Presynaptic Potentials and Facilitation of Transmitter Release in the Squid Giant Synapse

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ABSTRACT Presynaptic potentials were studied during facilitation of transmitter release in the squid giant synapse. Changes in action potentials were found to cause some, but not all, of the facilitation during twin-pulse stimulation. During trains of action potentials, there were no progressive changes in presynaptic action potentials which could account for the growth of facilitation. Facilitation could still be detected in terminals which had undergone conditioning depolarization or hyperpolarization. Facilitation could be produced by small action potentials in low $[Ca^{++}]_o$ and by small depolarizations in the presence of tetrodotoxin. Although the production of facilitation varied somewhat with presynaptic depolarization, nevertheless, approximately equal amounts of facilitation could be produced by depolarizations which caused the release of very different amounts of transmitter.

INTRODUCTION

Successive action potentials often trigger the release of increasing amounts of transmitter from presynaptic terminals. This phenomenon, called facilitation,[1] has been described for squid synapses in the preceding paper (Charlton and Bittner, 1978). In most synapses, facilitation has not been associated with changes in action potentials which were recorded extracellularly from the presynaptic terminal (see references in Zucker, 1974 *b*). However, focal extracellular electrodes do not detect certain changes in membrane potential important in transmitter release. For example, both the level of hyperpolarization immediately before an action (foot voltage) and the absolute voltage that action potential attains (peak voltage) are known to have large effects on transmitter release (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966; Bloedel et al., 1966; Katz and Miledi, 1967). Neither of these parameters can be measured by focal extracellular electrodes. Because we wished to measure intracellular membrane potentials in a presynaptic terminal during facilitation, we used the

[1] In this paper, the term facilitation refers to an increased probability for transmitter release occurring for ~2 ms to several seconds after a single impulse or brief train of impulses. The relationship between this type of facilitation and "very early" facilitation (Katz and Miledi, 1968), long term facilitation (Sherman and Atwood, 1971), or posttetanic potentiation (Gage and Hubbard, 1966) is not yet clear.

squid giant synapse, one of the few preparations in which this technique is possible.

We have recorded presynaptic membrane potentials using intracellular electrodes during facilitation[1] in nonfatigued squid synapses bathed in solutions containing lowered extracellular calcium $[Ca^{++}]_o$—conditions which should enhance the detection of facilitation. We report that an increase almost always occurs in the total amplitude of the second spike in a train. This increase in total amplitude consists of an increased hyperpolarization of the membrane potential at the foot and an increase in the voltage at the peak of the second action potential relative to the first. Although these changes might account for up to one-half of the facilitation observed at the second pulse in a train, changes in these parameters can account for little, if any, of the facilitation at subsequent pulses in the train.

In the second part of this paper, we report attempts to probe the facilitation mechanism by manipulations of presynaptic membrane potentials. We have examined the effects on facilitation of conditioning presynaptic hyperpolarization and depolarization. Further experiments were performed to determine how much depolarization was required to initiate the facilitation and whether facilitation was graded with the amplitude of the depolarization producing it.

MATERIALS AND METHODS

All of these experiments were performed at the Marine Biological Laboratories, Woods Hole, Mass., using the squid, *L. pealei*. Dissection techniques and other procedures were as described previously (Charlton and Bittner, 1978). Microelectrodes were placed into both presynaptic terminals and postsynaptic cells in their region of synaptic overlap (Fig. 1). We used fresh, unfatigued giant synapses perfused by artificial seawater with lowered $[Ca^{++}]_o$ of 2–5 mM and small amounts (1–6 mM) of $[Mn^{++}]_o$ to depress transmitter release. Data were collected and averaged by a computer of average transients (CAT, Mnemetron Corp., Pearl River, N.Y.) and plotted on a chart recorder (Figs. 3, 11) or photographed directly from an oscilloscope screen (Figs. 2, 5, 6, 7, 10). When using the CAT, we averaged 20–40 data sets and allowed 10 s or more between each train of stimuli.

Facilitation at the n^{th} pulse (f_n) was defined as:

$$f_n = \frac{V_n - V_o}{V_o}, \tag{1}$$

where V_o was the amplitude of the postsynaptic potential (PSP) evoked by the first or conditioning pulse, and V_n was the amplitude of the n^{th} PSP.

We used a modified Howland current pump (New, 1972) to apply constant intracellular currents (depolarizing or hyperpolarizing) to nerve terminals or to initiate and modify action potentials by short pulses of depolarizing current.

RESULTS

Action Potential during Pairs and Trains of Stimuli

Using a stimulus paradigm in which facilitation evoked by one stimulus train decayed before a second train was presented and in which transmitter release did not fatigue with repetitive stimulation (see Materials and Methods), we have

observed (Fig. 2 A) that the second of a pair of normal action potentials[2] in a squid presynaptic terminal usually has a greater total amplitude than the first. Also, the greatest facilitation was usually produced by short intrapair intervals at which time the total amplitude, prespike hyperpolarization, and peak voltage of the second action potential were all maximally increased compared to the first spike. The peak voltages of the second and successive action potentials in a train were somewhat higher than the peak voltage of the first action potential, but the peak voltages did not continue to increase as the train progressed (Figs. 3 A, 4). In fact, increasing facilitation during trains was accompanied by decreases in the total amplitude of successive action potentials after the second pulse in the train. Each action potential in a train always traveled to the tip of the terminal.

Inasmuch as there is some average value of hyperpolarization during trains of action potentials (Figs. 3 A, 4), and inasmuch as hyperpolarization acts over

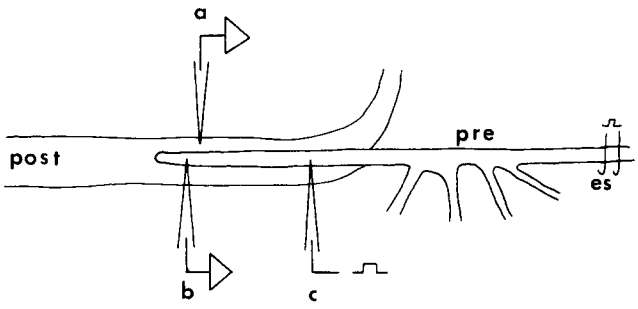

FIGURE 1. Diagram of electrode placement. All microelectrodes were placed within the zone of synaptic overlap of the presynaptic (pre) and postsynaptic (post) giant axons. Microelectrodes *a* and *b* recorded intracellular potentials from post- and presynaptic cells while microelectrode *c* was used for intracellular stimulation of the presynaptic terminal. The presynaptic giant axon could also be stimulated by a pair of extracellular stimulating electrodes (*es*).

several seconds to increase transmitter release (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966; Dudel, 1971), its is possible that the growth of PSP's during repetitive stimulation could have been due to a gradually developing effect of after-hyperpolarization following each spike. Furthermore, changes in the total spike amplitude or in the voltage level at the foot or peak of each action potential might have had independent effects on facilitation, and each might have been affected in a rather complex fashion by the maintained after-hyperpolarizations. Consequently, we designed experiments to manipulate these variables in order to determine their relative effects on the ability of squid synapses to release transmitter and to facilitate at the second and subsequent pulses of a stimulus train.

[2] Note that all experiments were performed using low $[Ca^{++}]_o$ salines often having increased $[Mn^{++}]_o$. Action potentials labeled as "normal" occurred in terminals having no artificial depolarization or hyperpolarization.

