Presynaptic Facilitation at the Crayfish Neuromuscular Junction

Role of Calcium-activated Potassium Conductance

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ABSTRACT   Membrane potential was recorded intracellularly near presynaptic terminals of the excitor axon of the crayfish opener neuromuscular junction (NMJ), while transmitter release was recorded postsynaptically. This study focused on the effects of a presynaptic calcium-activated potassium conductance, $g_{K(Ca)}$, on the transmitter release evoked by single and paired depolarizing current pulses. Blocking $g_{K(Ca)}$ by adding tetraethylammonium ion (TEA; 5–20 mM) to a solution containing tetrodotoxin and aminopyridines caused the relation between presynaptic potential and transmitter release to steepen and shift to less depolarized potentials. When two depolarizing current pulses were applied at 20-ms intervals with $g_{K(Ca)}$ not blocked, the presynaptic voltage change to the second (test) pulse was inversely related to the amplitude of the first (conditioning) pulse. This effect of the conditioning prepulse on the response to the test pulse was eliminated by 20 mM TEA and by solutions containing 0 mM Ca^{2+}/1 mM EGTA, suggesting that the reduction in the amplitude of the test pulse was due to activation of $g_{K(Ca)}$ by calcium remaining from the conditioning pulse. In the absence of TEA, facilitation of transmitter release evoked by a test pulse increased as the conditioning pulse grew from −40 to −20 mV, but then decreased with further increase in the conditioning depolarization. A similar nonmonotonic relationship between facilitation and the amplitude of the conditioning depolarization was reported in previous studies using extracellular recording, and interpreted as supporting an additional voltage-dependent step in the activation of transmitter release. We suggest that this result was due instead to activation of a $g_{K(Ca)}$ by the conditioning depolarization, since facilitation of transmitter release increased monotonically with the amplitude of the conditioning depolarization, and the early time course of the decay of facilitation was prolonged when $g_{K(Ca)}$ was blocked. The different time courses for decay of the presynaptic potential (20 ms) and facilitation (>50 ms) suggest either that residual

free calcium does not account for facilitation at the crayfish NMJ or that the transmitter release mechanism has a markedly higher affinity or stoichiometry for internal free calcium than does $g_{K(Ca)}$. Finally, our data suggest that the calcium channels responsible for transmitter release at the crayfish NMJ are not of the L, N, or T type.

INTRODUCTION

Evoked transmitter release from a nerve terminal is widely believed to be due to the transient rise in intracellular calcium during an action potential (reviewed by Katz, 1969; Augustine et al., 1987). Recently, it has been suggested that presynaptic voltage can itself modulate transmitter release induced by a given amount of calcium influx at the squid giant synapse (Llinas et al., 1981b) and the crayfish neuromuscular junction (NMJ) (Dudel et al., 1983; Parnas et al., 1986; Dudel, 1989a; cf. Zucker and Lando, 1986). In the crayfish, this "voltage hypothesis" of release has had no direct support since presynaptic voltage has not been measured.

Transmitter release at the crayfish NMJ is facilitated by previous activity (Dudel and Kuffler, 1961; Bittner, 1968; Zucker, 1974; Wojtowicz and Atwood, 1985). Prominent among theories to explain this phenomenon is the residual calcium hypothesis, which states that the probability of transmitter release is increased by an accumulation of calcium which occurs in the terminal during repetitive stimulation (Katz and Miledi, 1968; Rahamimoff, 1968; Zucker and Lara-Estrella, 1983). This hypothesis predicts that the time course of decay of facilitation is determined by the time course of decay of residual free calcium in the presynaptic terminal. As with the voltage hypothesis of release, the residual calcium theory has only indirect support at the crayfish NMJ, since intracellular calcium measurements at release sites have been difficult to obtain during facilitation.

In the previous paper (Sivaramakrishnan et al., 1991), we demonstrated the presence of a calcium-activated potassium conductance, $g_{K(Ca)}$, in presynaptic terminals of the crayfish opener excitor axon and showed that $g_{K(Ca)}$ strongly affects the repolarization of natural action potentials or artificial depolarizing pulses when other potassium conductances, such as the delayed rectifier potassium conductance, g_K, are blocked by the aminopyridines. This paper discusses the effects of $g_{K(Ca)}$ on transmitter released by a single pulse and facilitation of transmitter release measured with twin pulses.

We find that the presynaptic membrane potential during twin pulse facilitation is strongly affected by activation of $g_{K(Ca)}$. This conductance change is sufficient to explain some of the results that have been attributed to a voltage effect on release. Furthermore, we use the decline of $g_{K(Ca)}$, as determined in twin pulse experiments, to estimate the time course of decay of intracellular calcium near presynaptic release sites. The time course of decay of facilitation differs markedly from this estimate of the decay of free internal calcium, since significant facilitation persists even after $g_{K(Ca)}$ has returned to control values. These results suggest either that the mechanisms responsible for $g_{K(Ca)}$ and facilitation are spatially separated, that they have quite different calcium dependencies, or that the simplest forms of the residual calcium hypothesis of facilitation are wrong.

Some of these results have already appeared in preliminary form (Sivaramakrishnan et al., 1988).

MATERIALS AND METHODS

Experiments were performed on the opener excitor nerve–muscle preparation in the first walking leg of the crayfish *Procambarus clarkii*. Methods for recording from the opener excitor axon and muscle fibers were the same as those described in the previous paper (Sivaramakrishnan et al., 1991). Briefly, two intracellular microelectrodes were inserted into a secondary branch of the excitor axon within a fraction of a space constant from the nearest synaptic terminals in the presence of potassium channel blockers. One of these intracellular electrodes was used to pass current into the presynaptic terminal and the other electrode was used to record presynaptic membrane potential. A third electrode inserted into the subsynaptic muscle fiber was used to record postsynaptic potentials. The muscle fibers used in these experiments were located in the central portion of the opener muscle since the excitatory synapses made on these central muscle fibers are generally of the high facilitating type (Bittner, 1968; Bittner and Segundo, 1989).

