Use-Dependent Decline of Paired-Pulse Facilitation at *Aplysia* Sensory Neuron Synapses Suggests a Distinct Vesicle Pool or Release Mechanism

Xue-Ying Jiang and Thomas W. Abrams

Departments of Pharmacology and Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland 21201-1559

We have characterized paired-pulse facilitation at *Aplysia* sensory neuron-to-motoneuron synapses. This simple form of very short-term synaptic plasticity displayed an unusual feature: it decreased dramatically with repeated testing. Synaptic depression at these synapses and this use-dependent decrease in paired-pulse facilitation occurred independently of each other. Paired-pulse facilitation was inversely correlated with the size of the initial synaptic connection and was absent at stronger synapses. The use-dependent decrease in paired-pulse facilitation occurred at the same rate at large synapses as at small synapses, although the initial paired-pulse facilitation at large synapses was substantially smaller. Rates of synaptic depression were also independent of initial synaptic strength. Paired-pulse facilitation was blocked by presynaptic EGTA injection, but not by postsynaptic EGTA or BAPTA injection. These results indicate that presynaptic Ca\(^{2+}\) influx plays a critical role in paired-pulse facilitation. However, the persistence of the decrease in paired-pulse facilitation for longer than 15 min suggests that Ca\(^{2+}\) from the first paired action potential produces facilitation via a modulatory mechanism rather than by sum-mating with Ca\(^{2+}\) influx during the second paired action potential in activating the Ca\(^{2+}\) binding sites that initiate exocytosis. This modulatory mechanism may not involve protein phosphorylation because paired-pulse facilitation was unaffected by the protein kinase inhibitors H7 and KN-62. These findings further suggest that release by the second paired action potential occurs at sites distinct from those that mediate release by the first action potential.

**Key words:** synaptic plasticity; facilitation; synaptic depression; *Aplysia*; paired-pulse facilitation; calcium; phosphorylation


The experimental results that are cited were obtained with support from National Institutes of Health Grant MH 55880 to T.W.A. We thank Jonathan Cohen for critically reading and commenting on this manuscript and Marc Klein and Steve Siegelbaum for their helpful discussions of these findings. Allison Lin provided extensive assistance with data analysis and figures.

Correspondence should be addressed to Dr. Thomas W. Abrams, Department of Pharmacology, University of Maryland School of Medicine, BRB 4-002, 655 West Baltimore Street, Baltimore, MD 21201-1559.

Copyright © 1998 Society for Neuroscience 0270-6474/98/1810310-10$05.00/0

---

Paired-pulse facilitation is a very short-lived form of use-dependent synaptic plasticity observed at synapses across diverse phylogenetic groups (Katz and Miledi, 1968; Zengel et al., 1980; Zengel and Magleby, 1982; Atwood and Wojtowicz, 1986; Zucker, 1989; Fisher et al., 1997). In paired-pulse facilitation, transmitter release evoked by a second presynaptic action potential is enhanced when it follows a previous action potential within tens or hundreds of milliseconds. Because paired-pulse facilitation has been believed to represent a mechanistically simple presynaptic effect of residual Ca\(^{2+}\) (Katz and Miledi, 1968; Younkin, 1974; Regehr et al., 1994) and because its brief duration enables it to be induced repeatedly, this phenomenon is commonly monitored as an index of presynaptic alterations during more persistent and complex forms of synaptic plasticity. For example, more persistent enhancement of transmitter release may be accompanied by a decrease in paired-pulse facilitation (McNaughton, 1982; Muller et al., 1988; Staubli et al., 1990; Zalutsky and Nicoll, 1990). A decline in the paired-pulse ratio after the induction of some forms of long-term potentiation (LTP) in hippocampus is believed to suggest an increase in release probability. One way this might occur is if both of the paired action potentials access the same limited pool of releasable vesicles, so that an increase in release evoked by the first action potential would be accompanied by a decrease in release evoked by the second. Alternatively, if the probability of release by the second action potential is already very high, any substantial increase in the probability of release by the first action potential will result in a decrease in paired-pulse facilitation (Stevens and Wang, 1995), even though the amplitude of the second EPSP may remain unchanged. The paired-pulse ratio frequently increases with synaptic depression, either because less of the releasable pool is depleted by the first stimulus or because the probability of release by the second stimulus remains high.

The synaptic connections from mechanosensory neurons (SNs) to motoneurons (MNs) in *Aplysia* have provided an attractive and extensively studied system for analyzing a number of forms of associative and nonassociative synaptic plasticity, including posttetanic potentiation (Walters and Byrne, 1984; Schacher et al., 1990; Goldsmith and Abrams, 1991; Clark and Kandel, 1993; Bao et al., 1997). However, paired-pulse facilitation has not been characterized at these synapses. In the present study we have found that paired-pulse facilitation at *Aplysia* SN synapses is an extremely labile phenomenon, which is altered dramatically by the previous history of testing. These results suggest that, at least in this system, paired-pulse facilitation is a more complex phenomenon than has been understood previously. In addition, these results provide new insights into both the process of synaptic depression at these SN synapses and the mechanisms underlying variability in the initial strength of these connections.
MATERIALS AND METHODS

Electrophysiology. Aplysia californica, weighing 70–200 gm [obtained from Alacrity (Redondo Beach, CA) or Marinus (Long Beach, CA)] were anesthetized by injection with isotonic MgCl₂. Abdominal ganglia were removed and the ventral surface of the left hemiganglion was desheathed in a 1:1 mixture of MgCl₂ and artificial seawater. Ganglia were superfused at room temperature with high-divalent saline (6 × normal Ca²⁺; 1.6 × normal Mg²⁺) (Goldsmith and Abrams, 1991) to reduce spontaneous synaptic activity: (in mM) 328 NaCl, 10 KCl, 66 CaCl₂, 88 MgCl₂, and 10 Na-HEPES, pH 7.6, supplemented with nutrients [7 mM glucose, MEM essential and nonessential amino acids (0.2 × normal concentration; Life Technologies, Gaithersburg, MD), and MEM vitamin solution (0.7 × normal concentration; Life Technologies)]. This high-divalent saline did not alter synaptic strength; in synapses in which monosynaptic SN-to-MN connections were compared by switching back and forth between modified saline and normal saline after the synapses first were depressed to stable levels, mean EPSPs were 4.0 ± 1.2 mV in high-divalent saline versus 4.0 ± 1.1 mV in normal saline (n = 5).