After-Hyperpolarization and Facilitation

If the after-hyperpolarization following a single conditioning pulse was largely responsible for the facilitation detected by the first test pulse, then the amplitude of a "facilitated" PSP evoked by the second of a pair of normal action potentials should equal that of a nonfacilitated PSP evoked by an action potential during a conditioning hyperpolarization of comparable amplitude. For example, the second normal action potential (N') in Fig. 5 A started from a potential 4 mV

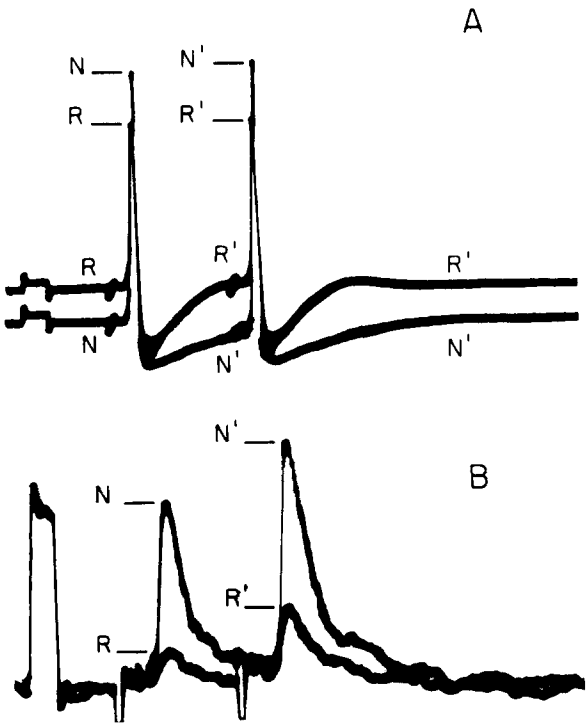

FIGURE 2. (A) Oscillographic records of a pair of normal action potentials (N, N') and a pair of reduced-amplitude action potentials (R, R') produced during a conditioning depolarization of the same nerve terminal. (B) Pairs of PSP's resulting from the action potentials in (A). Notice that the peak voltage of the second normal action potential (N') was somewhat higher than that of the first normal action potential (N) and that the foot of N' was at a more negative voltage than at the foot of N. After the normal action potentials were recorded, the terminal was depolarized 5–6 mV by the injection of steady depolarizing current into the terminal. When the depolarization had reached a steady level, two action potentials (R, R') were elicited by extracellular stimulation. The peak voltages of these action potentials were reduced about 10 mV (compared to the normal action potentials) and the foot of the second action potential (R') was not hyperpolarized with respect to the foot of the first (R). PSP's produced by the reduced action potentials (R, R') were smaller than those produced by normal action potentials (N, N'). Facilitation at N' was 0.25 and 1.16 at R'. Calibration pulse preceding all pairs represents 2 mV, 2 ms. Twin-pulse interval = 10 ms. $[Ca^{++}]_o$ = 4 mM, $[Mn^{++}]_o$ = 4mM, 15°C.

hyperpolarized with respect to the start of the first normal action potential (N). However, the second normal action potential evoked a PSP (N' in Fig. 5 B) of about the same amplitude (± 0.2 mV) as a PSP (H_{13} in Fig. 5 B) produced when the terminal was artificially hyperpolarized by 13 mV (H_{13} in Fig. 5 A). Similar data were obtained in other preparations (compare N' and H_{11} in Fig. 6). In other words, to make the first (nonfacilitated) PSP produced during an artificial

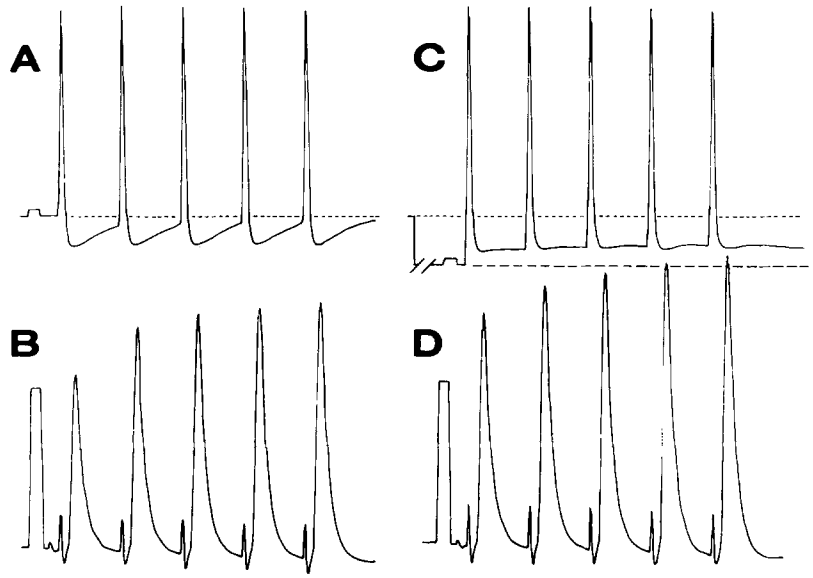

FIGURE 3. Intracellularly recorded presynaptic action potentials (A, C) and PSP's (B, D) produced by repetitive stimulation (10-ms interval) when the terminal was at its normal resting potential (A, B), and in the same terminal during the application of 24 mV of conditioning hyperpolarization (C, D). The short-dashed line (A) and (C) represents the normal resting membrane potential. In (C) the membrane potential was hyperpolarized by 24 mV for 10 s before the arrival of the first action potential. The hyperpolarization was maintained for > 5 min and 40 trains were elicited and averaged in a computer (the hyperpolarized level is represented by a long-dashed line). Notice in both trains that PSP amplitude continued to increase after the second spike, even though the total amplitude of action potentials decreased and the peak voltage and spike durations remained unchanged. Also note that the first PSP produced by the hyperpolarized terminal (D) was smaller than the last PSP produced by a series of normal action potentials (B). All records traced from CAT outputs. Each train was preceded by a calibration pulse of 2 mV, 2 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 6 mM, 15°C.

hyperpolarization equal in amplitude to the facilitated PSP produced by the second of a pair of normal action potentials, we had to use artificial hyperpolarization of greater amplitude and duration than the after-hyperpolarization that was observed to follow the first normal action potential.[3]

[3] Injection of hyperpolarizing current was begun > 10 s before action potentials were elicited; the current was maintained as long as action potentions were generated during a particular experiment

If the maintained average hyperpolarization during a short train of action potentials acts with a slow time-course to increase transmitter release (Dudel, 1971) then the maximum amount of this effect should be mimicked by a maintained artificial hyperpolarization applied to the terminal. When 24 mV of hyperpolarization was applied 10 s before stimulation of a presynaptic terminal (Figs. 3 C and D), it was obvious that the first PSP produced by the artificially polarized terminal (Fig. 3 D) was smaller than the PSP evoked by the last spike in a normal train (Fig. 3 B), even though the artificial hyperpolarization had a much greater amplitude and duration than the naturally occurring after-hyperpolarizations following each spike in the train.[3] These data show that the naturally occurring hyperpolarization during a train of action potentials could not have produced more than a few percent of the observed facilitation.

Changes in Peak Voltage and Facilitation

Although there were no progressive increases in the peak voltages of action potentials in a train, we often observed a small increase in peak voltage at the second action potential of a pair or train (Figs. 3 A, 4). Hence, we attempted to determine whether this increase accounted for facilitation in twin-pulse experiments. For example, in one preparation shown in Table I, a pair of normal action potentials having peak voltages above resting potential of 73 and 76 mV produced PSP's of 1.2 and 2.2 mV, respectively; that is, an increase of 3 mV in peak voltage at the second spike was associated with an 83% increase in PSP amplitude for a normal pair of action potentials. However, the peak voltage (73 mV) of a single action potential (Table I, dAP_1) had to be artificially increased to 83 mV by super-position of a brief depolarizing current pulse to produce a nonfacilitated PSP of similar amplitude (2.3 mV); that is, it was necessary to increase the peak voltage of the first spike by 10 mV in order to increase the transmitter output of that spike by 84%. Furthermore, the average amplitudes of the artificially increased action potentials in the whole terminal were greater than those presented in Table I, whereas normal action potentials underwent

using one stimulus interval (such an experiment could last as long as 5 min). Inasmuch as hyperpolarization acts over several seconds to increase transmitter release (Miledi and Slater, 1966, Dudel, 1971), the effect of these artificially imposed hyperpolarizations should have been maximal by the time the first test pulse was given. In fact, no consistent differences were noted in the effects of hyperpolarization on trains of PSP's recorded at various times during this maintained hyperpolarization; this observation indicates that the effects of hyperpolarization were indeed constant after the first 10 s had elapsed. Furthermore, increasing amounts of conditioning hyperpolarization produced increasing amounts of transmitter release and the largest conditioning hyperpolarizations were much larger than naturally occurring after-hyperpolarizations. Hence, the effect of hyperpolarization was not maximal at the voltage levels which occurred after a normal action potential(s). Finally, the current-passing and voltage-recording electrodes were at opposite ends of the region of synaptic overlap with the recording electrode nearer the tip of the terminal (see Fig. 1). Experiments using three electrodes in the presynaptic terminal indicated that the voltage gradient down the length of the terminal was, at most 2 mv. Consequently, the average hyperpolarization given to the entire synaptic region was, due to the spatial decrement of the imposed current, more than the measured hyperpolarization indicated for each figure. Most of these considerations would result in a tendency to overestimate the effects of naturally occurring hyperpolarizations on transmitter release. The last condition discussed would also result in our overestimating the effects of maintained artificial hyperpolarizations.