Measurements of Transmitter Release and Facilitation in the Absence of Action Potentials

Excitatory postsynaptic potentials (EPSPs) recorded in the muscle fiber were used as measures of transmitter release from the presynaptic terminal. EPSPs were evoked with artificial depolarizations of the terminal produced by injecting current pulses intracellularly into a secondary branch of the excitor axon. These experiments were performed in the presence of 0.1 μM tetrodotoxin (TTX) to block the regenerative sodium conductance (g_{Na}) and 1 mM 3,4-diaminopyridine (3,4-DAP) or 4-aminopyridine (4-AP) to block the delayed rectifier potassium conductance (g_K). The presynaptic membrane potential could be changed by changing the strength of an injected current pulse (pulse durations = 2–5 ms). The amplitudes of EPSPs evoked by presynaptic depolarization were plotted against the membrane potential to produce synaptic input–output curves. EPSP recordings often contained an artifact due to capacitative coupling between the presynaptic current passing electrode and the postsynaptic recording electrode. To eliminate the possibility that such an artifact might be responsible for incorrect interpretations of our postsynaptic voltage recordings, we decreased the artifact in some experiments by shielding the current passing electrode with silver paint, over which a coat of Q-dope (General Electronics) was applied. EPSPs recorded in such experiments did not show significantly different peak values from those in which the capacitative artifact was not eliminated.

Twin pulse facilitation was studied by injecting two depolarizing current pulses into the excitor axon. The first pulse is referred to as the conditioning pulse and the second pulse as the test pulse. Facilitation produced by the conditioning pulse was measured by the test pulse. Transmitter release that occurred in response to the test pulse when it was preceded by the conditioning pulse was compared with transmitter release in response to a control pulse (which was generated by a current pulse of the same strength as the test pulse) given without the conditioning pulse. Facilitation (F) at a given pulse interval was determined by the relation

$$F = (V - V_o)/V_o$$

where V_o is the amplitude of the EPSP in response to a single control pulse, and V is the amplitude of the EPSP in response to the test pulse preceded by the conditioning pulse (Mallart and Martin, 1967).

RESULTS

$g_{K(Ca)}$ and Transmitter Release

We have shown in the previous paper (Sivaramakrishnan et al., 1991) that the $g_{K(Ca)}$ present in presynaptic terminals of the excitor axon accounts for a large part of the rectification of the membrane current–voltage relationship. Since factors that affect membrane potential also affect transmitter release (Llinas et al., 1981a, b; Augustine et al., 1985a, b), synaptic input–output curves (see Materials and Methods) were examined in the presence and absence of $g_{K(Ca)}$. Fig. 1 A shows a typical experiment
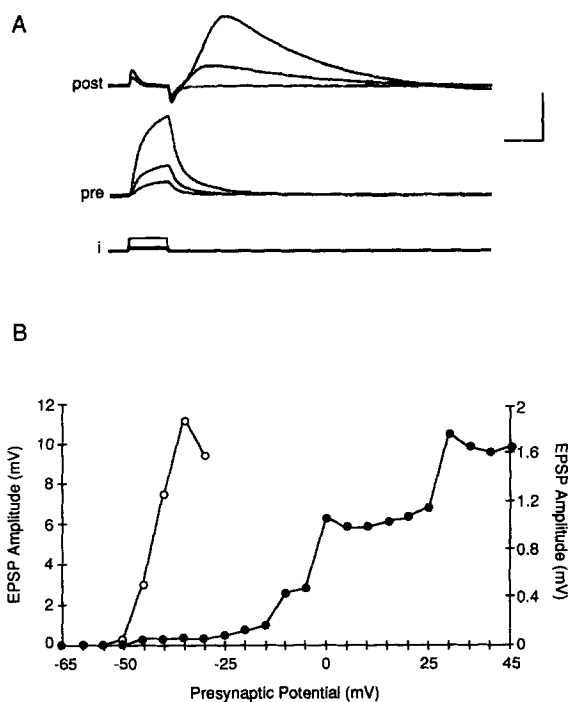

FIGURE 1. (A) Computer traces of pre- and postsynaptic recordings at the crayfish NMJ in presence of 0.1 μM TTX + 1 mM 4-AP + 20 mM TEA. Top trace, EPSPs recorded from a subsynaptic muscle fiber in response to presynaptic depolarizations. Middle trace, presynaptic membrane potential changes during transmitter release. Bottom trace, current pulses injected into the secondary branch. Scale: 5 ms, 200 nA, 30 mV (middle trace), 2 mV (top trace). (B) Input–output plots of presynaptic depolarization versus transmitter release measured as EPSP amplitude from a subsynaptic muscle fiber. Open circles and left abscissa, Van H containing TTX + 3,4-DAP + 20 mM TEA. Filled circles and right abscissa, Van H containing TTX + 3,4-DAP. Note different abscissa scales on left and right. Resting potential of terminal = −70 mV. External calcium = 13.5 mM (normal level in crayfish saline). Both curves were generated in the same preparation and from the same set of presynaptic terminals.

from which data were used to generate input–output curves. In this and most other experiments, the current pulses used were 2–5 ms in duration, and transmitter release always began at the end of the current pulse, with the peak response occurring as the presynaptic membrane potential returned to rest. Most of the transmitter release occurred after the depolarizing pulse, suggesting that the "off response" (Katz and Miledi, 1970) constituted a major portion of the EPSP. Note that in Fig. 1 A the off response is accompanied by the decay of the presynaptic depolarization which is appreciable for the largest depolarizing pulses.