Siphon SNs and LFS MNs were penetrated with single 12–20 MΩ microelectrodes filled with 2 M K-acetate and 0.4 M KCl. During penetration, 0.5 nA hyperpolarizing current was injected to prevent SN firing. Injection of EGTA, BAPTA, or H₂O was by passive diffusion from the recording pipette; compounds were diluted into 20 mM K-HEPES and 400 mM KCl, and microelectrodes filled with this solution were beveled to 7–10 MΩ; control neurons were penetrated with beveled pipettes containing the same buffered saline. SN action potentials were elicited by the injection of 2 msec depolarizing current pulses. The membrane potential of postsynaptic MNs was maintained at 60 mV below the resting potential to prevent action potentials. After a synaptic connection was identified, the synapse was rested for a minimum of 10 min before an experimental protocol was begun. During experiments on synaptic depression, action potentials in SNs were elicited at a 15 sec interval. During paired-pulse stimulation two SN action potentials were elicited at a 50 msec interval. During paired-pulse and tetanic stimulation, the intensity of the injected depolarizing current was increased 20–40% above threshold to ensure that an action potential was elicited during each depolarizing pulse.

In experiments examining the effect of injection of EGTA, BAPTA, or H₂O, the synaptic stimulation protocols were begun 30, 30, or 40 min, respectively, after penetration to allow time for diffusion to presynaptic or postsynaptic regions of the SNs or MNs. KN-62 was dissolved in DMSO and was diluted to a final DMSO concentration of 0.1%; this concentration of DMSO had no detectable effect on synaptic connections that were initially <8 mV displayed facilitation (i.e., PPR > 1.1) the first time that they were tested with paired pulses (mean PPR = 2.75 ± 0.32; n = 34), whereas only 22% of the synaptic connections that were initially >8 mV showed paired-pulse facilitation (mean PPR = 0.83 ± 0.07; n = 27) (Fig. 2A). There was not, however, an inverse relationship between EPSP₁ and EPSP₂. When synapses were first tested, the amplitude of EPSP₂ was independent of EPSP₁ (Fig. 2B); at synapses that previously had been depressed by repeated activation with single spikes (see below), EPSP₂ was positively correlated with EPSP₁ (Fig. 2D). Thus, when synaptic connections of different strengths were compared, there was no tendency for greater release by the first stimulus to result in a decrease in release by the second stimulus. This lack of an inverse relationship between releases by the two paired action potentials has been observed in studies at hippocampal synapses where the probability of release by the second stimulus was either independent of, or positively correlated with, the probability of release by the first stimulus (Stevens and Wang, 1995; Dobrunz et al., 1997). Interestingly, the time course of decline of the paired-pulse ratio with repeated testing was nearly identical at large and small SN synapses (Fig. 3).

RESULTS

Paired-pulse ratio at SN synapses decreases dramatically with repeated testing

In our initial studies, we hoped that measurements of paired-pulse facilitation would provide simple insights into mechanisms underlying more persistent forms of plasticity at Aplysia SN synapses. However, when we stimulated siphon SNs to fire pairs of spikes at a 50 msec interpulse interval, we observed a high level of variability in the paired-pulse ratio, much of which occurred within individual synaptic connections. Because SN synapses displayed paired-pulse depression as well as paired-pulse facilitation, we present results as the paired-pulse ratio (PPR) instead of presenting data on facilitation above control,

\[
PPR = \frac{amplitude\ of\ EPSP₂}{amplitude\ of\ EPSP₁} + \frac{amplitude\ of\ EPSP₁}{amplitude\ of\ EPSP₂},
\]

where EPSP₁ and EPSP₂ are the synaptic responses to the first and second paired action potentials, respectively. Thus, a paired-pulse ratio <1 corresponds to paired-pulse depression and a paired-pulse ratio >1 corresponds to paired-pulse facilitation. When synapses were stimulated repeatedly to fire pairs of action potentials, a striking pattern emerged: the paired-pulse ratio declined abruptly after the first test trial (Fig. 1A, B). During the initial test of the paired-pulse ratio, we observed facilitation in the majority of synapses (mean initial PPR = 1.90 ± 0.22; n = 61), whereas with subsequent tests we usually observed paired-pulse depression (the second time that the paired-pulse ratio was tested, mean PPR = 0.74 ± 0.08; n = 39). This decrease in the paired-pulse ratio with repeated testing was highly significant, both with a single repeated test at a 15 min interval (p < 0.002; two-tailed, paired t test; n = 6) and with a series of 15 tests at 15 sec intervals (F₁₃,₄₁₆ = 23.72; p < 0.001; n = 33; repeated measures ANOVA) (Fig. 1C). A limited number of experiments were conducted at several different interpulse intervals. With intervals of 30 and 100 msec, paired-pulse facilitation was present in the first trial and then disappeared with repeated testing in a qualitatively similar manner. With an interpulse interval of 1 sec, minimal paired-pulse facilitation was observed.