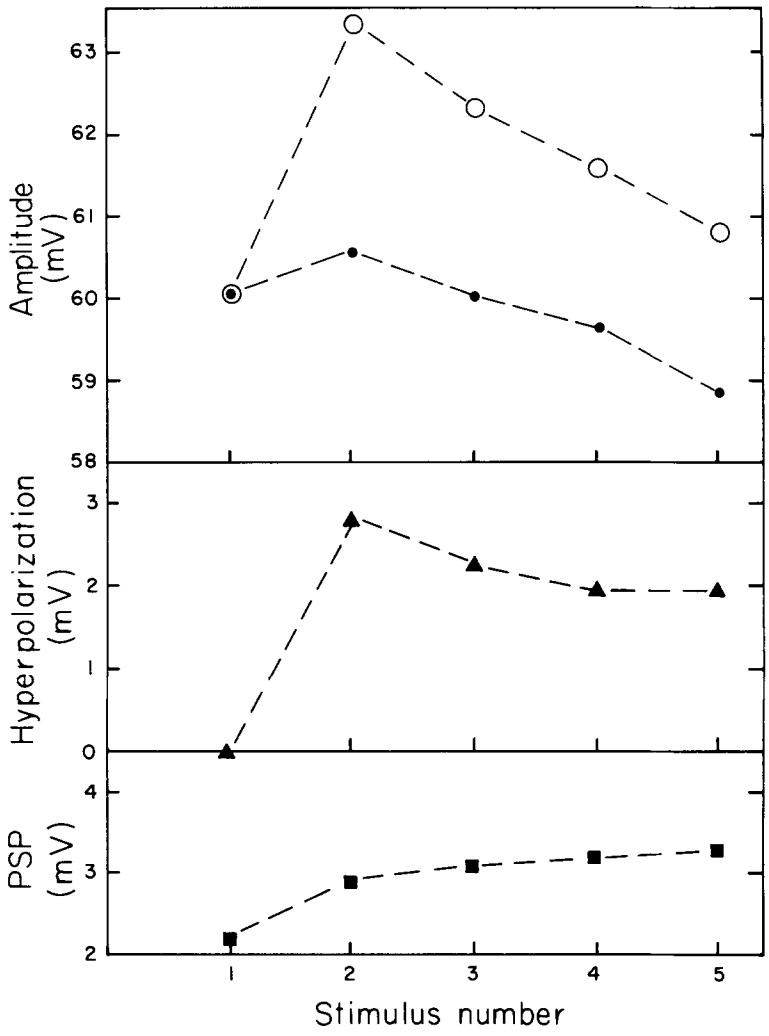

FIGURE 4. Changes in total amplitude (○), peak voltage (●), and hyperpolarization (▲) at each spike in a train of normal action potentials. (■) Increasing PSP amplitude. Data taken from Fig. 3 A and B. Stimulus interval = 10 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 6 mM, 15°C.

little changes in amplitude over the same length of the terminal.[4] Therefore, small changes in peak voltage occurring in normal action potentials would have had less effect on PSP amplitude than the effect calculated in Table I for action potentials increased by artificial depolarizing pulses. Hence, the results of all

[4] Increases in the peak voltage of action potentials produced by depolarizing current pulses should be greater at the current-passing electrode than the voltage-sensing electrode due to spatial decrement of current along the length of the terminal. This inaccuracy, due to spatial decrement, would have been much larger for depolarizing current than for hyperpolarizing current because we applied the depolarizing current during an action potential when membrane conductance was high. Conversely, when we applied hyperpolarizing current we tended to reduce membrane conductance and hence to reduce spatial decrement of current (Katz and Miledi, 1967).

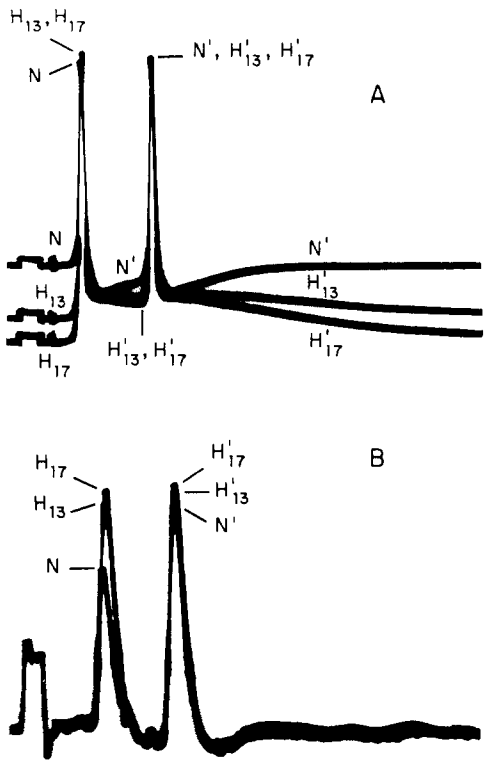

FIGURE 5. Superimposed oscillographic records of (A) action potentials and (B) PSP's recorded in a terminal at its normal (N, N') resting potential and in the same terminal when artificially hyperpolarized by 13 mV (H_{13}, H_{13}') and 17 mV (H_{17}, H_{17}'). Hyperpolarization was applied at least 10 s before the time when action potentials were elicited. Note that the foot of the second normal action potential (N') was hyperpolarized 4 mV with respect to the foot of the first normal action potential (N) and that the peak voltage of the second normal action potential (N') was somewhat higher than that of the first normal action potential (N). During conditioning hyperpolarization, the peak voltage of the first action potential (H_{13}, H_{17}) was somewhat higher than that of the normal action potential (N), but this increase in peak voltage was not sustained at the second hyperpolarized action potential (H_{13}', H_{17}'). The hyperpolarizations at the feet of action potentials are identified with the same symbols as the potentials. Note that in (B), PSP H_{13} was about the same amplitude as PSP N', and that facilitation decreased at greater levels of artificial hyperpolarization. Facilitation was 0.37 with no hyperpolarization and was 0.29 and 0.03 with 13 mV and 17 mV of hyperpolarization, respectively. Following the pair of action potentials, the membrane potential returned to its previous level at a much slower rate when artificially hyperpolarized (H_{13}', H_{17}') than when not artificially hyperpolarized (N'). The rising phases of action potentials and PSP's have been retouched. Same preparation as in Fig. 3. Calibration pulses of 2 mV, 2 ms precede each pair of potentials. Twin-pulse interval = 7 ms. $[Ca^{++}]_o$, = 5 mM, $[Mn^{++}]_o$ = 6 mM, 15 °C.

these inaccuracies strengthen our conclusion that naturally occurring changes in peak voltage cannot entirely account for facilitation at the second pulse.

Results using other experimental paradigms were in agreement with this conclusion. For example, when depolarizing pulses were applied to both the first and second action potentials so that the second pulse had a greater total amplitude but smaller peak voltage, the PSP at the second pulse was facilitated by 34% compared to the first pulse (Table I: dAP_1, dAP_2). This facilitation occurred even though the peak voltage of the second action potential was less than that of the first action potential.

TABLE I

CHANGES IN ACTION POTENTIALS, POSTSYNAPTIC POTENTIALS, AND FACILITATION

AP	Conditioning hyperpolarization	Total amplitude	Peak voltage above resting potential	PSP	*f*
	mV	*mV*	*mV*	*mV*	
nAP_1	0	73	73	1.2	0.83
nAP_2	0	84	76	2.2	
dAP_1	0	83	83	2.3	0.34
dAP_2	0	90	82	3.1	
dAP_1	0	101	101	2.6	0.23
dAP_2	0	109	103	3.4	
hAP_1	24	100	77	2.6	0.11
hAP_2	24	94	73	2.9	
hAP_1	24	100	76	2.7	0.05
hAP_2	24	98	77	2.8	

Abbreviations: AP, action potentials; nAP, normal action potentials; dAP, action potentials with increas d peak voltage due to superimposed, brief (1-2-ms) depolarizing current pulses; hAP, action potentials elicited during 24 mV of conditioning hyperpolarization. Twin-pulse interval equalled 7 ms in all cases. Data obtained from computer records using the preparation shown in Figs. 2 and 6.

