Calcium-activated Potassium Conductance in Presynaptic Terminals at the Crayfish Neuromuscular Junction

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ABSTRACT Membrane potential changes that typically evoke transmitter release were studied by recording intracellularly from the excitor axon near presynaptic terminals of the crayfish opener neuromuscular junction. Depolarization of the presynaptic terminal with intracellular current pulses activated a conductance that caused a decrease in depolarization during the constant current pulse. This conductance was identified as a calcium-activated potassium conductance, $g_{K(Ca)}$, by its disappearance in a zero-calcium/EGTA medium and its block by cadmium, barium, tetraethylammonium ions, and charybdotoxin. In addition to $g_{K(Ca)}$, a delayed rectifier potassium conductance (g_K) is present in or near the presynaptic terminal. Both these potassium conductances are involved in the repolarization of the membrane during a presynaptic action potential.

INTRODUCTION

The ionic mechanisms controlling transmitter release and its facilitation are of primary importance in the study of synaptic transmission. Release of transmitter from nerve terminals is controlled by the calcium concentration in presynaptic terminals (Katz, 1969), which in turn depends on the magnitude of the inward calcium current. Since entry of calcium ions into the presynaptic element is through voltage-dependent calcium channels (Llinas et al., 1981a; Augustine et al., 1985a), factors that affect the presynaptic membrane potential must also affect transmitter release. This presynaptic potential is determined by the conductances responsible for the action potential in the terminal region. In the squid giant synapse the presynaptic terminal is depolarized by a tetrodotoxin (TTX)-sensitive sodium conductance (g_{Na}) and a cadmium-sensitive calcium conductance (g_{Ca}). The presynaptic terminal is repolarized by delayed rectifier potassium conductances (g_K) sensitive to tetraethylammonium ions and the aminopyridines (Llinas et al., 1981a; Augustine et al., 1985a; Augustine, 1990).

At the crayfish opener neuromuscular junction (NMJ), we find that the presynaptic membrane potential (and hence transmitter release) is strongly influenced by a calcium-activated potassium conductance, $g_{K(Ca)}$, in addition to g_{Na} and g_K. The presence of $g_{K(Ca)}$ has been reported in other cell types (Marty, 1981; Latorre et al., 1982; Magleby and Pallotta, 1983) as well as in nerve terminals (Edgington and Stuart, 1981; Augustine and Eckert, 1982; Mallart, 1985; Anderson et al., 1987; Morita and Barrett, 1990), but its contribution to the total membrane conductance (g_m) of presynaptic terminals and its role in the control of transmitter release and facilitation have not been previously considered in detail. This paper outlines the evidence for the existence of a high conductance $g_{K(Ca)}$ in presynaptic terminals at the crayfish NMJ. The following paper (Sivaramakrishnan et al., 1991) examines the effect of $g_{K(Ca)}$ on transmitter release and facilitation.

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

MATERIALS AND METHODS

Small crayfish (*Procambarus clarkii*) 2–3 cm in carapace length were obtained from the Atchafalaya Biological Supply Company (Raceland, LA) and used within 2 wk of their arrival. The animals were induced to autotomize the first walking leg, which was then pinned out in a Sylgard-filled dish. The opener nerve and ventral surface of the opener muscle were exposed in the propodite segment of the leg (Dudel and Kuffler, 1961; Bittner, 1968). The preparation was viewed in a dissecting microscope using dark-field illumination from below.

Anatomy and Electrophysiology of the Walking Leg

The opener muscle in the propodite segment of the walking leg is innervated by the opener nerve bundle which contains three motor axons: two inhibitor axons and one excitor axon. One of the inhibitor axons is common to all the muscles of the walking leg but innervates only a few proximal fibers of the opener muscle (Wiens, 1987). The second inhibitor axon is specific to the opener muscle and innervates all opener muscle fibers. The excitor axon innervates both the stretcher and opener muscles (Wiersma, 1961). The specific inhibitor and excitor axons branch in parallel for most of their lengths in the propodite segment (Atwood and Morin, 1970; Jahromi and Atwood, 1974). All our experiments were carried out on the excitor axon, identified as described below, and particular care was taken not to stimulate or record from either of the inhibitor axons.

