored by 5-ms test pulses, under two experimental conditions. First we monitored the decay of the two parameters after a constant conditioning stimulus. Figure 5A illustrates superimposed $IPSP_{\text{cnt}}$ (---) and $IPSP_{\text{test}}$ (—) mediated by a 5-ms test pulse. The peak IPSP amplitudes, after the nonlinear summation was corrected for (Martin 1955), were then used to calculate F_n . Decay of F_n

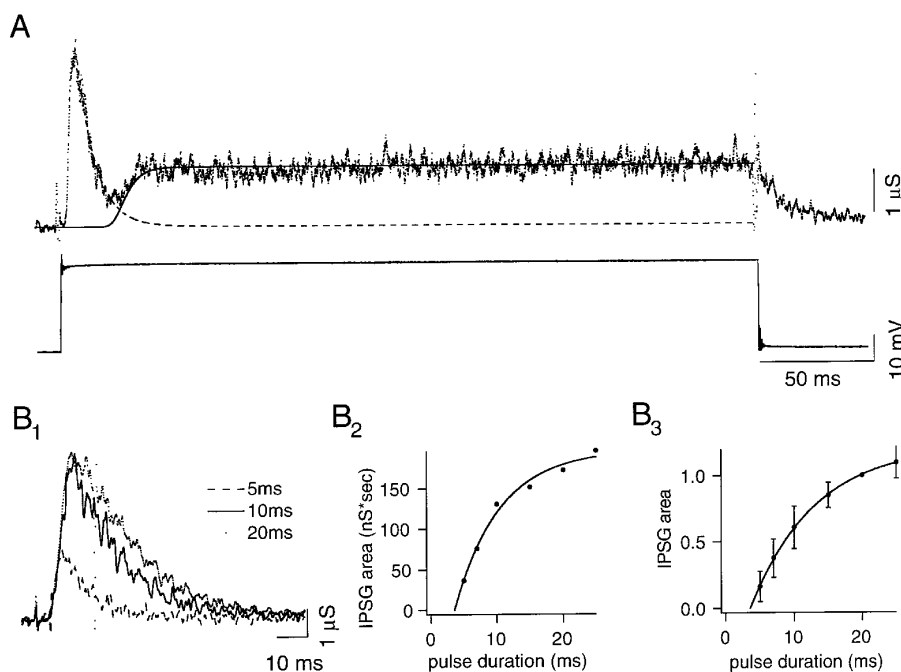


FIG. 4. Identification of the early component of a continuous transmitter release. A: IPSP (*top trace*) activated by a 300-ms presynaptic pulse (*bottom trace*) exhibits 2 distinct components of transmitter release: an early and transient component (---) and a steady-state component (—). Release time course of the early component is estimated by extrapolating the initial decline of the transient component by a single-exponential decay. Steady component was obtained by subtracting the early component from the IPSP trace. This experiment was performed at 23°C. $\Delta E_{\text{Cl}} = 9.2$ mV, $R_m = 0.9$ M Ω , $\tau_m = 10.1$ ms. B1: superimposed IPSPs activated by 5-, 10-, and 20-ms pulses. Total transmitter release does not increase proportionally with increasing test pulse duration. $\Delta E_{\text{Cl}} = 14.6$ mV, $R_m = 0.9$ M Ω , $\tau_m = 18.5$ ms. B2: total transmitter release plotted against presynaptic pulse duration. Total transmitter release is estimated by integrating the entire IPSP trace, i.e., IPSP area. Data were calculated from the same synapse as that shown in B1. B3: normalized total transmitter release, averaged from 12 preparations, plotted against presynaptic pulse duration. Data from individual experiments were normalized by the IPSP area activated by a 20-ms pulse before the average of individual duration points was calculated. Number of preparations used to calculate the 5-, 7-, 10-, 15-, 20-, and 25-ms points: 12, 8, 12, 12, 12, and 12, respectively.