Combined Effect of Naturally Occurring Changes in Peak Voltage and Hyperpolarization on Facilitation

For technical reasons, we did not directly determine whether a combination of the naturally occurring after-hyperpolarization and the naturally occurring increase in peak voltage of the second action potential (Figs. 3 A, 4) could account for all the facilitation at the second pulse in a pair or train. However, calculations made from the results of different experiments on the same terminal (such as the data given in Figs. 5 and 9, and Table I) showed that a combination of naturally occurring increases in hyperpolarization and peak voltage at the second action potential of a pair could have produced at most about 50% of the observed facilitation, if these two effects sum linearly.

For example, the pair of normal action potentials nAP_1 and nAP_2 from Table I produced PSP's of 1.2 and 2.2 mV, respectively ($f = 0.83$). The peak voltage of

the first action potential was 73 mV, whereas the peak voltage of the second action potential was 76 mV. At this same terminal shown in Table I, an artificially increased action potential of 83 mV peak voltage produced a nonfacilitated PSP of 2.3 mV whereas an artificially increased action potential

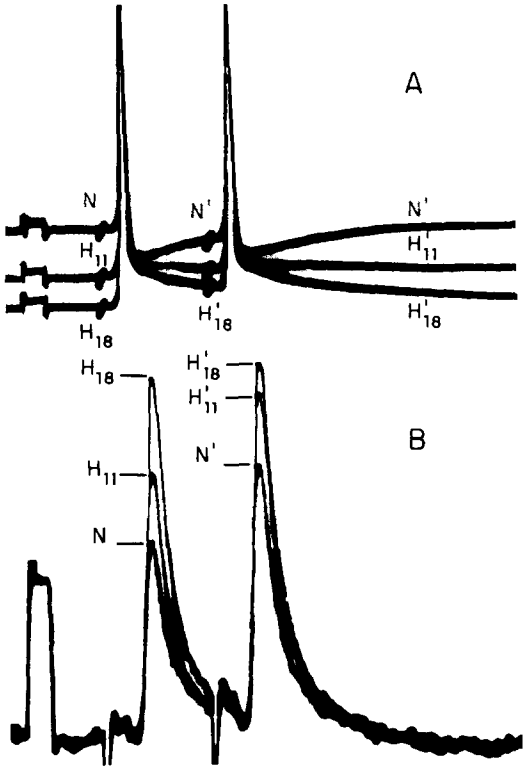

FIGURE 6. Superimposed oscillographic records of pairs of action potentials (A) and PSP's (B) elicited before (N, N') and during (H_{11}, H_{11}', H_{18}, H_{18}') conditioning artificial hyperpolarization of 11 mV (H_{11}) and 18 mV (H_{18}). The stimulus paradigm was identical to that of Fig. 5, except that the twin-pulse interval was 10 ms. Note that the feet of action potentials H_{11}' and H_{18}' were less hyperpolarized than the feet of action potentials H_{11} and H_{18}. PSP amplitude increased, but facilitation decreased with increased conditioning hyperpolarization. Facilitation was 0.43 with no hyperpolarization and was 0.30 and 0.05 with 11 and 18 mV of hyperpolarization, respectively. The rising phases of action potentials and PSP's have been retouched. Each pair of potentials is preceded by a calibration pulse of 2 mV, 2 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 4 mM, 15°C.

having 101 mV peak voltage produced a nonfacilitated PSP of 2.6 mv. By interpolation on a graph of PSP amplitude vs. peak spike voltage, a single action potential of 76 mV would have been expected to produce at most[4] a PSP of 1.5 mV. Therefore, the maximum amount of facilitation produced solely by the

increase in peak voltage from 73 to 76 mV in the pair of normal action potentials would have been about 0.25 (f = [1.5/1.2] − 1 = 0.25).

The facilitatory effect of postspike hyperpolarization for this same terminal could also be estimated. The foot of the second action potential (nAP_2 in Table I) was hyperpolarized 8 mV with respect to the resting potential. Using the approach described in Fig. 9, the effect of this naturally occurring hyperpolarization was calculated from the facilitation which would have been produced by a long-lasting artificial hyperpolarization of 8 mV; that is,

$$f = \left[\frac{\text{PSP produced by an action potential hyperpolarized 8 mV}}{\text{PSP produced by a normal action potential}}\right] - 1.$$

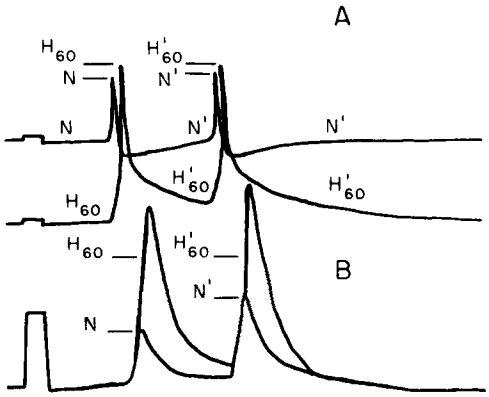

FIGURE 7. Facilitation during a very large conditioning hyperpolarization. Pairs of action potentials (A) and PSP's (B) were elicited before (N, N') and during (H_{60}, H_{60}') a conditioning artificial hyperpolarization of 60 mV. Facilitation was 0.53 with no hyperpolarization and 0.08 with 60 mV of hyperpolarization. Notice that the conduction velocity of the action potentials after conditioning hyperpolarization was slower than the velocity of a pair of normal action potentials. Same preparation as in Fig. 6. Twin-pulse interval = 10 ms. Calibration pulse = 2 mV, 2 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 4 mM, 15°C.

The maximum facilitation due to postspike hyperpolarization[3] calculated in this way was 0.16. Therefore, the maximum total facilitation which could have been produced by the simple summation of the 8 mV hyperpolarization and the increase of 3 mV in peak voltage at nAP_2 was 0.25 + 0.16 = 0.41. The actual facilitation measured at this twin-pulse interval was 0.83. In other words, a simple summation of the effects of the normally occurring after-hyperpolarization and the increase in peak voltage at the second action potential should have produced at most ~ 50% of the observed facilitation. Similar data were obtained in other preparations.

Effect of Conditioning Hyperpolarizations on Facilitation

When we applied artificial hyperpolarization to presynaptic terminals, PSP's became larger, but the amount of facilitation decreased compared to that