The paired-pulse ratio decreases with increasing strength of the initial synaptic connection

When we examined the relationship between the initial paired-pulse ratio and the amplitude of EPSP₁ at SN synapses, we observed an inverse correlation between initial synaptic strength and paired-pulse facilitation (“initial” refers to the time at which the paired-pulse ratio was first tested). For example, 88% of the synaptic connections that were initially <8 mV displayed facilitation (i.e., PPR > 1.1) the first time that they were tested with paired pulses (mean PPR = 2.75 ± 0.32; n = 34), whereas only 22% of the synaptic connections that were initially >8 mV showed paired-pulse facilitation (mean PPR = 0.83 ± 0.07; n = 27) (Fig. 2A). There was not, however, an inverse relationship between EPSP₂ and EPSP₁. When synapses were first tested, the amplitude of EPSP₂ was independent of EPSP₁ (Fig. 2B); at synapses that previously had been depressed by repeated activation with single spikes (see below), EPSP₂ was positively correlated with EPSP₁ (Fig. 2D). Thus, when synaptic connections of different strengths were compared, there was no tendency for greater release by the first stimulus to result in a decrease in release by the second stimulus. This lack of an inverse relationship between releases by the two paired action potentials has been observed in studies at hippocampal synapses where the probability of release by the second stimulus was either independent of, or positively correlated with, the probability of release by the first stimulus (Stevens and Wang, 1995; Dobrunz et al., 1997). Interestingly, the time course of decline of the paired-pulse ratio with repeated testing was nearly identical at large and small SN synapses (Fig. 3).
Aplysia mechanosensory neuron synapses display marked homosynaptic depression with repeated activation at intervals of several minutes or shorter (Castellucci and Kandel, 1974; Byrne, 1982). The decrease in paired-pulse ratio with repeated testing at a 15 sec intertrial interval might suggest a change caused by synaptic depression. However, several lines of evidence indicate that the use-dependent decrease in the paired-pulse ratio occurs independently of synaptic depression. First, with the longer, 15 min, interval, there was minimal depression (EPSP<sub>1</sub> at the time of the second test was 93.8% of EPSP<sub>1</sub> at the time of the first test), and yet there was also a dramatic decrease in the paired-pulse ratio (to 47 ± 8% of the initial value, from 1.53 ± 0.10 to 0.71 ± 0.13) (see Fig. 1). Second, when the paired-pulse ratio was tested repeatedly at a 15 sec intertrial interval, the change in the paired-pulse ratio was much more dramatic and rapid than the depression of EPSP<sub>1</sub> (data not shown). Third, to test directly whether the paired-pulse ratio decreases as a result of homosynaptic depression, we examined SN synapses after they first had been depressed with a series of 15 single stimuli at a 15 sec interval to, on average, 32.8 ± 1.6% of their initial amplitude (Fig. 4A).

When the paired-pulse ratio was first tested, 15 sec after the fifteenth single action potential, the paired-pulse ratio (2.23 ± 0.38; n = 30) was as large as the paired-pulse ratio at naive, nondepressed synapses (1.90 ± 0.22; n = 61) (see also Fig. 4B, in which the paired-pulse ratios at nondepressed and depressed synapses are separated according to the size of initial connection). Thus, repeated stimulation with single spikes did not decrease paired-pulse facilitation.

**Depression reduces EPSP<sub>2</sub> along with EPSP<sub>1</sub>**

Although synaptic depression with single action potentials did not reduce the paired-pulse ratio, the absolute amplitude of EPSP<sub>2</sub> did decrease. The decrease in EPSP<sub>2</sub> can be detected by comparing Figure 2, B and D. It is also evident that EPSP<sub>2</sub>, decreased with depression at small synapses (with initial amplitudes <8 mV) because, despite a threefold decrease in the size of EPSP<sub>1</sub>, there was not a significant increase in the paired-pulse ratio (Fig. 4B).

In the case of large synapses, although an increase in the paired-pulse ratio was observed after depression (PPR = 1.38; Fig. 4B), it was substantially less than what would be predicted if EPSP<sub>2</sub> had remained constant [initial PPR ÷ (EPSP<sub>1</sub>-depressed/EPSP<sub>1</sub>-initial)] gives a predicted PPR for large synapses after depression of 2.53 (where EPSP<sub>1-initial</sub> was determined without paired-pulse stimulation).

**Does synaptic depression change large synapses into small synapses?**

We wanted to know whether, after depression, initially large synapses showed paired-pulse facilitation comparable to that of initially small synapses. We addressed this issue in three ways. First, we compared the paired-pulse ratio for initially large synapses after depression with the paired-pulse ratio for initially small synapses. The paired-pulse ratios of initially large synapses (>8 mV initial amplitude) increased significantly after the synapse had been depressed, from 0.83 ± 0.07 (n = 27) to 1.38 ± 0.18 (n = 17) (p < 0.01; Fig. 4B); however, the average PPR after depression of 1.38 did not approach the average PPR of small synapses, either before or after depression (Fig. 4B). In contrast, the paired-pulse ratio at smaller synapses (<8 mV initial amplitude) did not change significantly after depression (Fig. 4B).