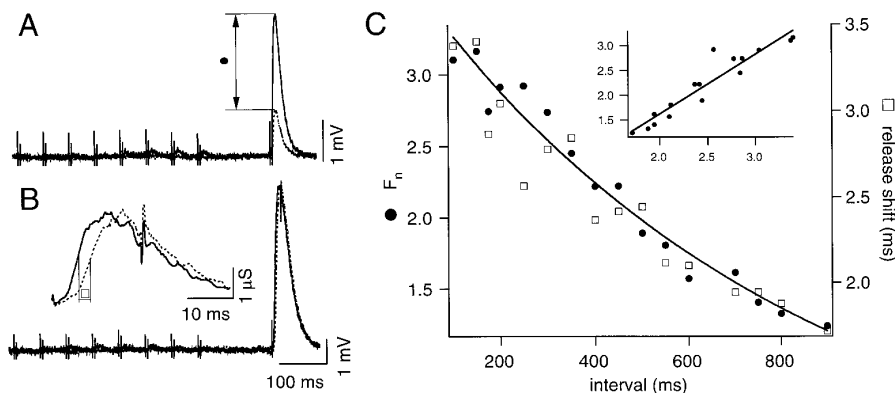


FIG. 5. Correlation between release shift and IPSP amplitude change during the decay of F_2 facilitation. *A*: facilitation activated by 8 subthreshold conditioning pulses and monitored by a 5-ms test pulse depolarized to -24 mV. $IPSP_{\text{cnt}}$ (---) and $IPSP_{\text{test}}$ (—) are superimposed. Filled circle: change in IPSP amplitude. The last 4 conditioning pulses triggered small IPSPs, but such small release does not affect the measurement of facilitation. *B*: facilitation activated by the same conditioning stimuli and monitored by a 20-ms test pulse depolarized to 0 mV. *B, inset*: IPSP traces converted from the IPSPs; open square identifies measurement of release shift. *C*: decay time course of release shift (open squares) and F_n (filled circles). Decay is fitted by a single-exponential function with a time constant of 800 ms. *C, inset*: F_n (*Y*-axis) plotted against release shift (*X*-axis). Straight line: linear regression fit. Effect of nonlinear summation was corrected for before F_n was calculated. $F_n = (IPSP_{\text{test}} - IPSP_{\text{cnt}})/IPSP_{\text{cnt}}$. $\Delta E_{\text{Cl}} = 11$ mV, $R_m = 0.70$ M Ω , $\tau_m = 7.5$ ms. Because of signal-to-noise ratio considerations, 5-ms test pulses were used to monitor amplitude changes. Test pulses of 2 ms provided similar results, but the IPSPs in this case required considerable averaging to generate measurable recordings. (See Fig. 1 legend and Vyshedskiy and Lin 1997b).

is plotted in Fig. 5C (filled circles). Figure 5B illustrates recordings in which facilitation was activated by the same protocol but a 20-ms test pulse was used to monitor the facilitation. The release shift (Fig. 5, B and C, open squares) follows a decay time course similar to that of F_n . The inset in Fig. 5C shows the correlation analysis, where F_n is plotted against release shift, of the two parameters with a correlation coefficient of 0.92 ($P < 0.001$). Similar correlation analysis in eight additional preparations provided consistent results. The values of the correlation coefficients ranged from 0.78 to 0.98 (all statistically significant; Table 1).

A second approach with which to establish the correlation between the release shift and F_n was to actively change the strength of the conditioning stimulus. Figure 6A shows an example in which a single conditioning stimulus, which trig-

gers a significant conditioning IPSP ($IPSP_{\text{cod}}$; \circ), activated a substantial increase in $IPSP_{\text{test}}$ (filled circle) mediated by a 5-ms test pulse. As the amplitude of the conditioning pulse is changed systematically, facilitation, monitored by the same 5-ms test pulse, also varies accordingly. In Fig. 6C, F_n (filled circles) is plotted against the level of depolarization of the conditioning pulse (V_{cod}). $IPSP_{\text{cod}}$ (\circ) is also plotted for comparison. Similar to results published previously (Vyshedskiy and Lin 1997a), significant facilitation appears before the conditioning pulse triggers a measurable release. Furthermore, facilitation reaches a plateau before the $IPSP_{\text{cod}}$ reaches its maximal level. Facilitation activated by the same series of conditioning stimuli was also monitored with a 20-ms test pulse (Fig. 6B). Release shifts measured from IPSPs are plotted in Fig. 6C (\square). F_n and the release shift exhibit an identical dependence on the level of V_{cod} . Similar to the results of the correlation analysis performed during the decay of facilitation, the correlation between F_n and the release shift is highly significant in this protocol (Fig. 6C, inset; correlation coefficient = 0.98, $P < 0.001$). Statistical results obtained from a total of eight preparations are shown in Table 1. The highly significant correlation between release shift and F_n observed under two experimental conditions strongly supports the hypothesis that the release shift and the increase in IPSP amplitude during facilitation are mediated by the same mechanism.