The shape of the input–output curve was greatly affected by the presence or absence of TEA which blocks $g_{K(Ca)}$. When g_{Na} and g_K were blocked with TTX and 3,4-DAP, but $g_{K(Ca)}$ was not blocked, the synaptic input–output curve often showed "steps" (Fig. 1 *B*, filled circles). The overall slope of the steepest portion of the curve ranged from 1.5 to 4 (EPSP mV/presynaptic mV) in 10 experiments. When 10–20 mM tetraethylammonium (TEA) was added to the bath to reduce $g_{K(Ca)}$ (Sivara-makrishnan et al., 1991), the input–output curve was smooth and did not show steps, the slope increased, and transmitter release began at lower presynaptic depolarizations. In TEA, the amplitude of the EPSP was 5–10-fold greater than in TTX and 4-AP alone (Fig. 1 *B*, open circles). These concentrations of TEA did not significantly affect the quantal size of spontaneous miniature potentials. In most preparations, transmitter release in response to short pulses (2–5 ms) did not decrease at depolarizations greater than +70 mV whether or not $g_{K(Ca)}$ was blocked. In some experiments ($n = 6$) transmitter release seemed to "saturate" at large depolarizations (greater than +20 to +30 mV), with the EPSP amplitude showing very little change over a 30–40-mV change in membrane potential (Fig. 1 *B*). In a few experiments ($n = 2$), transmitter release did decrease at large depolarizations, especially when TEA was present. This decrease was most probably due to a temporary depletion of transmitter stores, since reverting to smaller depolarizations produced less release than before and this decrease was not observed if at least 3–5 min were allowed to elapse between successive depolarizations of the presynaptic terminal.

Recovery from $g_{K(Ca)}$ Activation in a Two-Pulse Protocol

A two-pulse protocol was used to generate facilitation in our experiments, and therefore it was of interest to examine the effects of $g_{K(Ca)}$ on membrane potential during twin pulse depolarization of the presynaptic terminal. $g_{K(Ca)}$ has been shown to depend both on membrane voltage and intracellular free calcium (Magleby and Pallotta, 1983). Thus, if a conditioning pulse produces an increase in intracellular calcium which has not completely decayed at the time of a subsequent test pulse, then the test pulse should exhibit larger effects of $g_{K(Ca)}$ because of the accumulated calcium. Increased $g_{K(Ca)}$ should therefore cause a greater decline in potential during the test pulse (see relaxation phase of Fig. 3 of Sivaramakrishnan et al., 1991). Fig. 2 *A* shows the results of varying the peak conditioning depolarization in an experiment without TEA, i.e., when $g_{K(Ca)}$ is not blocked. As the peak depolarization of the conditioning pulse is increased, the peak test depolarization decreases. For example, the smallest conditioning pulse (labeled 1 in Fig. 2 *A*) is associated with the largest depolarizing response to a constant current test pulse. The data of Fig. 2 *A* are consistent with all experiments ($n = 9$) in which $g_{K(Ca)}$ is not blocked.

When $g_{K(Ca)}$ was partially blocked with 5 mM TEA, a relaxation was not observed during a single pulse or during a test pulse preceded by a conditioning depolarization less than 40 mV (e.g., pulse 1 of Fig. 2 *B*). As the peak conditioning depolarization was increased beyond 40 mV, the peak depolarization of the test pulse decreased and a relaxation became apparent during the test depolarization (e.g., pulses 3 and 4 of Fig. 2 *B*). As the interval between the conditioning and test pulses increased, the peak test depolarization gradually recovered with a time course of 20

ms to the value obtained during the control pulse (Fig. 2 C). This time course of recovery of the test pulse depended on the magnitude of the conditioning depolarization: the larger the conditioning depolarization, the longer the recovery time of the test depolarization.
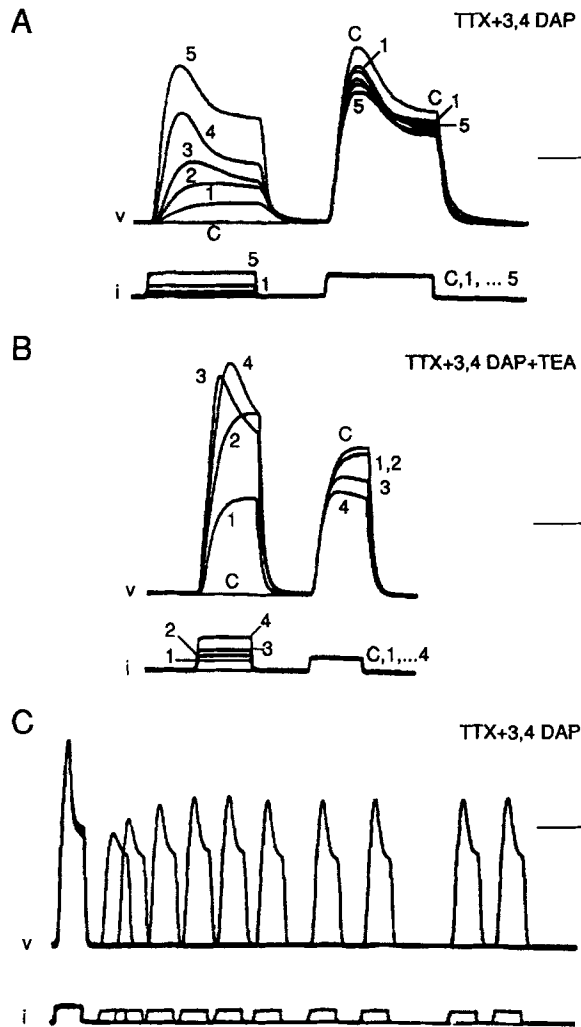