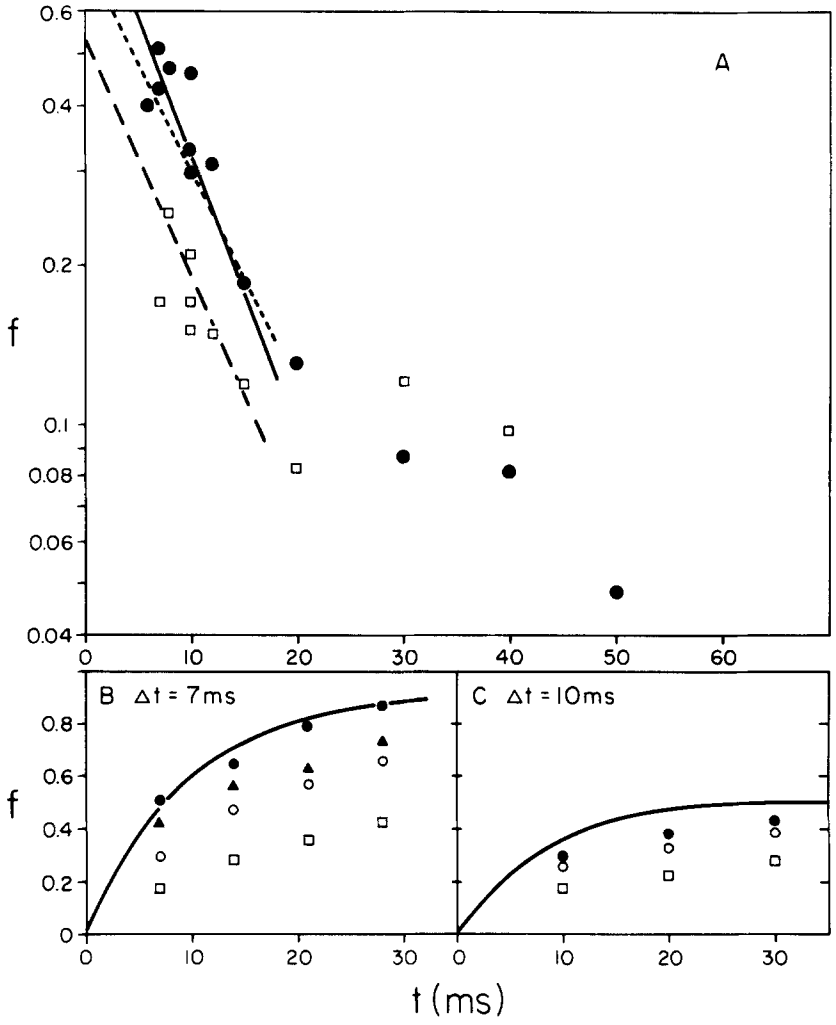

FIGURE 8. (A) Decay and (B, C) growth of facilitation before and during conditioning artificial hyperpolarization. In (A), the decay of facilitation (f) is plotted vs. the twin-pulse interval. The solid dots represent the decay of facilitation in the terminal at its normal resting potential, and the solid line represents the regression line through those points at twin-pulse intervals of < 20 ms. The squares in (A) represent the decay of facilitation in the same terminal during application of 20 mV of conditioning hyperpolarization as explained in the text. The long-dashed line is the regression line through the squares at intervals < 20 ms. The short-dashed line represents the decay of facilitation calculated to occur when allowance is made for loss of hyperpolarization at the second action potential of a pair (according to the method outlined in Fig. 9 and Table II). Note that the apparent loss of facilitation in the hyperpolarized terminal (A) was greater at short intra-pair stimulus intervals than at longer intervals. (B) and (C) represent growth of facilitation at stimulus intervals of 7 and 10 ms in the terminal with no conditioning voltage (●, ▲) and in the same terminal during 20 mV of conditioning hyperpolarization (□). The solid lines in (B) and (C) represent the growth of facilitation

produced by normal action potentials (Figs. 3, 5, 8). The decrease in facilitation was greater when larger conditioning hyperpolarizations were used (Figs. 5 and 6), but facilitation was never entirely abolished, even when terminals were artificially hyperpolarized as much as 60 mV (Fig. 7; cf. Miledi and Slater, 1966). Obviously, facilitation could still occur even though the total amplitude of action potentials in a pair had been greatly increased. Conditioning hyperpolarization produced a greater reduction in facilitation at short intrapair stimulus intervals than at longer intervals (Fig. 8).

PSP's continued to grow when trains of stimuli were given to artificially hyperpolarized terminals. The rate of increase of facilitation (but not its magnitude) appeared similar to that which occurred in normal terminals (Figs. 3, 4, and 8). It should also be noted that, as in normal terminals, facilitation in hyperpolarized terminals increased during repetitive stimulation despite progressive decreases in total amplitude, peak voltage, and postspike hyperpolarization. Inasmuch as the direction of the changes in all three parameters would be expected to reduce transmitter release, this result again implies that some factor other than these voltage changes must have been responsible for the observed facilitation.

We attempted to ascertain whether the effects of conditioning hyperpolarizations on three variables—(*a*) PSP amplitude, (*b*) peak voltage, or (*c*) voltage at the foot of successive action potentials—could directly account for the observed reduction in facilitation.

(*a*) The PSP's produced by artificially hyperpolarized terminals were larger than those produced by the same terminals with otherwise identical stimulus when it was not hyperpolarized (Figs. 3, 5, and 9). In that facilitation is reduced at higher levels of transmitter output in from neuromuscular synapses (Mallart and Martin, 1968; Rahaminoff, 1968), the possibility existed that a similar increase in transmitter release might account for the decreased facilitation in artificially hyperpolarized terminals. However, several lines of evidence indicate that this hypothesis is not correct for squid synapses.

Our experiments were done in low $[Ca^{++}]_o$ and the amount of transmitter released by hyperpolarized terminals was only a small fraction of that released at normal $[Ca^{++}]_o$. Hence, there should have been little depletion of the transmitter available for release by subsequent action potentials. Furthermore, as preparations became equilibrated to low $[Ca^{++}]_o$, facilitation at a given interval was often larger at higher levels of transmitter release in nonhyperpo-

predicted by the linear summation theory assuming that F_1 and T_1 are described by the solid line in (A) (Charlton and Bittner, 1978). The open circles in (B) and (C) represent the summation of facilitation during 20 mV of conditioning hyperpolarization when compensation was made for loss of hyperpolarization at the feet of action potentials as explained in text. The open circles in (B) should probably be compared to the filled triangles in (B) since these two sets of data were collected at more nearly the same time than the data represented by the open circles and the filled circles. All data in (A–C) represent computer averages of 40 stimulus presentations. Same preparation as used in Fig. 5. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 6 mM, 15°C.

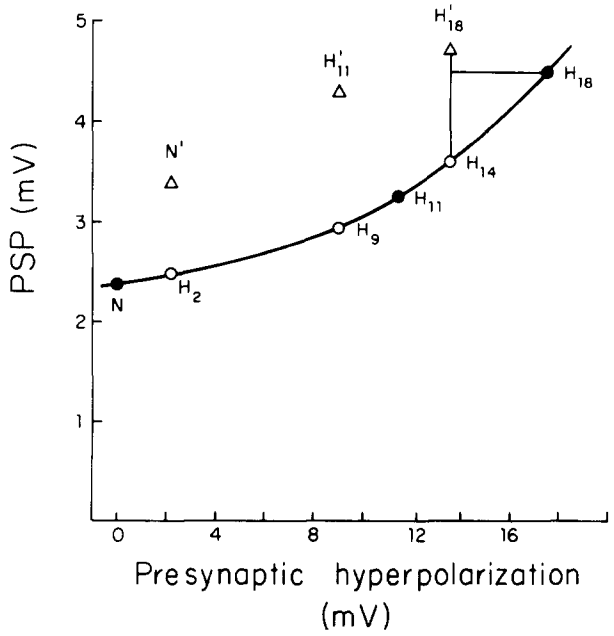

FIGURE 9. Effect of conditioning presynaptic hyperpolarization on PSP amplitude and facilitation. Data are from Fig. 6. The solid circles represent the amplitude of the PSP's produced by the first of a pair (10-ms interval) of normal action potentials (N, 0 mV hyperpolarization) and those produced by the first of a pair of action potentials given during conditioning hyperpolarization of 11 mV (H_{11}) and 18 mV (H_{18}). The solid curve was drawn through these points (●) by eye and represents the probable relation between conditioning hyperpolarization and amplitude for the first (nonfacilitated) PSP at this synapse. The triangles represent the observed amplitude of the second PSP in the pair produced by the normal terminal (N') and by the same terminal during conditioning hyperpolarization of 11 mV (H_{11}') and 18 mV (H_{18}'). The triangles have been placed at the level of hyperpolarization which was actually measured at the foot of the second action potential. For instance, the second normal action potential (N') was hyperpolarized by 2.2 mV at its foot and produced a PSP of 3.4 mV. Once the terminal had been conditioned with 11 mV of hyperpolarization, the second action potential in the pair had a hyperpolarization of 9 mV at its foot and produced a 4.3 mV PSP. The labels beside the open circles represent the intersection of the solid line (probable relation between PSP amplitude to the first pulse and amount of conditioning hyperpolarization) with the level of presynaptic hyperpolarization measured at the foot of the second (facilitated) action potential. These points (○) were used to predict the expected PSP amplitude which would have been produced by a single, nonfacilitated, action potential if the terminal had been hyperpolarized by 2(H_2), 9(H_9), or 14(H_{14}) mV. Facilitation was then calculated as the ratio (minus one) of the amplitudes of the actual second PSP's (N', N_{11}', H_{18}') to the amplitude of the nonfacilitated PSP's (H_2, H_9, H_{14}) which would have been produced had the presynaptic hyperpolarization been that which actually occurred at the second action potentials. For example, the vertical distance between H_{18}' and H_{18} represents the observed difference in transmitter release between the first (H_{18}) and the

larized terminals before equilibration than at the lower levels of release produced by the same terminals when artificially hyperpolarized after equilibration. Finally, when transmitter release was increased by the application of depolarizing current pulses of various strengths and durations during normal action potentials, facilitation was not reduced as much as when conditioning hyperpolarizing currents were used to increase transmitter release.