Second, we fit hyperbolic functions to the paired-pulse ratio data from depressed and nondepressed synapses of the form PPR = a + b/x, where x is the amplitude of EPSP<sub>1</sub> (see Fig. 2A, C). If after depression, large synapses simply became small synapses, then the hyperbola fit to the data from depressed synapses would be similar to the curve fit to the data from nondepressed synapses; this is not the case (see Fig. 2C). On the other hand, if the
paired-pulse ratios of large synapses change little after depression, then we should be able to predict the paired-pulse ratios of depressed synapses by scaling the hyperbola fit to the data for nondepressed synapses, multiplying the y values by the depression ratio, 0.328. The hyperbola fit to the actual data from depressed synapses is intermediate between the nondepressed curve and the scaled curve and somewhat closer to the latter. Third, we considered only the EPSPs that were initially >8 mV but that depressed to <8 mV. Although the average amplitude for EPSP1 when first tested after depression was 4.3 ± 0.4 mV for this group, the average paired-pulse ratio was 1.42 ± 0.21 (n = 11). In contrast, the paired-pulse ratio for nondepressed synapses <8 mV was significantly greater, averaging 2.75 ± 0.32 (p < 0.05; two-tailed t test), although the average amplitude for EPSP1 when it was tested initially was comparable (4.4 ± 0.3 mV; n = 34). Thus, EPSP size, independently of initial synaptic strength, is not an adequate predictor of the paired-pulse ratio.

Use-dependent plasticity of paired-pulse facilitation provides evidence that a separate set of release sites contributes to EPSP2.

In some experimental systems (e.g., Stevens and Wang, 1995), changes in the paired-pulse ratio may represent primarily a change in the probability of release evoked by the first stimulus. However, we observed a dramatic reduction in the release evoked by the second stimulus after a single paired-pulse trial, when there was minimal change in release evoked by the first. We consider here what synaptic organization is required for the observed decrease in the amplitude of EPSP2 with repeated testing that occurred independently of any substantial change in

![Figure 2](image2.png)

Figure 2. Relationship between the paired-pulse ratio and the amplitude of EPSP1. The paired-pulse ratio (A, C) and the amplitude of EPSP1 (B, D) are plotted versus the amplitude of EPSP2 for nondepressed synapses (A, B) and depressed synapses (C, D). Both sets of data are for the first trial in which a synapse was tested with paired stimulation. Paired-pulse data on nondepressed synapses were obtained 10–15 min after a synapse first was identified. Synaptic depression was achieved first by stimulating an SN to fire 15 single action potentials at a 15 sec ITI; this protocol reduced synaptic strength to an average of 32.8 ± 1.6% of the initial amplitude (see Fig. 4.4 for data on the time course of depression for these same synapses). The paired-pulse test was 15 sec after the fifteenth SN spike. For A and C, the data were fit with hyperbolic functions of the form a + b/x, where x is the amplitude of EPSP1 (solid lines). In C, the dotted curve is the hyperbolic fit for nondepressed synapses from A; the dashed curve is the curve from A transformed by multiplying x by the average depression ratio (i.e., 0.328). In B and D, the data were fit with straight lines with a slope of 0.18 (with a correlation coefficient of 0.18) for the nondepressed synapses (B) and with a slope of 0.71 (with a correlation coefficient of 0.61) for the depressed synapses (D). Note that, whereas there is an inverse correlation between the paired-pulse ratio and the amplitude of EPSP1, the amplitude of EPSP2 is not negatively correlated with EPSP1.

![Figure 3](image3.png)

Figure 3. Paired-pulse ratios for large and small synapses decline at the same rate with repeated testing. SN synapses were tested with paired-pulse stimulation at a 15 sec ITI. Note that, although the average initial paired-pulse ratio at synapses >8 mV (large synapses) was more than twofold smaller than the average initial paired-pulse ratio at synapses <8 mV (small synapses), the paired-pulse ratio at large synapses showed comparable decrement with repeated testing. The average initial EPSP amplitude was 4.7 ± 0.4 mV (n = 21) and 13.1 ± 1.1 mV (n = 12) for the small synapses and large synapses, respectively; these two groups of synapses initially had paired-pulse ratios of 2.60 ± 0.49 and 1.13 ± 0.26, respectively. Data are from the same experiments as in Figure 1C. Note that the paired-pulse ratios for the large and small synapses are plotted on two different scales. The time course of the decrease in paired-pulse ratios did not differ significantly between the two groups (F1,31 = 2.19; p = 0.149; repeated measures ANOVA testing trial × synapse size interaction; comparison of the second-order polynomial trend).
Figure 4. Effect of previous synaptic depression on the initial paired-pulse ratio. A. Time course of synaptic depression for large and small synapses. Synapses were depressed by stimulating SNs to fire single action potentials at a 15 sec ITI, and the paired-pulse ratio was then tested after the fifteenth trial. The mean amplitude of the EPSPs on trial number 15 was 32.8 ± 1.6% of the initial value. Note that the large (≥8 mV) and small synapses (<8 mV) depressed at the same rate. The time course of synaptic depression did not differ significantly between the two groups (F_{1,28} = 1.34; p = 0.258; repeated measures ANOVA testing trial × initial size interaction; comparison of the second-order polynomial trend). B. Group data for paired-pulse ratios of large and small synapses tested once, either without depression (NON-DEPRESSED) or 15 sec after a series of 15 single action potentials (DEPRESSED). Both before and after depression, the large synapses (≥8 mV) differed significantly from the small synapses (<8 mV) in their paired-pulse ratios (p < 0.001 for nondepressed synapses and p < 0.01 for depressed synapses; two tailed t test). After synaptic depression the paired-pulse ratio for large synapses increased significantly (p < 0.01; two tailed t test); in contrast, despite comparable synaptic depression, there was no significant change in the paired-pulse ratio of small synapses. The average initial EPSP amplitudes were 14.8 ± 1.1 mV (n = 27) and 4.4 ± 0.3 mV (n = 34) for the large and small synapses, respectively, in the nondepressed group; for synapses in the depressed group, the average EPSP amplitudes before depression were 16.7 ± 1.5 mV (n = 17) and 5.7 ± 0.4 mV (n = 13) for the large and small synapses, respectively. Depressed synapse data are from the same synapses as in A.