FIGURE 2. Effect of amplitude of conditioning pulse (A, B) or interpulse interval (C) on the maximum depolarization produced by a test pulse of constant current strength. In A and C the bathing solution contained 0.1 µM TTX + 1 mM 3,4-DAP. In B the bathing solution contained 0.1 µM TTX + 1 mM 3,4-DAP + 5 mM TEA. The interval between pairs of pulses was ≥5 min and 10 pulse pairs were superimposed in C. Top traces, membrane potential changes recorded in secondary branch of excitor axon. Bottom traces, current pulses injected into excitor axon. In A and B, each conditioning and test pulse is labeled from 1 to 4 (or 5) in increasing order of conditioning current strength. C, control trace (no conditioning pulse). Note that test pulse current strength is kept constant in A, B, and C. The control current strength (i.e., a test pulse lacking a preceding conditioning pulse) equals the test current strength. Note also that the largest conditioning pulse (5 in A, 4 in B) produces the smallest voltage response to the constant current test pulse. Scale: (A) 2 ms, 50 mV, 200 nA; (B) 5 ms, 20 mV, 200 nA; (C) 10 ms, 50 mV, 200 nA.

In contrast, when $g_{K(Ca)}$ was greatly reduced by increasing TEA to 20 mM, the conditioning pulse exerted no influence on the voltage response of the test pulse. There was no evidence of the relaxation during the test pulse (Fig. 3 A) and no decrease in the peak test depolarization even when it was preceded by very large conditioning pulses. Furthermore, the peak test depolarization remained the same at

all intervals between the conditioning and the test pulses (Fig. 3 *B*). Similar results were obtained in bathing media containing 0.5 mM cadmium or 0 mM $Ca^{2+}/1$ mM EGTA instead of 20 mM TEA (data not shown).

These data indicate that the peak test depolarization is reduced by increased activation of $g_{K(Ca)}$ during the test pulse, probably due to the increased intracellular calcium present during the test pulse as a result of increasing conditioning depolarizations.
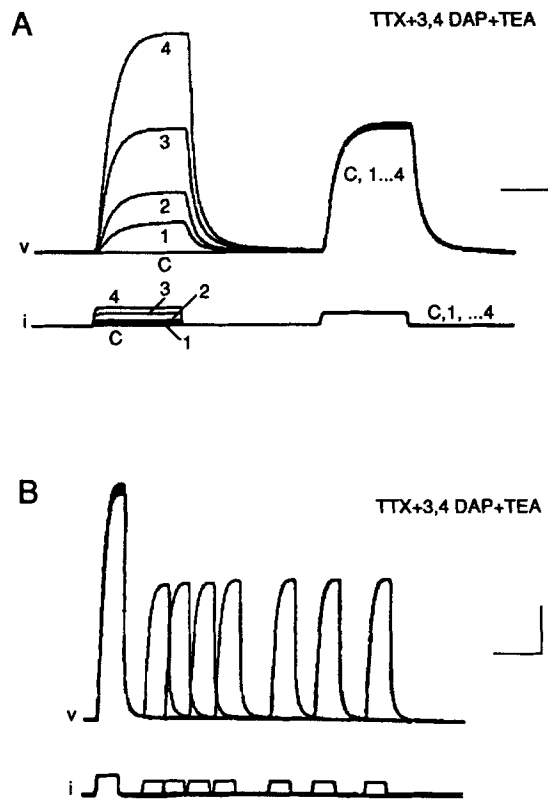

FIGURE 3. Protocol as in Fig. 2, except that $g_{K(Ca)}$ was greatly reduced with 20 mM TEA in the presence of 0.1 μM TTX + 1 mM 3,4-DAP. *Top traces,* potential changes in the axon. *Bottom traces,* current pulses injected into axon. (*A*) Strength of test current pulse kept constant while that of conditioning current pulse varied. The interpulse interval was also kept constant. Pulses labeled 1 . . . 4 are four conditioning and test pulse traces generated by conditioning pulses of increasing magnitude. *C,* control pulse (a pulse given without a conditioning pulse having a current strength equal to that of the test pulse). Note that the amplitudes of the conditioning pulses no longer influence the amplitude of the response to the test pulse. (*B*) Strengths of conditioning and test current pulses kept constant while the interval between pulses was varied. The interval between pairs of pulses was ≥ 5 min and seven pulse pairs were superimposed. Scale: (*A*) 2 ms, 50 mV, 200 nA; (*B*) 10 ms, 50 mV, 200 nA.