The primary branch of the opener nerve bundle containing the specific inhibitor and excitor axons bifurcates in a Y pattern into two secondary branches (Fig. 1), which in turn divide into tertiary and quarternary branches that synapse on opener muscle fibers (Fuchs and Getting, 1980; Baxter and Bittner, 1981; Wojtowicz and Atwood, 1984). A platform made out of a bent insect pin, lightly coated with Sylgard, was slipped under the opener nerve bundle in the propodite to lift it slightly off the underlying muscle fibers and to provide mechanical tension. Two intracellular electrodes (i and V_i in Fig. 1), having resistances of 11–15 MΩ when filled with 4 M potassium acetate, were placed 50–150 μm apart in a secondary or tertiary branch of the excitor axon, one on either side of the platform. The two presynaptic electrodes were within 100–200 μm of the nearest axon terminals (Wojtowicz and Atwood, 1984). To determine whether both intracellular electrodes were in the excitor axon and not in the specific inhibitor axon, action potentials were generated in the excitor axon by extracellularly stimulating the lateral nerve bundle in the more proximal meropodite segment (not shown). The lateral nerve bundle contains the excitor but neither of the inhibitor axons. If both intracellular electrodes

were in the excitor axon, they both recorded action potentials conducted from the meropodite to the secondary or tertiary branch in the propodite.

The only preparations used were those in which the excitor action potentials recorded by both intracellular electrodes were at least 90 mV in amplitude (Fig. 1, *inset*). The more proximal of the two intracellular electrodes (*i* in Fig. 1) was used to pass current and the distal electrode was used to record membrane potential (V_1 in Fig. 1). The current passing electrode was always the more proximal of the two electrodes because voltage decrement is severe proximal to the
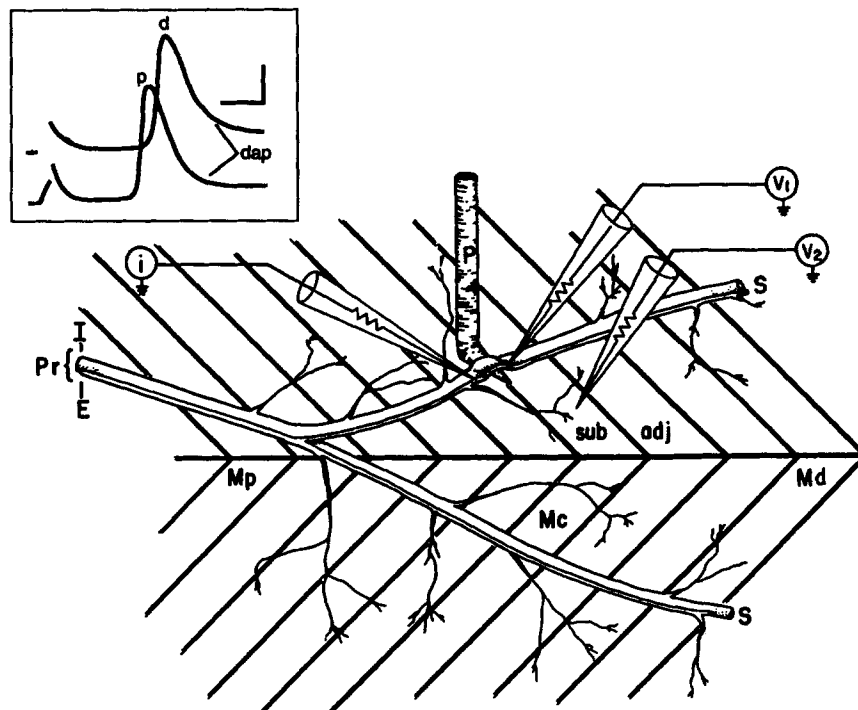