(*b*) During applied hyperpolarization, the second and subsequent action potential in a pair or train also had lower peak voltages than the first (Figs. 3, 5, and 7), but this effect was not sufficient to account for much of the loss of

TABLE II

CALCULATION OF THE EFFECT OF LOSS OF ARTIFICALLY MAINTAINED HYPERPOLARIZATION AT THE SECOND ACTION POTENTIAL OF A PAIR

Column #2 membrane potential at foot of first pulse	Column #1 action potential number (Fig. 6)	Hyperpolarization at foot of each pulse	PSP evoked by each pulse	f	f'
mV		*mV*	*mV*		
−70	1st (N)	0	2.4	0.43	0.35
	2nd (N')	2.2	3.4		
−81	1st (H_{11})	11	3.3	0.30	0.46
	2nd (H_{11}')	9.0	4.3		
−88	1st (H_{18})	18	4.5	0.05	0.32
	2nd (H_{18}')	14	4.7		

f' = calculated facilitation derived from Figs. 6 and 9 as explained in text. Twin-pulse interval = 10 ms.

facilitation. For example, in one terminal during conditioning hyperpolarization (Table I), the peak voltage of the second action potential was reduced to 73 mV compared to the peak voltage of 77 mV reached by the first action potential; this reduction was associated with a facilitation of 0.11 compared to 0.83 at the same terminal without conditioning hyperpolarization. However, changes in the peak voltage of action potentials in either normal or artificially hyperpolarized terminals were generally only ~ 2–5 mV and, we have calculated, by using a graph derived from data in Table I, that such small changes in peak voltage could reduce facilitation by only ~0.1.

second (H_{18}') PSP's when the terminal had been conditioned with 18 mV of hyperpolarization. The vertical distance between H_{18}' and H_{14} represents the difference between the second PSP in the terminal which had been conditioned with 18 mV of hyperpolarization and the expected (nonfacilitated) PSP amplitude at the first pulse had the terminal been conditioned by only 14 mV of hyperpolarization (i.e., the level of hyperpolarization which actually occurred at the foot of the second action potential). The facilitation at the second pulse was then calculated (H_{18}'/H_{14}) − 1 or (4.7/3.6) − 1 = 0.32. Table II gives the values of facilitation for the normal and two hyperpolarized pairs of action potentials in this synapse. The data in Table II are calculated from Fig. 6 using Fig. 9 as shown above. Data from other synapses yielded similar results. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 4 mM, 15°C.

(*c*) After an action potential in artificially hyperpolarized terminals, the membrane potential took longer to return to its base-line hyperpolarized level than in normal terminals, and successive action potentials began at progressively less hyperpolarized voltages (Figs. 3–7). For example, after an action potential in the artificially hyperpolarized terminal shown in Fig. 5, the membrane potential regained its original hyperpolarized level exponentially with a time constant of 17 ms. However, when the same terminal was not hyperpolarized, the resting potential was regained exponentially with a time constant of 5–6 ms. Inasmuch as the amount of transmitter released is affected by the level of hyperpolarization (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966), a graphical method was used to determine what effect the loss of hyperpolarization in the second or successive action potentials in an artificially hyperpolarized terminal might have had on decrease in facilitation.

The effect of conditioning hyperpolarization on transmitter release was determined at a few different levels of artificial hyperpolarizations measured from records of a single oscilloscope sweep (Figs. 5 and 6) or computer averages of 20–40 pulses; both methods gave similar results. From a graph of conditioning hyperpolarization vs. PSP amplitude (such as that shown in Fig. 9 using data taken from Fig. 6); we estimated, for each preparation, an "expected" amplitude for a single, nonfacilitated PSP which would have been produced if the level of presynaptic hyperpolarization had been equal to that observed to occur at the foot of the second action potential. Facilitation was then calculated by comparing the observed second PSP to the "expected" PSP. When facilitation was calculated in this manner for several different levels of conditioning hyperpolarization in each of four different preparations, we found that artificially hyperpolarized action potentials could have produced an average of 85% (range = 60–110%) of the facilitation produced by nonhyperpolarized action potentials. For example, in Figs. 6 and 9 the expected facilitation at a 10-ms stimulus interval was 0.35 if all spikes had arisen from the original resting potential. The expected facilitation values calculated by taking into account the effects on foot voltage of 11 and 18 mV conditioning hyperpolarizations were 0.46 and 0.32, respectively. Most of the apparent deficit in facilitation during short trains of stimuli in hyperpolarized terminals could also be accounted for by this calculation (Fig. 8).

Several other approaches were used to estimate the effects on facilitation of presynaptic voltage changes during conditioning hyperpolarizations. For example, we drew a curve through N', H_{11}', and H_{18}' (triangles in Fig. 9) to predict facilitated PSP amplitudes associated with various conditioning hyperpolarizations. By using this curve, we could estimate the expected PSP amplitude at the second pulse if the hyperpolarization at the foot of that second pulse were the same as that at the foot of the first pulse. We then compared these "expected facilitated PSP amplitudes" for the second pulse with the PSP amplitudes actually recorded for the first pulse (N, H_{11}, and H_{18} in Fig. 9). The result of this and other approaches was the same; that is, the effect of conditioning hyperpolarization on facilitation can largely or entirely be accounted for by changes in the voltage at the foot of the second or subsequent spikes in a brief train.

The Magnitude of Presynaptic Depolarizations Necessary to Produce Facilitation

It is known that transmitter release can be initiated by presynaptic depolarizations which are much smaller than those of normal action potentials and that the amount of transmitter release is drastically affected by the magnitude of the presynaptic depolarization (Bloedel et al., 1966; Katz and Miledi, 1967; Charlton and Atwood, 1977). It is not known whether the mechanism which produces the facilitation can similarly be initiated by small depolarizations or indeed whether the production of facilitation is graded with the amplitude of the depolarization producing it.

To determine this relationship, in one set of experiments we reduced the amplitude of presynaptic action potentials by application of a maintained artificial depolarization of a few millivolts before the initiation of an action potential (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966). This reduction in spike amplitude is caused by a decrease in the membrane potential at the foot voltage and the peak voltage. This latter effect is presumably due to an increase in sodium inactivation, (Hodgkin and Huxley, 1952). Fig. 2 shows one such experiment in which facilitation increased when the peak voltage of both action potentials of a pair was reduced about 10 mV. In other preparations, reductions of the peak voltage of action potentials by 16 mV reduced PSP amplitude by $> 50\%$ but left facilitation unchanged. We do not know why the results differ in different preparations, but in all cases it was evident that small action potentials could elicit facilitation.

In a second type of experiment, pairs of depolarizing pulses were delivered to a terminal poisoned by tetrodotoxin to eliminate action potentials (Fig. 10). Pairs of artificial depolarizations as small as 21 mV above resting potential (measured at the tip of the terminal) produced facilitation almost as large as that produced by normal action potentials. Experiments using three electrodes in the preterminal showed that, for these small depolarizations, the voltage gradient down the terminal would be no more than 4–5 mV. In other experiments, facilitation, although reduced, was substantial when the total amplitude of action potentials

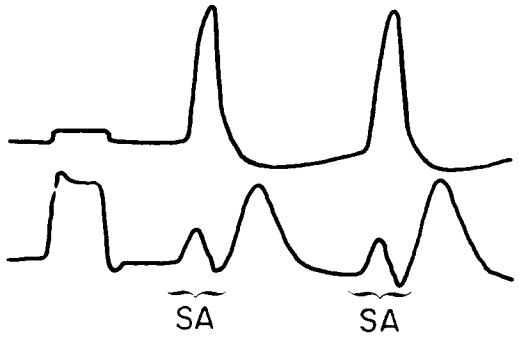

FIGURE 10. Facilitation produced by small (~20 mV) artificial depolarizations in the presence of tetrodotoxin (0.5 μg/ml.). Upper trace: presynaptic depolarizing pulses. Lower trace: recorded from the postsynaptic giant axon, PSP's, and stimulus artifact (SA). Facilitation = 0.25 at this twin pulse interval of 7 ms. Calibration pulse = 2 mV, 2 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 0 mM, 16°C.

was greatly increased by brief, appropriately timed, depolarizing pulses (Table I).