$g_{K(Ca)}$ and Facilitation

We examined twin pulse facilitation in the presence and absence of $g_{K(Ca)}$. In one set of experiments, facilitation at the test pulse relative to a control pulse (see Materials and Methods) was plotted against the peak conditioning depolarization. In these experiments, only the strength of the conditioning current pulse was varied. The strength of the test current pulse, the strength of the control current pulse, and the interval between the conditioning and test pulses were all kept constant. When $g_{K(Ca)}$ was not blocked, the peak test depolarization decreased as the peak conditioning depolarization increased (Fig. 2 *A* and right-hand ordinate of Fig. 4 *A*). In such cases

(n = 6), facilitation initially increased as the peak depolarization of the conditioning pulse increased from −40 to −20 mV. However, facilitation then decreased with further increases in the conditioning depolarization (left-hand scale of Fig. 4 A). When $g_{K(Ca)}$ was blocked with 15–20 mM TEA, the peak test depolarization remained unchanged as the peak conditioning depolarization increased (Fig. 3 A). In such cases (n = 7), facilitation showed no decrease at high amplitudes of the conditioning depolarization but continued to increase, reaching a plateau at conditioning depolar-
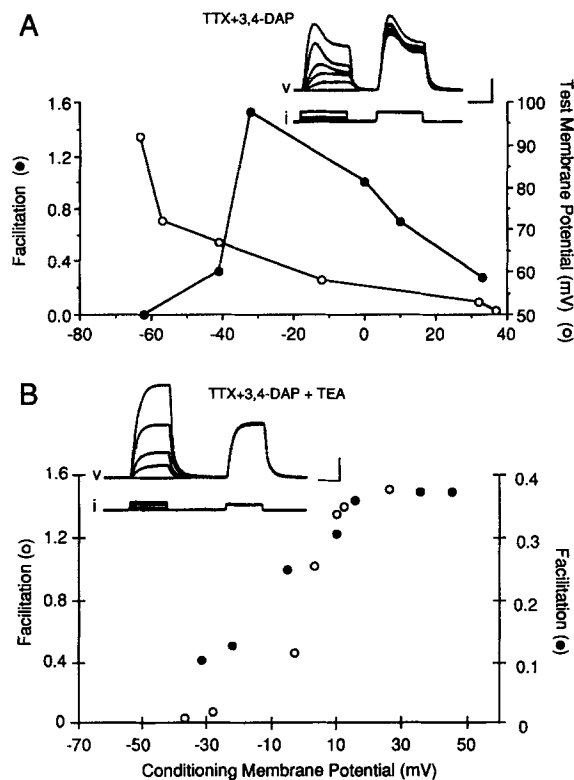

FIGURE 4. Growth of twin pulse facilitation in the absence (A) and presence (B) of TEA to block $g_{K(Ca)}$. Test pulse current strength was kept constant while conditioning current strength varied. Facilitation of EPSP amplitudes recorded in a subsynaptic muscle fiber is plotted as a function of peak amplitude of conditioning depolarization in a secondary branch. (A) Same experiment as Fig. 2 A. $g_{K(Ca)}$ not blocked; 0.1 μM TTX and 1 mM 3,4-DAP used to block g_{Na} and the delayed rectifier g_K. Left-hand abscissa, facilitation of EPSP amplitudes (filled circles); right-hand abscissa, maximum depolarization of the membrane potential above resting levels during the test pulse (open circles). Note that although the current strength used to elicit the test pulse remains constant, the maximum depolarization decreases as

$g_{K(Ca)}$ activation by the control pulse increases. (B) Facilitation of EPSP amplitudes when $g_{K(Ca)}$ is greatly reduced with 20 mM TEA in the presence of 0.1 μM TTX + 1 mM 3,4-DAP. Left-hand scale, open circles; right-hand scale, filled circles. Data for filled and open circles are taken from different preparations. Data from filled circles came from preparation shown in Fig. 3 A and the insert of Fig. 3 B. Scale: 10 ms, 50 mV, 200 nA for A and B.

izations greater than about +20 mV (Fig. 4 B). In 15–20 mM TEA, the overall relationship between facilitation and peak conditioning depolarization was sigmoidal.

In another set of experiments, the decay of facilitation was examined by plotting the facilitation at different intervals between the conditioning and test pulses. In these experiments only the time interval between the conditioning and test pulses was varied. The current strengths of the conditioning and test pulses were not necessarily equal to each other, but both were kept constant. In the presence of $g_{K(Ca)}$,

the peak test depolarization decreased as the interval between the conditioning and test pulses decreased (Figs. 2 C, 5 A). In such cases, an initial rapid decay of facilitation (defined here as the fast component) with a time course of 20 ms was followed by a slight increase in facilitation (Fig. 5 A). This increase or "hump" was followed by a slower decay of facilitation (defined here as the slow component), which had a time course of 50 ms or more in different preparations ($n = 6$). The time course of the fast component of decay corresponded closely with the decrease in the peak test depolarization at small interpulse intervals and the hump corresponded with the return of the test depolarization to its control value. After the hump, facilitation continued to decay even though the peak test depolarization was now constant (Fig. 5 A). When $g_{K(Ca)}$ was blocked with TEA, the peak depolarization of the
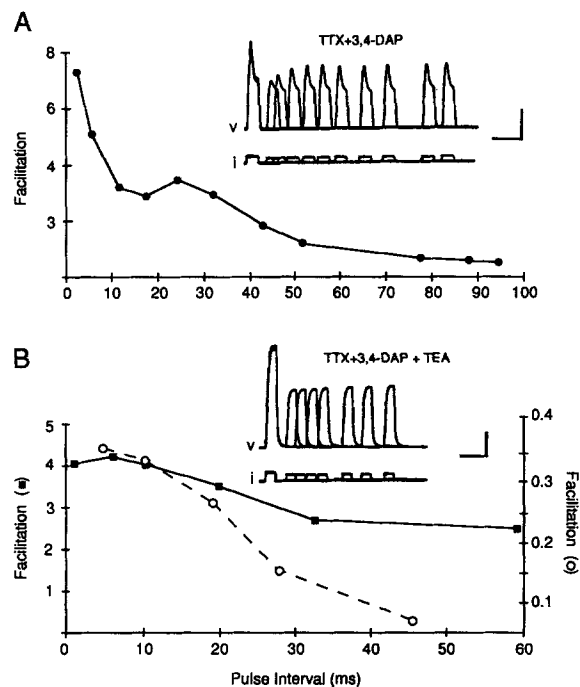