DISCUSSION

We have exploited the flexibility of the presynaptic voltage-control method to investigate the effects of test pulse duration on the expression of F_2 facilitation. The main finding in this report is that prolonged test pulses uncover an acceleration in transmitter release kinetics during facilitation. Although previous studies performed with the use of action potential or brief presynaptic pulses have shown no detectable change in release kinetics during facilitation, our

TABLE 1. Statistical analysis of the correlation between release shift and IPSP amplitude changes during facilitation

Experiment	Type of Correlation	Number of Data Points	Correlation Coefficient	Statistical Significance
41896	Activation	14	0.942	<0.001
50996	Activation	15	0.975	<0.001
53096	Activation	15	0.94	<0.001
61896	Activation	17	0.924	<0.001
70296	Activation	16	0.95	<0.001
70996	Activation	17	0.98	<0.001
73096	Activation	16	0.96	<0.001
73196	Activation	19	0.98	<0.001
61395	Decay	15	0.86	<0.001
61995	Decay	20	0.9	<0.001
71195	Decay	18	0.91	<0.001
50996	Decay	16	0.982	<0.001
51696	Decay	16	0.894	<0.001
61396	Decay	7	0.78	<0.05
61896	Decay	16	0.94	<0.001
70296	Decay	16	0.92	<0.001
70996	Decay	15	0.943	<0.001

IPSP, inhibitory postsynaptic potential.

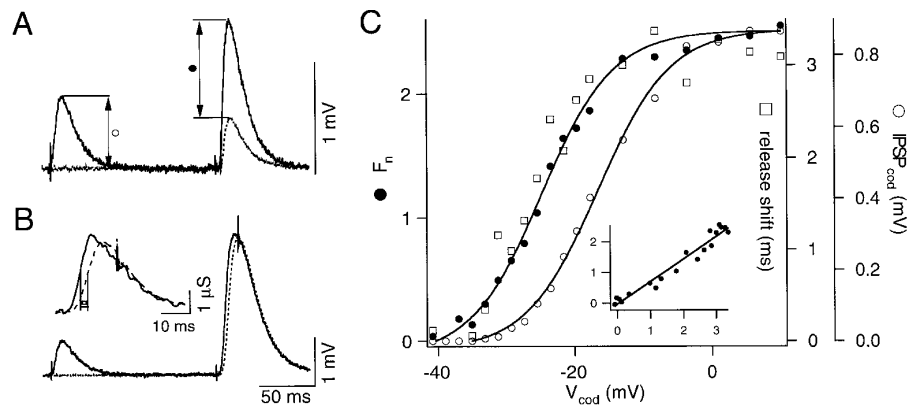


FIG. 6. Correlation between release shift and facilitation magnitude as the magnitude of facilitation is modulated by conditioning pulse amplitude. *A*: facilitation activated by a suprathreshold conditioning pulse and monitored by a 5-ms test pulse depolarized to -13 mV. The conditioning pulse activates a significant IPSP (\circ). IPSP_{ent} (---) and $\text{IPSP}_{\text{test}}$ (—) are superimposed; filled circle identifies the change in IPSP amplitude. *B*: facilitation activated by the same conditioning pulse and monitored by a 20-ms test pulse depolarized to 0 mV. *B*, inset: IPSP traces converted from IPSPs. *C*: dependence of release shift (\square) and F_n (filled circles) on the level of conditioning pulse depolarization (V_{cod}). F_n and release shift exhibit an identical dependence on V_{cod} . Amplitude of conditioning IPSPs (\circ) is also plotted for comparison. *C*, inset: F_n (Y -axis) plotted against release shift (X -axis). Straight line: linear regression fit. Effect of nonlinear summation was corrected for before F_n was calculated. $\Delta E_{\text{Cl}} = 6.4$ mV, $R_m = 0.37$ M Ω , $\tau_m = 8.8$ ms.

results do not contradict these findings. This is because the facilitation process does not change the minimal synaptic delay. Therefore it would have been difficult for the previous studies to detect significant changes in release kinetics within the brief time window of observation allowed by action potentials or brief presynaptic steps. In addition, the facilitation process did not increase the total amount of transmitter release activated by 20-ms pulses. The analysis of the relationship between total transmitter release and test pulse duration suggests that a 20-ms pulse is sufficiently long to release up to 90% of an early component of continuous transmitter release. We conclude that the facilitation process accelerates release kinetics without modifying the total amount of transmitter released by the early component.