Experiments were also designed to determine whether the production of facilitation varies systematically with the amplitude of the conditioning action potential. The results of one such experiment are detailed in Figs. 11 and 12. In this experiment, a brief, variable amplitude pulse of depolarizing current was injected into a presynaptic terminal 8 ms before a pair of action potentials (intrapair interval = 6 ms) were elicited by extracellular stimulation. In this terminal, low intracellular current strengths produced graded depolarizations

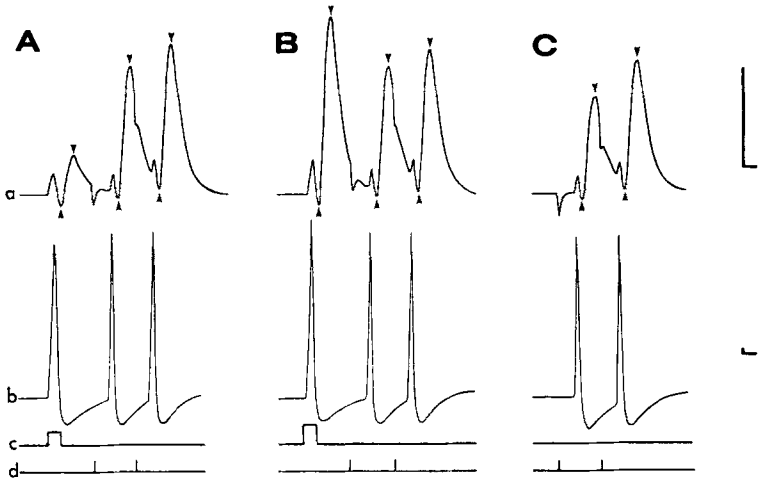

FIGURE 11. Facilitation after presynaptic pulses having variable amplitudes. In the top trace (a), the foot and peak of each PSP are marked by an arrow whereas other deflections represent stimulus artifacts and crosstalk between pre-and postsynaptic electrodes; (b) presynaptic depolarization and action potentials; (c) current injected into presynaptic terminal; (d) extracellular stimulation of presynaptic axon. Traces (a) and (b) taken from computer averages of 40 responses plotted on a chart recorder. Traces (c) and (d) have been added for clarity. In (A) and (B), note that the first PSP was produced by a variable-amplitude presynaptic depolarization whereas the second and third PSP's were produced by normal action potentials elicited by extracellular stimulation. In (A), the first PSP was ~1 mV and the presynaptic depolarization was slightly smaller than an action potential. In (B), the first PSP was about 3.8 mV and presynaptic depolarization was larger than an action potential. In (C), two PSP's were elicited by extracellular twin-pulse stimulation of the presynaptic axon (6-ms interval). Several trials similar to (A) and (B) were performed using various amplitudes for the first presynaptic depolarization. The extracellularly evoked action potentials were invariant and were considered to be standard testing pulses which tested for facilitation remaining after the variable "conditioning" pulse. In these trials, the facilitation which followed the first depolarization was determined by finding the ratio of the amplitude of the second PSP to the amplitude of the first PSP in (C). Note that the second and third PSP's in (A) are virtually the same amplitude as the second and third PSP's in (B) despite the fact that the first PSP was much larger in (B) and than in (A). Interval between variable conditioning pulse and first test pulse = 8 ms. Interval between test pulses = 6 ms. Calibration: 2 mV, 2 ms. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 4 mM, 20°C.

in the presynaptic terminal, but higher currents produced action potentials which were somewhat larger and longer than most normal action potentials in these terminals. Therefore, this stimulus paradigm produced three PSP's in which the first PSP varied in amplitude according to the amplitude of the

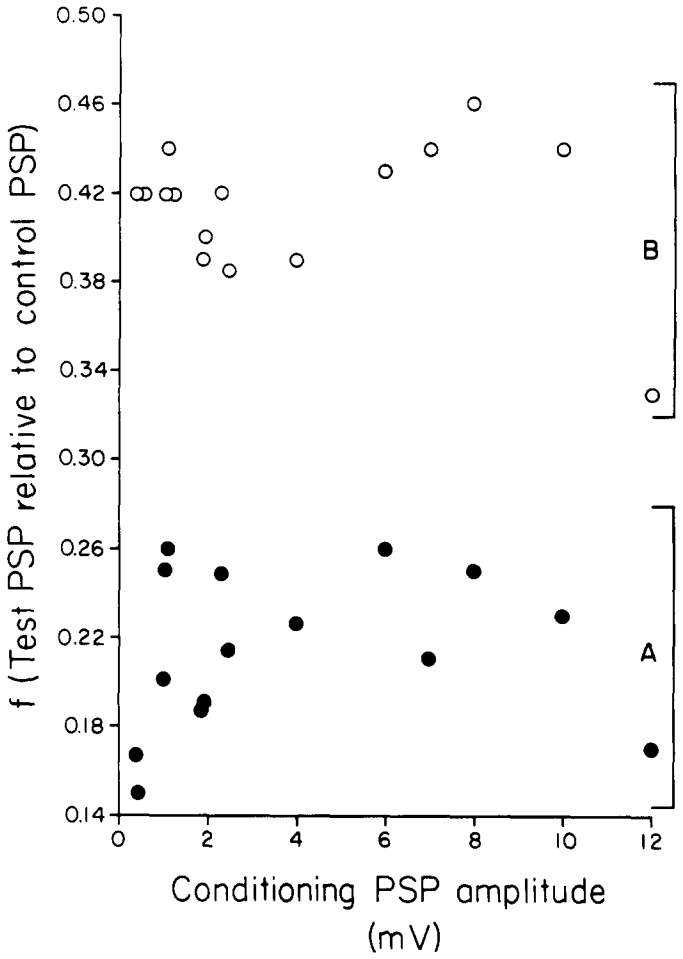

FIGURE 12. Facilitation after variable presynaptic depolarizations. Results of the experiment in Fig. 11. The amplitude of a variable "conditioning" PSP is plotted vs. the amount of facilitation which was detected by a standard "test" action potentials. (A) The solid circles represent the facilitation at first test pulse in trials similar to (A) and (B) in Fig. 11; (B) the open circles represent the facilitation at the second test pulse. Facilitation was as defined in Fig. 11. $[Ca^{++}]_o$ = 5 mM, $[Mn^{++}]_o$ = 4 mM, 20°C.

variable conditioning depolarizing pulse while the second and third PSP's were produced by "test" action potentials of constant amplitude and duration.

The amplitudes of the PSP's evoked by the two "test" potentials were compared with the amplitude of a control (nonfacilitated) PSP elicited by a

single action potential given at some time before or after the above sequence. Facilitation at each test PSP was measured as

$$f = \frac{\text{test PSP (mV)}}{\text{control PSP (mV)}} - 1.$$

The facilitation detected by the first test pulse (A in Fig. 12) consisted of the facilitation produced by a variable conditioning pulse. The facilitation detected by the second testing pulse consisted of the facilitation remaining from that produced by the variable conditioning pulse and the facilitation produced by the first test pulse. These facilitation values are plotted against the amplitude of the PSP (x axis in Fig. 12) produced by the variable conditioning pulse. (We plotted PSP amplitude rather than the depolarization of the variable test pulse because we could not be certain what fraction of the conditioning depolarization reached all the presynaptic release sites.[2–4])

The data suggest that the facilitation detected by the first test pulse (group A in Fig. 12) increased with increases in conditioning presynaptic depolarization and PSP amplitude from 0.4 to 6 mV and then declined slightly as the conditioning PSP amplitude approached 12 mV. (The 0.4 mV PSP in Fig. 12 was about 1/90 of the amplitude of the PSP which would have been produced by a normal action potential in normal $[Ca^{++}]_o$; a normal action potential in this low $[Ca^{++}]_o$ solution produced a PSP of about 2 mV.) Changes in facilitation measured by the second test pulse (group B in Fig. 12) seemed roughly parallel to those measured by the first test pulse. However, it is remarkable that the observed facilitation at the first or second test pulse changed at most by 20% while PSP amplitude, produced by the variable conditioning pulses, varied by over 3,000%. In fact, facilitation was virtually identical after conditioning pulses which produced conditioning PSP amplitudes of 0.4 and 12 mV.

The interpretation of this type of experiment is complicated by the fact that both the magnitude of the conditioning depolarization and the amplitude of the resultant PSP vary together. Furthermore, it is likely that parts of the terminal near the current passing electrode release more transmitter than other parts of the terminal which are not depolarized to the same extent.[2–4] The advantages of this paradigm are that the conditioning and testing depolarizations do not have to be the same amplitude, and that the amplitude of the testing depolarizations ceases to be an experimental variable.

DISCUSSION

The first part of this paper described attempts to determine whether voltage differences between "conditioning" and "testing" presynaptic action potentials account for facilitation.