FIGURE 5.   Effect of $g_{K(Ca)}$ on decay of twin pulse facilitation. Facilitation, calculated as in Fig. 4, is plotted as a function of interpulse interval which is measured from end of conditioning depolarization to start of test depolarization. (A) Same preparation shown in Fig. 2 C bathed in 0.1 μM TTX + 1 mM 3,4-DAP ($g_{K(Ca)}$ not blocked). (B) Decay of facilitation plotted for two preparations with $g_{K(Ca)}$ greatly reduced with 20 mM TEA in the presence of 0.1 μM TTX + 1 mM 3,4-DAP. *Left-hand abscissa,* filled squares taken from preparation shown in the inset; *right-hand abscissa,* open circles. Inset scale: 10 ms, 50 mV, 200 nA for A and B.

test pulse did not decrease from its control value even at small intervals from the conditioning pulse (Fig. 3 B). In such cases ($n = 5$), the decay curve of facilitation (Fig. 5 B) lacked the initial fast component and the hump, but retained a slower component with a time course of 50 ms or more.

DISCUSSION

In the presence of TTX, transmitter released by artificial presynaptic depolarizations is greatly enhanced when $g_{K(Ca)}$ is blocked by TEA and g_K is blocked by the aminopyridines. Under these conditions, the synaptic input–output curve is shifted toward smaller depolarizations (Fig. 1). This shift probably results from an increased

space constant of the presynaptic membrane when $g_{K(Ca)}$ is blocked with TEA (see Figs. 2 and 4 of Sivaramakrishnan et al., 1991). Since $g_{K(Ca)}$ is responsible for ~60% of the g_m activated by presynaptic depolarizations as determined with long (50 ms) pulses (Fig. 4 of Sivaramakrishnan et al., 1991), the use of TEA to block $g_{K(Ca)}$ should allow a much greater percentage of the depolarization produced at the current injection site in the secondary branch to reach the terminals made by the excitor axon on the subsynaptic muscle fiber. The larger depolarization should increase the inward calcium current, thereby increasing the amount of transmitter released and shifting the synaptic input–output curve toward smaller depolarizations. The steps in the synaptic input–output curve generated in the absence of TEA would seem to be due to the recruitment of additional synaptic terminals by depolarizations of increasing strengths. This suggestion is borne out by the disappearance of these steps when TEA is present, indicating that, in TEA, depolarizing current spreads more uniformly to all the presynaptic terminals made on a single muscle fiber.

$g_{K(Ca)}$ and Facilitation: Implications for the Voltage Hypothesis of Release

When $g_{K(Ca)}$ is present, the twin pulse facilitation measured at a given conditioning–test pulse interval decreases as the maximum depolarization of the conditioning pulse increases (Fig. 4 A). We hypothesize that this decrease in facilitation is a consequence of the decrease in peak depolarization of the test pulse (test current strength kept constant) because of the increased residual calcium and hence $g_{K(Ca)}$ during the test pulse. Increased activation of $g_{K(Ca)}$ during the test pulse causes the membrane potential to move toward the potassium equilibrium potential, decreasing the magnitude of the test depolarization and hence the amount of transmitter released in response to the test pulse. Facilitation would therefore be less. When $g_{K(Ca)}$ is blocked by TEA, there is no decrease in the maximum depolarization of the test pulse when it is preceded by a conditioning pulse (Fig. 3 A) and consequently no decrease in facilitation (Fig. 4 B).

A decrease in the growth of twin pulse facilitation with increasing strengths of the conditioning current pulse, similar to that observed in Fig. 4 A, has been reported previously in crayfish (Dudel et al., 1983). Since the residual calcium theory of facilitation suggests that the degree of facilitation measured by the test pulse is a function of calcium entry during the conditioning pulse (Katz and Miledi, 1968; Zucker and Lara-Estrella, 1983), this decrease in twin pulse facilitation produced by larger conditioning pulses has been interpreted as being due to a decrease in calcium entry during the conditioning pulse (Dudel et al., 1983). Since transmitter release is directly proportional to some power of the presynaptic calcium concentration (Llinas et al., 1981b; Augustine et al., 1985b), reduced calcium entry during a single pulse should decrease the amount of transmitter released by that pulse. However, as evident from Fig. 1, transmitter release in response to a single current pulse increases as the strength of the current pulse continues to increase (see also Dudel et al., 1983) even though calcium entry supposedly (Dudel et al., 1983) decreases. To explain this apparent paradox, Dudel et al. (1983) invoked a direct effect of voltage on transmitter release. We propose instead that, as is the case at the squid giant synapse (Llinas et al., 1981b; Augustine et al., 1985b), transmitter release in response to a single pulse of increasing depolarization continues to increase because calcium entry

increases with increasing depolarization. It is this increase in internal calcium that results in an increased activation of $g_{K(Ca)}$ during the test pulse and, consequently, a decrease in the peak depolarization (see Fig. 4 A, right ordinate), calcium entry, and transmitter release during the test pulse. Facilitation would then decrease. Our observation that TEA eliminates this effect of large conditioning pulses on facilitation is consistent with this explanation. In light of this effect of $g_{K(Ca)}$ on twin pulse facilitation, the voltage hypothesis of transmitter release at the crayfish NMJ (Parnas et al., 1982*a*, *b*, 1984, 1986; Dudel et al., 1983; Parnas and Segel, 1984) should be reconsidered.