Possible sources of error

One possible explanation for the observed change in release kinetics during facilitation is that the conditioning stimulus could significantly modify the membrane properties of the inhibitor such that a “sagging” occurs, at the release sites, during the later phase of the 20-ms test pulse. Such sagging, activated by the conditioning stimulus, could truncate transmitter release during the later phase of a 20-ms pulse. As a result, $\text{IPSP}_{\text{test}}$ would appear to have accelerated release kinetics. This scenario could be caused by residual voltage- or calcium-activated potassium (K^+) current activated by the conditioning stimulus. This hypothesis is unlikely for three reasons. First, the amplitude of 20-ms test pulses (Fig. 2) and associated voltage-clamp current (data not shown) did not exhibit any significant change during facilitation. Our observations are consistent with a recent study of presynaptic calcium current in the excitor and inhibitor of the crayfish opener muscle (Wright et al. 1996b). The latter study reported that there is no change in the leakage current measured with a paired-pulse protocol with an interpulse interval of 15 ms. This observation suggests that there must be minimal residual K^+ current within the short

interpulse interval. Because the study was performed in an extracellular solution that was virtually identical to that used in our experiments, it would then be reasonable to suggest that the likelihood of a significant amount of residual K^+ current being present 150 ms later is also minimal. Second, pharmacological evidence suggests that there is very little residual K^+ current in our system that would be capable of altering the cable properties of the inhibitor. For example, BK-type calcium-dependent potassium current [$I_{\text{K}(\text{Ca})}$] is unlikely to play a role in our experiments, because our recordings were performed in the presence of 40 mM TEA, which should completely block BK-type $I_{\text{K}(\text{Ca})}$ (Sivaramakrishnan et al. 1991). In addition, studies at the crayfish excitor have demonstrated that the decay time constant of BK-type current is in the range of 20 ms, and should therefore have decayed to an insignificant level by the time our test pulses were delivered 150 ms later (Blundon et al. 1993). Although it remains possible that long-lasting SK-type $I_{\text{K}(\text{Ca})}$ could cause a significant modification of presynaptic membrane resistance 150 ms after the conditioning stimulus, bath application of 5 μM apamin, which is known to block SK-type K^+ channels, did not change our experimental results. Third, using the depolarization-release coupling relationship as a guide, we have shown that there is no change in the presynaptic space constant during the time window of F_2 facilitation (Vyshedskiy and Lin 1997a). Because one of the physiological variables that determines the space constant is membrane resistance, the unchanged space constant during F_2 facilitation suggests that there is no change in membrane resistance during the same period. These independent lines of evidence make it reasonable to suggest that the waveform of 20-ms depolarizing pulses is not changed by the conditioning stimulus. As a result, the release shift activated by presynaptic pulses with an invariant waveform must arise from mechanisms underlying the facilitation process.

A second possible cause for concern is that the exact events that occur during a 20-ms pulse are not understood. A continuous calcium influx is able to trigger continuous

secretion of synaptic vesicles. Meanwhile, the calcium influx that occurs at the beginning of the pulse may facilitate the release process that happens near the end of the pulse (Augustine et al. 1985). In addition, vesicular depletion and resupply processes may also occur before the end of a 20-ms pulse. A precise description of such dynamic and complex events is beyond our current understanding of synaptic physiology. By comparing IPSP_{cnt} and $\text{IPSP}_{\text{test}}$ activated by the same test pulse, we have treated the underlying complexity as a "constant background." In other words, as long as the test pulse waveforms at release sites remain constant before and during facilitation, the changes in release kinetics must be uniquely attributed to the process underlying F_2 facilitation.

Early component of transmitter release

Prolonged presynaptic depolarization of the crayfish inhibitor activates at least two components of transmitter release; a transient early component and a persistent late component. Results presented in this report only involve the early component. Our results, however, do not allow us to formulate the specific mechanism that underlies the transient nature of the early component. An understanding of the mechanism should provide insights to the facilitation process because the total release mediated by this component is not modified during facilitation. An obvious hypothesis to explain the transient nature of the early component would be that it is mediated by depletion of a readily available pool of vesicles. However, other competing hypotheses cannot be ruled out. For example, the decline in transmitter release before the end of a 20-ms pulse may be due to inactivation of calcium current (Wright et al. 1996a) or activation of a calcium-sequestering mechanism (Herrington et al. 1996). Both mechanisms could effectively lower the intracellular calcium concentration near the end of a prolonged depolarization. Alternatively, a recent study in the squid giant synapse suggests that the vesicular secretion machinery exhibits an adaptation process that terminates the release process regardless of the level of release or intracellular calcium concentration (Hsu et al. 1996). Any of these hypotheses, or a combination of them, could explain the time course of the early component. A resolution of these competing hypotheses would require further experiments. At this point, we can only conclude that the facilitation process does not involve a change in the mechanism that determines the total amount of transmitter released by the early component.