\[
m_2 = n_1 \times P_{1b} + n_2 \times P_2,
\]

where \(m_2\) is the mean quantal content for EPSP\(_2\), \(n_1\) is the number of release sites available to contribute to EPSP\(_1\), \(P_{1b}\) is the probability of release during the second action potential at these sites that mediate EPSP\(_1\), \(n_2\) is the number of release sites that constitute a hypothetical separate class that is activated exclusively by the second paired action potential, and \(P_2\) is the probability of release at these hypothetical release sites that contribute selectively to EPSP\(_2\). By definition, the \(n_2\) sites are not effectively activated by a single action potential and do not contribute substantially to EPSP\(_1\). \(P_{1b}\) can be considered to be a function of \(P_1\) and \(F_1\), where \(P_1\) is the probability of release during the first action potential at the \(n_1\) sites, and \(F_1\) is a \(Ca^{2+}\)-dependent facilitatory factor initiated by the first paired action potential that acts transiently to increase \(P_{1b}\) (see Results below for evidence of \(Ca^{2+}\) dependence). \(P_2\) also is enhanced by a \(Ca^{2+}\)-dependent facilitatory factor \((F_2)\); however, in the absence of the \(Ca^{2+}\) signal from the first action potential, \(P_2\) is very low.

In principle, EPSP\(_2\) could be simply a direct function of the same factors that control release by the first action potential (which together determine \(P_2\)), in combination with the \(Ca^{2+}\)-dependent facilitatory factor \(F_1\). If this were the case, the product \(n_2 \times P_2\) would be negligible, and the only significant release during the second action potential would occur at that same population of sites that can be effectively activated by the first action potential, i.e., the \(n_1\) sites. Use-dependent changes in the paired-pulse ratio then would be explained entirely by changes in the \(Ca^{2+}\)-dependent facilitatory factor \(F_1\) because changes in either \(n_1\) or \(P_1\) would alter EPSP\(_1\). However, this facilitatory factor \(F_1\) is an unlikely site for the persistent use-dependent plasticity that was observed here because repeated single stimuli do not decrease paired-pulse facilitation (Fig. 4); if \(F_1\) changed simply by being activated, then it would decrease incrementally during a series of single presynaptic spikes. This leaves the remaining two parameters, \(n_2\) and \(P_2\), either one or both of which could change in a manner that explains the use-dependent decrease in the paired-pulse ratio. Even if it is a decline in \(P_2\) that is responsible for the decrement in paired-pulse facilitation with repeated testing, there must exist a unique set of release sites that is accessed primarily by the second paired spike, with which \(P_2\) is associated. The second paired action potential also may activate some of the \(n_1\) sites; however, there must be very low-probability activation of these sites given that EPSP\(_1\) can show little depression, whereas EPSP\(_2\) depresses dramatically or fails completely (e.g., Fig. 1A).

We do not know whether a single action potential evokes release at a low probability from the same sites that are accessed by the second paired action potential. We observed that EPSP\(_2\) was reduced in size during a series of single spikes that depress EPSP\(_1\). There are at least two ways that this could occur. During a single unpaired action potential there may be modest, very low-probability activation of the sites that contribute primarily to EPSP\(_2\) (the \(n_2\) sites). Alternatively, there may be a component of synaptic depression that occurs independently of release (Dobrunz et al., 1997), as suggested by the high-\(Mg^{2+}\) saline results of Castellucci (1974), for example, a depression of \(Ca^{2+}\) current.

Post-tetanic potentiation persists through repeated testing

Because paired-pulse facilitation has some similarities with post-tetanic potentiation (PTP), we asked whether PTP also disappeared after testing. Before examining PTP at each synapse, we
repeatedly stimulated the SN at a 15 sec interval to depress the connection to a stable level to allow for comparisons of multiple episodes of PTP. The SN was then stimulated at 20 Hz for 2 sec to induce PTP. After five post tests at 15 sec intervals, the synapse was rested 10 min and the protocol was repeated. PTP decreased significantly with repeated testing ($F_{2,12} = 7.37; p = 0.008; n = 7$) (Fig. 5). Nevertheless, PTP remained highly significant even during the third episode ($F_{2,12} = 6.40; p < 0.001; n = 7$; repeated measures ANOVA, testing the trial effect for the last five pretests and the five posttests). Thus, unlike paired-pulse facilitation, PTP did not disappear after initial testing. A number of differences between PTP and paired-pulse facilitation, particularly in various aspects of their Ca$^{2+}$ dependence, have been observed previously (e.g., Kamiya and Zucker, 1994; Fisher et al., 1997), which also suggests that facilitation is not simply a modest form of PTP.