More recently, data suggesting that calcium-induced transmitter release at the crayfish NMJ is greater than expected at small depolarizing currents or low external calcium concentrations have been used as additional support for the voltage hypothesis of transmitter release (Dudel, 1989*a–d*). However, these data can more readily be explained by the following two observations. First, activation of $g_{K(Ca)}$ is both voltage and calcium dependent (Barrett et al., 1982; Methfessel and Boheim, 1982; Wong et al., 1982; Moczydlowski and Latorre, 1983) and, at the crayfish NMJ, the decrease in depolarization caused by activation of $g_{K(Ca)}$ does not occur at depolarizations <40–50 mV (Sivaramakrishnan et al., 1991, Fig. 3). Therefore, transmitter release is greater than expected at small depolarizations or low calcium concentrations because, under these conditions, the effect of $g_{K(Ca)}$ on reducing the depolarization is minimal. Second, when the delayed rectifier g_K and $g_{K(Ca)}$ are not blocked, and the presynaptic membrane is depolarized by >50 mV, the current–voltage curve is no longer linear (Sivaramakrishnan et al., 1991, Fig. 4). This nonlinearity implies that, when the rectifier g_K and $g_{K(Ca)}$ are not blocked, the variation of transmitter release and facilitation with current injected into the presynaptic terminal (as plotted in Dudel et al., 1983; Dudel, 1989*a–d*) differs greatly from the variation of transmitter release and facilitation with the actual voltage across the presynaptic terminal membrane (as plotted in Figs. 1 and 4). Dudel (1989*a*) also mentions, but gives no details about, a lack of effect of TEA and 3,4-DAP on transmitter release and facilitation. This result contradicts our own observations (Figs. 1 and 4) in which we intracellularly measure the potential near the presynaptic terminal. We have no explanation for this discrepancy unless Dudel never used these potassium channel blockers in combination.

As another alternative to the voltage hypothesis, the apparent decrease in facilitation with large, extracellularly applied, conditioning pulses (Dudel et al., 1989*a–c*) has been attributed to poor spatial control of voltage over the several nerve terminals made by the excitor axon on a single opener muscle fiber (the "rim effect," Zucker and Lando, 1986). This hypothesis is unnecessary in view of our data showing the modulation of depolarization by $g_{K(Ca)}$ (Sivaramakrishnan et al., 1991).

$g_{K(Ca)}$ as an Assay of Internal Free Calcium: Implications for the Residual Calcium Theory of Facilitation

During a twin pulse experiment (e.g., Fig. 2 C), increasing the interpulse interval should decrease the contribution of residual free calcium to the activation of $g_{K(Ca)}$ during the test pulse. This process would explain why both cadmium (which blocks a rise in internal calcium during depolarization) and TEA (which blocks $g_{K(Ca)}$) eliminate

the time-dependent effects of a conditioning depolarization on a test depolarization (Fig. 3 B). As a corollary, recovery of the peak depolarization of the test pulse (and hence the decrease of $g_{K(Ca)}$) at longer twin pulse intervals may reflect the decay of internal free calcium in the terminal. Hence, $g_{K(Ca)}$ may be a useful bioassay of internal free calcium in the dependence of facilitation on residual free calcium may be estimated by comparing the time courses of decay of $g_{K(Ca)}$ and facilitation.

Fig. 2 C suggests that $g_{K(Ca)}$ recovers to control values with a time course of ~20 ms. Since $g_{K(Ca)}$ depends on internal free calcium (Barrett et al., 1982; Methfessel and Boheim, 1982; Wong et al., 1982; Moczydlowski and Latorre, 1983), these data suggest that within ~20 ms residual free calcium in the terminal has declined to a level too low to activate $g_{K(Ca)}$. This time course of decay of residual free calcium should be the same when $g_{K(Ca)}$ is blocked. However, when we block $g_{K(Ca)}$, facilitation decays more slowly (50 ms or more) than this estimate for the decay of residual free calcium (Fig. 5 B).

If $g_{K(Ca)}$ and facilitation are both dependent on free calcium, at least two factors need be considered when comparing their time courses. The first is the relative positions of the channels responsible for $g_{K(Ca)}$ and the machinery responsible for facilitation. If these structures are located close together they should see similar free calcium concentrations. If not, then calcium ions that enter the nerve terminal during depolarization of the presynaptic membrane would have to diffuse unequal distances before activating the machinery responsible for facilitation and the channels responsible for $g_{K(Ca)}$. Hence, even if facilitation and $g_{K(Ca)}$ were both governed by the same free internal calcium, they could exhibit different time courses governed by the different diffusion times of calcium ions. However, our observations of the times of onset of $g_{K(Ca)}$ and transmitter release at the crayfish NMJ suggest that the temporal discrepancy between $g_{K(Ca)}$ and facilitation is not due to differing diffusion times of calcium ions in the presynaptic terminal. For example, relaxation in membrane voltage caused by activation of $g_{K(Ca)}$ begins as early as 2 ms after the onset of the depolarization (Sivaramakrishnan et al., 1991, Fig. 3). This latency is of the same magnitude as the latency of transmitter release evoked by natural action potentials, suggesting, albeit indirectly, that the calcium ions that enter the presynaptic terminal upon depolarization travel similar distances to reach the calcium-activated potassium channels and the release machinery. The second factor, about which we have no information, is whether $g_{K(Ca)}$ and facilitation have the same sensitivity to presynaptic free calcium. An apparent temporal discrepancy between the decays of $g_{K(Ca)}$ and facilitation could occur if these two processes have different stoichiometries or affinities to calcium.