Mechanisms of synaptic facilitation as revealed by prolonged presynaptic pulses

An obvious explanation for the accelerated transmitter release reported here is the facilitation of calcium current kinetics. This hypothesis is unlikely for three reasons. First, it has been shown that P-type calcium channels mediate transmitter release at the crayfish neuromuscular junction (Araque et al. 1994; Wright et al. 1996a). Activity-dependent facilitation of P-type channels is not commonly observed. On the two occasions when such facilitation did occur, an extreme facilitation protocol with a +80-mV conditioning pulse was needed to activate a small facilitation

(Rheinallt and Lansman 1996; Song and Surmeier 1996). More importantly, it has been shown that the type of sub-threshold conditioning pulses used in our report failed to facilitate calcium channels of any type (Rheinallt and Lansman 1996). Second, calcium imaging studies of the crayfish excitor and inhibitor have not revealed any indication of activity-dependent facilitation of calcium current (Delaney and Tank 1994; Tank et al. 1995). Although action-potential-activated calcium influx examined in the imaging studies is different from the I_{Ca} activated by 20-ms steps, the comparison is not inappropriate for the present purpose. It should be noted that changes in the IPSP time course during facilitation happen on the rising phase of IPSPs. The facilitation of I_{Ca} must also happen during the initial rising phase if this mechanism is to be responsible for the accelerated release during facilitation. This type of facilitation of I_{Ca} must also increase calcium influx activated by action potentials because the number of calcium channel openings activated by an action potential is mainly determined by the initial rate of calcium channel opening (Llinás et al. 1982). Therefore the absence of calcium influx facilitation demonstrated by imaging methods strongly suggests that our results are not likely to be caused by calcium current facilitation. Furthermore, we have demonstrated that the characteristics of F_2 facilitation monitored by brief presynaptic pulses are similar to those activated by action potentials in terms of the decay time constant (Vyshedskiy and Lin 1997a) and the absence of changes in IPSP waveforms (Fig. 1). Therefore the findings reported here are intimately related to facilitation activated by action potentials and should be regarded as an extension of action-potential-based protocols. Third, direct measurement of calcium current in the crayfish excitor and inhibitor has demonstrated that there is no facilitation of presynaptic calcium current during the F_1 time window (Wright et al. 1996a). It seems reasonable to extrapolate this finding to the F_2 time window. These independent lines of evidence suggest that facilitation of calcium channel kinetics is an unlikely explanation for our results. It is then reasonable to attribute the release shift to an acceleration in the kinetics of transmitter release.

Although all existing experimental results suggest that facilitation is driven by residual $[\text{Ca}^{2+}]_i$, the exact molecular events involved in facilitation remain unknown (Zucker 1996). Recent mathematical models of synaptic facilitation, in which the presence of high-affinity calcium-binding sites of the secretion process is assumed to be responsible for facilitation (Bertram et al. 1996; Yamada and Zucker 1992), are not inconsistent with our results. For example, these models predict an insignificant change in synaptic delay during facilitation tested by brief pulses, which is similar to the results reported here and in other studies (Datyner and Gage 1980; Parnas et al. 1989). In addition, if the duration of presynaptic depolarization is increased in these models, it is possible to generate results similar to the release shift reported here. This line of logic is also consistent with recent findings that there is a decrease in the apparent calcium cooperativity of the release process during F_1 and F_2 facilitation (Vyshedskiy and Lin 1997a; Wright et al. 1996b). Although the consistency between our results and the mathematical models is compelling, a critical test of the models

will require a better understanding of the dynamics of continuous transmitter release.

The analysis of neurotransmitter release mediated by 20-ms depolarizations provides two new physiological parameters that are potentially useful for further investigation of facilitation mechanisms: release shift and total release mediated by the early component. The former approximates changes in release kinetics, whereas the latter provides a measurement for possible modulation in the total amount of transmitter released by the early component. In the analyses of synaptic facilitation based on action potential driven protocols, the amplitude of the postsynaptic response is the only parameter that can be measured. The two parameters reported here dissect the amplitude of postsynaptic responses into separate mechanistic components. If all the physiological manipulations known to enhance transmitter release were associated with only the release shift, then the utility of the two new parameters would be quite limited. Our preliminary studies on the effects of serotonin and okadaic acid have shown that these modulators achieve an increase in transmitter release by simultaneously creating a release shift and increasing total release (unpublished observations). These observations then suggest that each of the two physiological parameters could be mediated by a separate molecular mechanism and that the F_2 facilitation process selectively utilizes the one causing the release shift. Therefore the release shift and total release may prove to be highly discriminating parameters in the dissection of mechanisms underlying different types of synaptic enhancement.

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