FIGURE 1. Schematic drawing of ventral surface of the opener muscle in the propodite segment and the Y branch formed by the primary branch of opener nerve (*pr*) bifurcating into two secondary branches (*S*). *E*, excitor axon; *I*, inhibitor axon; *Mp*, proximal muscle fiber; *Mc*, central muscle fiber; *Md*, distal muscle fiber; *P*, platform under opener nerve; *i*, intracellular current passing electrode; V_1, intracellular voltage recording electrode; V_2, intracellular postsynaptic recording electrode; *sub*, subsynaptic muscle fiber; *adj*, adjacent muscle fiber. (*Inset*) Action potentials recorded in Van H with two intracellular electrodes in a secondary branch of the excitor axon. *p*, recording from proximal electrode; *d*, recording from distal electrode; *dap*, depolarizing afterpotential. Scale (inset): 0.5 ms, 30 mV.

current passing electrode (See also Katz and Miledi, 1970; Charlton and Bittner, 1978*a*, *b*; Augustine et al., 1985*a*). A model 8800 high impedence amplifier (Dagan Corp., Minneapolis, MN) was used to pass constant current pulses and an M707-A intracellular amplifier (World Precision Instruments, Sarasota, FL) was used to record membrane potential. A third electrode (V_2 in Fig. 1) of resistance 1–3 MΩ, also filled with 4 M potassium acetate, was inserted into the subsynaptic or adjacent muscle fiber (Fig. 1) to record excitatory postsynaptic potentials (EPSPs), which were used as a measure of transmitter release.

In some experiments EPSPs were produced by artificial depolarizations "sculpted" to resemble natural action potentials. The artificial action potentials were produced in the presence of TTX in the secondary branch of the excitor axon by passing a depolarizing current pulse through the proximal electrode (i in Fig. 1), followed by a hyperpolarizing current pulse. The amplitude, duration, and delay between the two current pulses were varied so that the artificial action potential resembled as closely as possible a natural action potential recorded at the same site in the secondary branch (V_1 in Fig. 1). In these experiments a natural action potential was generated by stimulating the excitor axon bundle in the meropodite segment in the absence of TTX and other drugs.

Data were collected and stored at a sampling frequency of 20 kHz using an IBM PC-AT computer equipped with an A/D board and digital oscilloscope software (R. C. Electronics Inc., Goleta, CA). When recording EPSPs, 25–50 traces were averaged using an on-line signal averaging program.

Solutions

The preparation was perfused externally with modified Van Harreveld's (Van H) solution (a physiological saline for crayfish) containing (in mM): 206 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$, and 10 HEPES, pH 7.4. The volume of the bath was ~2 ml and complete exchange of solution took place in ~1 min. In experiments in which the action potential was blocked, the walking leg was perfused with Van H containing 0.1 μM TTX and, in some experiments, 1–2 mM 3,4-diaminopyridine (3,4-DAP) or 0.1–1 mM 4-aminopyridine (4-AP) were present in addition to TTX. Tetraethylammonium chloride (TEA) at concentrations of 1–20 mM was added to solutions by equimolar substitution for sodium chloride. Solutions containing barium were made by replacing the external calcium chloride (13.5 mM) on a mole-per-mole basis with barium chloride. Cadmium-containing solutions were made by adding 0.5 mM cadmium chloride to Van H without altering the concentration of any other ions. Apamin, a toxin from honey bee venom (Habermann, 1972), was added to Van H in 1-μM concentrations. Charybdotoxin (CTX), a toxin from the venom of the scorpion *Leiurus quinquestriatus* (Miller et al., 1985), was dissolved in Van H to a concentration of 50 nM before use. CTX was a kind gift from Dr. C. Miller (Brandeis University, Boston, MA); other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All experiments were performed at room temperature (22–24°C).