It is also possible that the time courses of $g_{K(Ca)}$ and facilitation do not coincide because facilitation is due to some factor other than residual free calcium, while $g_{K(Ca)}$ depends on free calcium. As one alternative, residual *bound* calcium may be responsible for facilitation at the crayfish opener NMJ. A similar model has been proposed by Balnave and Gage (1974) for the frog NMJ. In this model, calcium enters the terminal and binds to receptors R (perhaps two classes of receptors, one with a slow and the other with a fast off-rate constant of binding to calcium),

$$nCa^{2+} + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ca_nR \overset{?}{\longrightarrow} Exocytosis$$
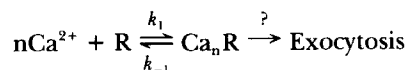
In the multiply liganded form the reaction either proceeds to exocytosis, or releases the calcium with an off-rate, k_{-1}. We propose that the off-rate constant is sufficiently slow for the incompletely liganded receptor system, so that the calcium on the receptor remains bound while the free calcium decays rapidly to low values. When a second pulse raises the level of free calcium, that calcium then binds to the receptor to produce a completely liganded receptor and exocytosis. Since $g_{K(Ca)}$ is dependent on the second or third power of the internal free calcium concentration (Barrett et al., 1982; Methfessel and Boheim, 1982; Wong et al., 1982; Moczydlowski and Latorre, 1983; but see Gorman and Thomas, 1980; Hermann and Hartung, 1982; Lando and Zucker, 1989), it decays rapidly (within 20 ms) to low values, whereas facilitation, which depends on bound calcium, decays more slowly (50 ms or more).

Saturation of Synaptic Input–Output Curves

As the presynaptic membrane potential exceeds 0 mV and approaches the calcium equilibrium potential, E_{Ca}, we would expect the calcium current, and hence transmitter release, to decrease. Therefore, saturation of the input–output (Fig. 1 *B*) and facilitation (Fig. 4 *B*) curves at large depolarizations seems contrary to expectation. Several factors could account for saturation of the input–output curves. The presynaptic terminal may be one or more space constants removed from the point where the depolarization is initiated in the secondary branch of the excitor axon. This is almost certainly the case for large depolarizations of the secondary branch in the absence of TEA, when activation of $g_{K(Ca)}$ in the presynaptic terminal causes the presynaptic membrane to rectify. Current–voltage curves of the presynaptic membrane show strong evidence of this rectification (Sivaramakrishnan et al., 1991, Fig. 4). However, when $g_{K(Ca)}$ is greatly reduced with 10–20 mM TEA, the current–voltage curve is linear even at high depolarizations, so that, in TEA, saturation of the input–output curve cannot be explained by a decreasing space constant. Second, the input–output curve could appear to saturate if the depolarizations produced are not sufficient to take the presynaptic membrane potential to E_{Ca}. This seems an unlikely explanation, since the plateau (or saturation) of the input–output and facilitation curves in TEA is broad (that is, the amplitude of the EPSP changes very little over 20–30 mV of depolarization). This long plateau is inconsistent with previously published current–voltage relations for calcium currents in soma or in presynaptic terminals, where the calcium current reaches a peak at or near a membrane potential of 0 mV, and begins to decrease immediately with any further depolarization (Llinas et al., 1981*a*, *b*; Augustine et al., 1985*a*, *b*; Nowycky et al., 1985).

The most likely explanation for this saturation is that the main inward presynaptic calcium flux occurs at the termination of, rather than during, the short depolarizing current pulse. Such tail currents have been demonstrated at the squid giant synapse (Llinas et al., 1981*a*; Augustine et al., 1985*a*) and result from the fact that a large depolarizing pulse can activate the presynaptic calcium conductance, g_{Ca}, but little calcium current would flow when the driving force is small. Upon repolarization of the membrane, g_{Ca} is still high (though rapidly deactivating), but the driving force on calcium ions is enormous. Under such conditions, transmitter release is small during the depolarizing current pulse, but large at the termination of the current pulse, the "off response" (Katz and Miledi, 1970). At the crayfish opener NMJ, we suggest that

transmitter release evoked by short depolarizing pulses is an off response since it seems to occur only after the current pulse is terminated (Fig. 1 A). Saturation of the input–output curve represents a saturation of the off response.

If the explanation for the saturation of transmitter release is as described above, we can make interesting conclusions regarding the voltage dependence of g_{Ca} in these crayfish terminals. In the presence of TTX + 3,4-DAP + TEA, the tail currents will mainly be a function of the g_{Ca} that is activated during the short depolarizing pulse. Figs. 1 B and 4 B therefore reflect an approximation of the voltage activation curve of g_{Ca}. Transmitter release occurs at relatively low voltages, unlike the activation of L or N type calcium channels in chick dorsal root ganglia (Nowycky et al., 1985). Crayfish presynaptic calcium channels do not behave like T type calcium channels either, since the calcium current (as judged by the magnitude of transmitter release) does not seem to inactivate at large depolarizations. If inactivation was present during the depolarizing pulse, and the rate of inactivation increased with depolarizations, the total calcium conductance activated at the end of the depolarizing pulse would be smaller at larger depolarizations, and transmitter release would be expected to decrease. Instead, transmitter release appears to reach a stable plateau at larger depolarizations, precluding the possibility of calcium current inactivation. We therefore conclude that the presynaptic calcium channels at the crayfish NMJ may not fit into the present classification scheme for calcium channels found in chick dorsal root ganglion neurons. Calcium channels in the presynaptic terminal of the squid giant synapse have also been found to differ from the T, N, or L type channel in their pharmacological sensitivity and activation–inactivation kinetics (Charlton and Augustine, 1987).

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