**Interaction of serotonin-induced facilitation with the paired-pulse ratio**

Serotonin (5-hydroxytryptamine, 5HT) acts to increase release at *Aplysia* SN synapses by at least two presynaptic mechanisms: modulation of K$^+$ currents in the SNs, which results in increased Ca$^{2+}$ influx, and modulation of either the exocytosis mechanism itself or the releasable pool (Byrne and Kandel, 1996). We asked whether 5HT-induced synaptic facilitation was accompanied by the expected decrease in the paired-pulse ratio, which is associated with several other forms of transmitter release (e.g., McNaughton, 1982; Muller et al., 1988). Because the initial paired-pulse ratio at naive synapses is quite variable (see Fig. 2A) and because it decreases dramatically with testing, it was difficult to determine the effects of 5HT on the paired-pulse ratio at minimally stimulated synapses by using either between-synapse or within-synapse comparisons; therefore, we did not analyze 5HT-induced facilitation at rested synapses. Instead, we examined 5HT facilitation of moderately depressed synapses at which the paired-pulse ratio had been tested repeatedly. A 3 min exposure to 5HT produced reliable and maintained enhancement of EPSP$_1$ that developed gradually and reached an amplitude more than twice the initial nondepressed amplitude ($F_{9,72} = 10.66; p < 0.001; n = 9$; repeated measures ANOVA) (Fig. 6A). In contrast, EPSP$_2$, which was facilitated only transiently ($F_{9,72} = 3.40; p = 0.002$) and did not approach its initial amplitude (Fig. 6B). During the period of initial facilitation, because EPSP$_2$ was increased in...
Figure 7. Effect of Ca\(^{2+}\) chelators injected pre- or postsynaptically on paired-pulse facilitation. Either EGTA (50 mM in pipette) was injected into the presynaptic SNs, or EGTA (100 mM in pipette) or BAPTA (200 mM in pipette) was injected into the postsynaptic MNs. Paired-pulse ratios were tested 30 min after penetration to allow the chelators to diffuse to presynaptic or postsynaptic regions of the neurons. Control neurons were injected with vehicle. Presynaptic EGTA significantly decreased the paired-pulse ratio, whereas postsynaptic EGTA or BAPTA had no significant effect (F\(_{2,20} = 8.36\) and \(p = 0.001\) for overall comparison among the five groups; pairwise comparisons revealed that EGTA-injected SNs were significantly different from vehicle-injected SN controls, \(p = 0.015\), whereas EGTA-injected MNs and BAPTA-injected MNs were not significantly different from MN controls).

parallel with EPSP\(_1\), there was no significant change in the paired-pulse ratio, which remained below 15% of the initial value; however, when EPSP\(_2\) again declined, the paired-pulse ratio decreased to a very low level (~5% of the initial PPR). Because after repeated testing EPSP\(_2\) was extremely small and often undetectable, it is not meaningful to compare quantitatively the facilitation of EPSP\(_1\) and EPSP\(_2\) nor to quantify the change in the paired-pulse ratio. Nevertheless, during the period of initial facilitation, there was no evidence that the paired-pulse ratio declined.

Why was the facilitation by 5HT of EPSP\(_2\) short-lived compared with the facilitation of EPSP\(_1\)? It could be that, although 5HT effectively facilitated EPSP\(_1\) throughout the exposure, facilitation at the sites mediating EPSP\(_2\) was for some reason more transient. Alternatively, once the second spike again produced a moderate level of transmitter release in the presence of 5HT, there may be rapid additional depression of EPSP\(_2\); this could be a consequence of the same mechanism that produced profound depression of EPSP\(_2\) when it was initially tested by using the paired-pulse protocol. According to this proposal, whenever release is effectively activated at the sites mediating EPSP\(_2\), this release is depressed rapidly. This second alternative is attractive because it does not require a 5HT-induced facilitatory process that is unique to the second spike but, rather, results from the same dramatic depression of EPSP\(_2\) that is observed in the absence of 5HT.

**Contribution of Ca\(^{2+}\) and phosphorylation to paired-pulse facilitation**

The observation that during paired-pulse facilitation, Ca\(^{2+}\) influx during the first action potential was necessary for facilitation of the release evoked by the second action potential led to the residual Ca\(^{2+}\) hypothesis (Katz and Miledi, 1968; Younkin, 1974). In its simplest form, this model proposes that small amounts of residual Ca\(^{2+}\) summate with Ca\(^{2+}\) influx during a second action potential to increase exocytosis; such powerful, nonlinear summation could, in principle, occur because Ca\(^{2+}\)-triggered release of transmitter apparently depends on the binding of approximately four Ca\(^{2+}\) ions (Zucker, 1989). However, several lines of evidence suggest that this early model might be too simple (e.g., Yamada and Zucker, 1992; Van der Kloot, 1994; Winslow et al., 1994); thus, Ca\(^{2+}\) initiates facilitation via a mechanism that is not fully understood (Fisher et al., 1997).

We tested the effect on paired-pulse facilitation of presynaptic injection of EGTA. Because EGTA is a relatively slow Ca\(^{2+}\) buffer compared with the exocytosis process itself, in a number of systems presynaptic EGTA can block some forms of plasticity with either little effect or a modest effect on the release process itself (Adler et al., 1991; Winslow et al., 1994; Bao et al., 1997). After 30 min to allow for diffusion to synaptic sites, recording with 50 mM EGTA in the presynaptic electrode reduced EPSP amplitude by a highly variable amount (in 17 synapses in which measurable transmitter release persisted, EPSP amplitude decreased significantly to 50.93 ± 4.2% of the initial value, compared with a decrease to 89.4 ± 7.8% of the initial value for vehicle-injected SNs, \(n = 8\), \(p < 0.001\), two-tailed \(t\) test; in three other synapses, no EPSP was detectable after 30 min of EGTA injection). This reduction in transmitter release indicated that the chelator had diffused to presynaptic terminals. In these experiments, after EGTA injection, the paired-pulse ratio was reduced dramatically (PPR = 0.27 ± 0.04 for EGTA-injected SNs, \(n = 17\), vs 1.44 ± 0.22 for vehicle-injected SNs, \(n = 8\); \(p < 0.001\); two-tailed \(t\) test) (Fig. 7). Thus, presynaptic Ca\(^{2+}\) appears to be necessary for paired-pulse facilitation.

Bao et al. (1997) demonstrated that a component of PTP at these *Aplysia* SN synapses depends on postsynaptic Ca\(^{2+}\). We tested the importance of postsynaptic Ca\(^{2+}\) in paired-pulse facilitation by recording from MNs with pipettes filled with 100 mM EGTA or 200 mM BAPTA. Postsynaptic injection of either of these Ca\(^{2+}\) chelators did not significantly affect paired-pulse facilitation (Fig. 7). The concentrations of these chelators injected in MNs were two to four times the concentration of EGTA injected presynaptically, which was sufficient to reduce release substantially; therefore, it is likely that the concentrations of EGTA and BAPTA that diffused to postsynaptic sites would have been adequate to interfere with any modulatory effects of Ca\(^{2+}\).