We conclude that the independent effects of after-hyperpolarizations or increases in peak voltage cannot account for all the observed facilitation in twin pulse experiments. In fact, a combination of their effects could produce at most half of the facilitation at the second pulse. Takeuchi and Takeucki (1962) claimed that "facilitation of the PSP seems to be due mainly to the change in amplitude of the presynaptic action potential." The present results, however,

place an upper limit on the contribution of action potential changes to facilitation. Our data are also in agreement with the conclusion (Miledi and Slater, 1966; Martin and Pilar, 1964) that changes in amplitude of presynaptic action potentials are not necessary for facilitation. However, Miledi and Slater (1966) and Takeuchi and Takeuchi (1962) rapidly and repetitively stimulated the squid giant synapse to depress transmitter release to subthreshold levels at normal $[Ca^{++}]_o$. By the use of this experimental paradigm the facilitation measured by these authors may have been complicated by recovery from depression (Charlton and Bittner, 1978). This explanation could account for the fact that the time-course of the facilitation reported by Miledi and Slater (1966) differed greatly from the time-course of the first and second phases of facilitation measured in low $[Ca^{++}]_o$ (Charlton and Bittner, 1978).

The naturally occurring variations in prespike amplitude at the second pulse would lead to an underestimate of the rate of decay (T_1) of the underlying facilitation (i.e., that component of the facilitation not produced by changes in action potentials) and an overestimate of its magnitude (F_1) in twin-pulse studies. Consequently, the use of these parameters (T_1, F_1) in the linear summation model (Mallart and Martin, 1967; Charlton and Bittner, 1978) could lead to an overestimate in the predictions for the rate of growth and the final value of facilitation during short trains of stimuli. It is therefore interesting to note that in those few cases in which our data deviated substantially from the predicted curve generated by the linear summation hypothesis for squid synapses, the observed facilitation values were usually less than the predicted values. The deviations from linear summation are much greater in crustacean neuromuscular synapses than in squid synapses (Zucker, 1974 *b*; Bittner and Sewell, 1976), but there is no evidence that such gross deviations are explainable by variations in presynaptic voltage levels. Twin-pulse facilitation also appears to decay less rapidly at lower temperature and (Charlton and Bittner, 1978) and part of this effect could be due to changes in total amplitude, peak voltage, or after-hyperpolarization at the foot of the second action potential at lower temperatures.

Studies in preparations where intracellular recordings cannot be made report that PSP amplitude and facilitation during trains of stimuli are not associated with changes in the amplitude or duration of extracellularly recorded presynaptic action potentials (Hubbard and Schmidt, 1963; Katz and Miledi, 1965; Braun and Schmidt, 1966; Linder, 1973; Zucker, 1974 *b*). However, the interpretation of extracellularly recorded action potentials is difficult because such data do not indicate slow changes in membrane resulting potentials or changes in peak voltage of action potentials but only slow local membrane currents proportional to the second derivative of intracellular action potentials (Katz and Miledi, 1965). Our direct recordings of intracellular action potentials during repetitive stimulation circumvent these difficulties associated with extracellular records and show that various parameters of prespike voltage do undergo small changes during repetitive stimulation. However, in agreement with the papers cited above, we conclude that the growth of PSP's after the second pulse in a short train of stimuli is not entirely due to any combination of presynaptic

voltage changes such as progressive increases in duration, peak voltage, level of hyperpolarization, or total amplitude of spikes during trains of presynaptic action potentials. In fact, the small changes in presynaptic voltage which occur after the second pulse may oppose the detection of facilitation inasmuch as the total amplitude and intraspike hyperpolarization of the successive presynaptic action potentials decline as the PSP's increase (Figs. 3 and 4).

The second part of this paper examined two factors which could control the production of facilitation initiated by a conditioning depolarization. In particular, we examined the effects of conditioning hyperpolarizations on facilitation produced by action potentials. We also examined the effect of a variable amplitude conditioning pulse on the facilitation detected by constant amplitude test pulses.

First, we find that facilitation is reduced in terminals that are artificially hyperpolarized as reported by Hubbard and Willis (1962) and Miledi and Slater (1966).[5] However, we have observed that the apparent decrease in facilitation during conditioning hyperpolarizations is accompanied by decreases in peak voltage and hyperpolarization at the foot of the second or successive action potentials. The decreases in foot-hyperpolarization in particular should reduce transmitter release and thus obscure the ability of the facilitatory process to increase transmitter release. When the depressive effects of these presynaptic voltage changes are taken into account (Fig. 9 and associated discussion), we conclude that the magnitude and decay of twin-pulse facilitation and summation of facilitation during brief trains is similar in normal and hyperpolarized terminals. It is therefore likely that artificial hyperpolarization has no direct effect on the mechanism which produces facilitation but only interferes with the detection of facilitation.

Second, we report that an action potential can produce facilitation even though its total amplitude is drastically increased by conditioning hyperpolarization (Figs. 3–7, Table I) or by increase in peak voltage (Figs. 11 and 12). On the other hand, small action potentials (Figs. 2 and 11) and small artificial depolarizations (Fig. 10) can also elicit facilitation of similar magnitude to that produce by normal action potentials. All of these manipulations produce wide fluctuations in presynaptic potentials and in the amount of transmitter released by these potentials, yet have small effects on the amount of facilitation produced. It has been known for some time that the relationship between PSP amplitude and facilitation is complex (see discussion in Charlton and Bittner, 1978) and that production of facilitation is not dependent on the ability of an action potential to release transmitter (del Castillo and Katz, 1954; Dudel and Kuffler, 1961; Bittner and Harrison, 1970). However, the present results (Figs.

[5] In contrast to the results of Miledi and Slater (1966), we never found that the second PSP of a pair produced by a hyperpolarized terminal was smaller than the first PSP. This disparity may again be explained by differences in experimental paradigms; that is, we used low $[Ca^{++}]_o$ salines to reduce transmitter release to subthreshold levels whereas Miledi and Slater (1966) used repetitive stimulation at normal $[Ca^{++}]_o$ to depress transmitter release. Because transmitter stores may have been partially exhausted in Miledi and Slater's experiments, there may not have been sufficient transmitter available to allow for facilitation when the amplitude of the first PSP was increased by conditioning hyperpolarization.

11 and 12) show that although the amount of facilitation is affected somewhat by the amplitude of the presynaptic depolarization which produced it, nevertheless, similar amounts of facilitation are produced by large or small depolarizations which evoke large and small PSP's.

Possible Mechanisms of Facilitation

Although our data clearly indicate that naturally occurring voltage changes in successive presynaptic action potentials do not cause much of the observed facilitation after the second pulse, they do not rule out the possibility that naturally occurring changes in certain ionic currents could produce facilitation. Facilitation at these squid synapses appeared to be relatively unaffected by maintained artificial depolarization of terminals (Fig. 2). It is thus unlikely that facilitation is directly related to the increase in sodium conductance or to the influx of sodium that occurs during action potentials because sodium conductance is partially inactivated by conditioning depolarization (Hodgkin and Huxley, 1952). This conclusion is strengthened by the fact that we (Fig. 10), and others (Bloedel et al., 1966; Katz and Miledi, 1967), have found that facilitation can still occur in the presence of tetrodotoxin, a poison which eliminates the voltage sensitive sodium conductance.

However, if a particular membrane current was small in relation to the other currents flowing during an action potential, changes in this small current would not be expected to affect the shape or amplitude of action potentials (Katz and Miledi, 1969). For instance, if a calcium current ($I_{Ca^{++}}$) were to increase in successive action potentials, one would not expect to see any changes in the action potentials inasmuch as $I_{Ca^{++}}$ forms only a small fraction of the total ion current flowing during an action potential (Katz and Miledi, 1969). In fact, studies employing the Ca^{++}-sensitive photoprotein aequorin show that successive action potentials in *Aplysia somata* (Stinnakre and Tauc, 1973) or successive equal amplitude voltage-clamp pulses in other molluscan somata appear to admit increasing amounts Ca^{++} (Eckert et al., 1977; Lux and Heyer, 1977; c.f. Thomas and Gorman, 1977).

Since increases in $I_{Ca^{++}}$ have been postulated to account for the increased transmitter release seen during facilitation (Stinnakre and Tauc, 1973; Zucker, 1974 *a*), it is interesting to compare the results of our manipulations of membrane potentials with attributes of the $I_{Ca^{++}}$ found in squid terminals by Katz and Miledi (1971) and Llinas and Nicholson (1975). Facilitation and $I_{Ca^{++}}$ are not inactivated by maintained depolarization and are not activated by conditioning hyperpolarization. Facilitation and $I_{Ca^{++}}$ are not sensitive to tetrodotoxin and both can be elicited by large and small depolarizations. It is therefore evident that there are similarities in the response of both the facilitation mechanism and $I_{Ca^{++}}$ to several manipulations. However, our data do not rule out other possible mechanisms for facilitation such as residual calcium, mobilization of transmitter, Ca^{++}-mediated release of Ca^{++}, or the saturation of Ca^{++}-buffering sites.

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