It recently has been suggested that Ca\(^{2+}\)-dependent phosphorylation might mediate paired-pulse facilitation (Winslow et al., 1994). To test this possibility, we injected the broad-spectrum
protein kinase inhibitor H7 into the presynaptic SN. H7 inhibits a number of kinases, including cAMP-dependent kinase, protein kinase C (PKC) (Hidaka et al., 1984), and calmodulin-dependent kinase II (CaMKII) (Waxham et al., 1993). Braha et al. (1990) observed that, at *Aplysia* SN synapses, H7 applied extracellularly at a concentration of 200 μM significantly reduced the facilitation initiated by either cAMP-dependent kinase or PKC. We found that intracellularly injected H7 (50 mM in pipettes) had no effect on the paired-pulse ratio (Fig. 8A). In the majority of these experiments we verified that adequate concentrations of H7 diffused to the presynaptic terminals by examining an H7-sensitive form of synaptic plasticity that we had recently identified, burst-dependent protection from synaptic depression (Jiang and Abrams, 1997). Siphon SN synapses typically depress rapidly to less than one-half of their initial amplitude when activated repeatedly at an intertrial interval briefer than 1 min (e.g., Goldsmith and Abrams, 1991; Eliot et al., 1994); however, if the SNs fire brief bursts of several spikes, rather than single action potentials, these bursts almost completely protect the synapses from depression. (Burst-dependent protection is evident in Fig. 6 in the data for EPSP₁, which, despite a series of 12 previous paired-pulse stimuli, had depressed only 15%.) In those paired-pulse experiments in which we subsequently examined synaptic depression when SNs were activated with bursts of four spikes, control synapses decreased minimally, to 87.7 ± 6.9% (n = 6) of their initial amplitude, whereas synapses from H7-injected SNs decreased significantly more, to 41.5 ± 9.4% (n = 6) of their initial size (p < 0.01).

The inhibition of CaMKII requires several-fold higher concentrations of H7 than does the inhibition of PKC (Hidaka et al., 1984; Waxham et al., 1993; Schweitzer et al., 1995; Sitges et al., 1995) [for example, in rat CNS protein kinase preparations, Neil Waxham (personal communication) determined Ki values for H7 of 8 μM for PKC and 20–32 μM for CaMKII]. Thus, it seemed possible that, although H7 reached sufficient concentrations at the presynaptic terminals of SNs after intrasomatic injection to inhibit PKC and to block burst-dependent protection, these concentrations may have been insufficient to inhibit CaMKII effectively. We therefore also conducted experiments with KN-62, a specific CaMKII inhibitor. Nakanishi et al. (1997) found at *Aplysia* SN synapses that 10 μM KN-62, applied extracellularly for 15 min, blocked a component of short-term 5HT-induced synaptic facilitation. There was no reduction in the paired-pulse ratio after ganglia were incubated for 30 min with 20 μM KN-62 (Fig. 8B), indicating that paired-pulse facilitation does not depend on CaMKII.

**DISCUSSION**

We observed that paired-pulse facilitation at *Aplysia* SN synapses is a complex and plastic phenomenon that can be altered dramatically simply as a result of minimal paired-pulse testing. After a single paired-pulse trial, paired-pulse facilitation decreased abruptly and paired-pulse depression predominated for at least 15 min. This unusual plasticity of the paired-pulse ratio provides some novel insights into both paired-pulse facilitation and the regulation of synaptic strength at these SN connections.

The decrease in paired-pulse facilitation is independent of both synaptic depression and initial synaptic strength, suggesting that the second EPSP is mediated by a separate set of release sites

Three changes in synaptic strength appeared to occur independently of each other: (1) use-dependent decrease in paired-pulse facilitation, (2) synaptic depression, and (3) variation in initial synaptic size, which was inversely correlated with the initial paired-pulse ratio. Synaptic depression occurred without an associated decrease in paired-pulse facilitation; moreover, paired-pulse facilitation decreased dramatically with repeated testing, with little change in the amplitude of EPSP₁. Synapses that were initially large had small paired-pulse ratios; however, the decrease in the paired-pulse ratio with repeated testing occurred at the same rates at initially small and initially large synapses (see Fig. 3), as did synaptic depression (see Fig. 4A). To account for these several aspects of synaptic strength changing independently, there must be at least three independent parameters that influence transmitter release during paired action potentials. The plasticity of EPSP₂ argued for the existence of a separate set of release sites that are the primary loci mediating release evoked by the second spike. Such an independent set of release sites would provide the additional parameters, n₂ and P₂ (see Results), that would enable these three aspects of synaptic strength to vary independently. This separate set of release sites that is hypothesized to contribute selectively to EPSP₂ could be part of a continuum of release sites with different probabilities of release, such that the probability of release at these sites during a single action potential is particularly low. Release at these sites may have a distinct dependence on Ca²⁺ compared with sites that release with a single spike, as is the case for release of neuromodulators (Whim and Lloyd, 1989; Peng and Zucker, 1993; Vilim et al., 1996). Thus, this second set of release sites may normally have a very low probability of release, which increases transiently when Ca²⁺ is elevated after the first paired spike. One explanation for the abrupt shift from paired-pulse facilitation to paired-pulse depression after a single pair of spikes is that, at this subset of release sites selectively activated by the second spike, the release process recovers very slowly.

**Changes in the number of synaptic sites cannot account alone for the variability in the strength of SN synapses**

Long-term changes in the strength of these SN synapses that are induced by both behavioral or cellular training are accompanied by changes in the number of synaptic interaction, i.e., active zones and presynaptic varicosities (Bailey and Chen, 1988a,b; Martin et al., 1997). One might therefore predict that much of the variability in initial synaptic strength at SN synapses would be accounted for by differences in the number of synaptic

**Figure 8.** Inhibition of protein phosphorylation with either H7 or KN-62 did not decrease paired-pulse facilitation. A, SNs were injected with H7 (50 mM in pipettes) for 40 min before testing. B, KN-62 (20 μM) was applied in the bath for 30 min before testing. Neither inhibitor significantly affected the PPR as compared with the controls.
contacts. If increased initial synaptic strength were primarily explained by an increased number of synaptic contacts that had, on average, the same properties as the varicosities between neurons with small synaptic connections, then we would expect that paired-pulse ratios would be similar at initially large and initially small synapses. However, the observation that paired-pulse facilitation showed a strong inverse correlation with initial synaptic strength suggests that active zones of initially large synapses have, on average, a higher probability of release than those of initially small synapses. Bailey and Chen (1983, 1989) observed that after long-term synaptic facilitation there is an increased number of vesicles associated with each active zone in SNs; such an increase in the number of docked vesicles could underlie an increase in release probability. Substantial heterogeneity in release probability has been described in hippocampal synapses where release sites of stronger synaptic connections tend to have higher release probabilities (Malinow and Tsien, 1990; Bolshakov and Siegelbaum, 1995; Dobrunz and Stevens, 1997). Moreover, hippocampal synapses with higher probabilities of release tend to have larger releasable pools, which suggests that the number of vesicles at the active zone may contribute to release probability (Dobrunz and Stevens, 1997; Murthy et al., 1997).

**Synaptic depression alters a characteristic of synapses distinct from the synaptic property that influences initial synaptic strength**

Large and small synapses displayed similar rates of synaptic depression. The observation that the rate of depression is independent of initial synaptic strength suggests that depression may result from the alteration of a synaptic property distinct from the property that underlies the difference in paired-pulse facilitation between large and small synapses; as suggested above, this difference in paired-pulse facilitation is likely to result from a difference in release probabilities. If release probability were to decrease during synaptic depression, the paired-pulse ratios of large synapses should, after depression, become comparable to the paired-pulse ratios of initially small synapses. However, depressing large synapses did not convert them to small synapses. Thus, large synapses must differ from small synapses in some factor that remains different even after short-term synaptic depression. If, as proposed above, initially large synapses have a greater percentage of high-probability release sites, then depression must result primarily from a reduction in the number of release sites rather than from the conversion of high-probability sites to low-probability sites. Consistent with this hypothesis, synaptic depression of small synapses was not accompanied by an increase in the paired-pulse ratio. If synaptic depression resulted from a decrease in the probability of release, then one would expect that it might be accompanied by a reciprocal increase in the paired-pulse ratio (e.g., Mallart and Martin, 1968).

This proposed loss of release sites with depression could actually represent a series of abrupt changes in the probability of release at individual sites, such that individual sites become functionally inactive. Recently, Klein et al. (1997) have concluded, on the basis of quantal analysis, that synaptic depression at these same SN synapses may result from the switching of individual release sites from a high-probability release state to a very low-probability, or inactive, state, rather than from a gradual decrease in the probability of release at all sites. Such large changes in the probability of release of individual sites, such that the contributions of some sites become negligible, could be functionally equivalent to the decrease in the number of release sites during depression suggested by these paired-pulse ratio results.

**Nature of the Ca$^{2+}$ effect that underlies paired-pulse facilitation**

The original “residual Ca$^{2+}$” hypothesis proposed that, during paired-pulse facilitation, low levels of elevated Ca$^{2+}$ persisting after the first spike summed with the Ca$^{2+}$ increase during the subsequent spike to activate greater numbers of the Ca$^{2+}$ binding sites that trigger exocytosis (e.g., Younkin, 1974). Alternatively, Ca$^{2+}$ from the first spike could bind to a site separate from those that normally initiate vesicle release to modulate the exocytotic mechanism transiently, thereby increasing release by the second spike. Our findings argue against the traditional view that residual Ca$^{2+}$ produces facilitation by more effectively activating those sites normally involved in triggering exocytosis, because persistent alteration of paired-pulse facilitation occurred with minimal change in release by the first spike. Any alteration related to Ca$^{2+}$ binding to the sites that normally trigger release should have parallel effects on EPSP$_1$ and EPSP$_2$. Thus, Ca$^{2+}$ from the first spike may enhance release by modulating release probability, e.g., by altering vesicle docking or priming. This modulatory mechanism probably does not involve phosphorylation because the protein kinase inhibitors H7 and KN-62 did not affect facilitation. As mentioned above, it is possible that the release sites that primarily mediate EPSP$_2$ require priming by Ca$^{2+}$ from the first spike in order for release to be activated with a substantial probability by the second spike. The possibility that Ca$^{2+}$ acts at heterogeneous sites in initiating release and facilitation was suggested previously by computer simulations (Yamada and Zucker, 1992).

**Paired-pulse facilitation as an index of presynaptic function**

If the second of two paired action potentials releases from a different set of sites than activated by the first action potential, then clearly plasticity at one set of release sites could occur independently of plasticity from a second set. This would make it difficult to use paired-pulse facilitation as a simple index of presynaptic function. These *Aplysia* SN synapses recover after activation unusually slowly. However, it is possible that, in other systems without such dramatic depression, release by the second paired spike could also occur at sites distinct from those that are activated by the first paired spike.

**REFERENCES**


Katz B, Miledi R (1968) The role of calcium in facilitation at the frog neuromuscular junction at 0°C is not maximal at time zero. J Neurosci 4:5722–5724.