RESULTS

Proximity of the Recording Site to the Nearest Synaptic Terminals

EPSPs that occurred in response to artificial action potentials delivered in Van H containing TTX were within 30–50% of the amplitude of EPSPs produced by natural action potentials delivered in drug-free Van H (Fig. 2). When 4-AP or 3,4-DAP was present in addition to TTX, EPSPs were 70–80% of those recorded in drug-free Van H. If TEA was present in addition to TTX and 3,4-DAP or 4-AP, EPSP amplitudes in response to artificial action potentials were within 95–98% of the amplitudes of EPSPs produced by natural action potentials in Van H. Similar results were obtained in four other preparations. The rise and decay times of EPSPs produced by natural and artificial action potentials did not appear to differ from each other. In these and other experiments ($n > 20$) we found that the terminals that gave the largest EPSPs to artificial action potentials lay on the subsynaptic muscle fiber. In the presence of TTX + 4-AP + TEA, EPSPs recorded from an adjacent muscle fiber (one to three
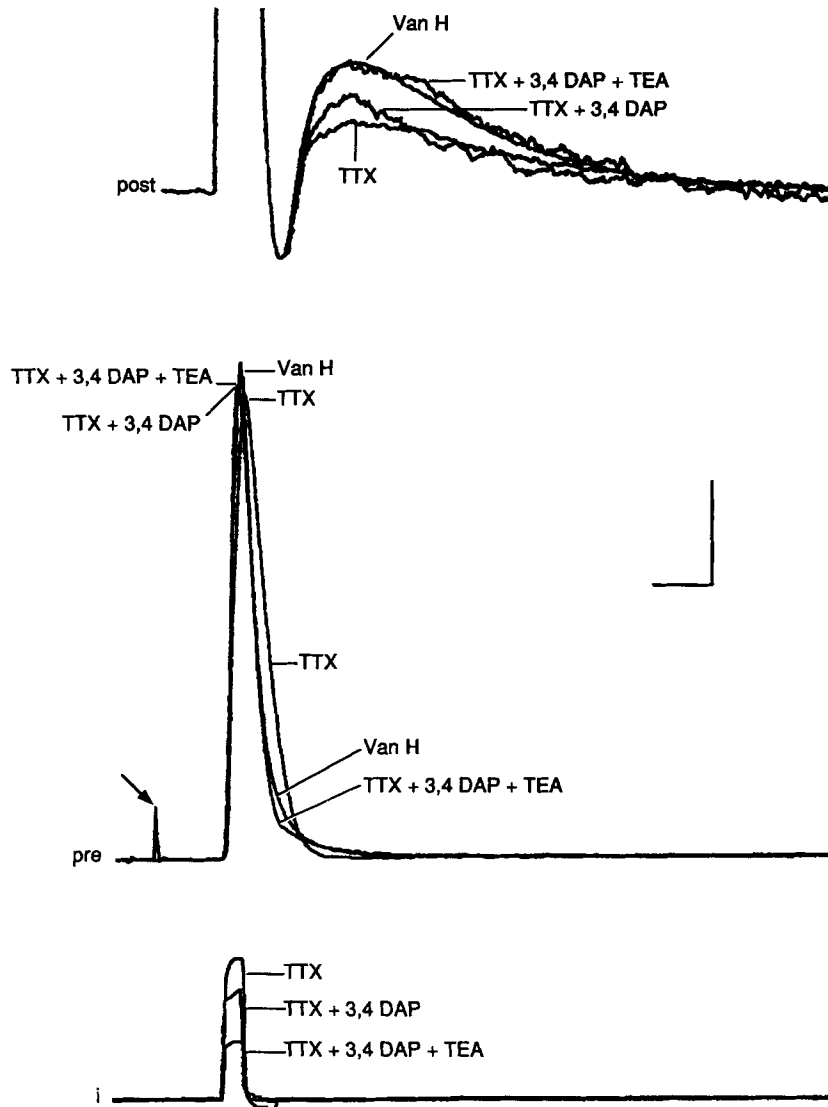
FIGURE 2. Transmitter release in response to natural (Van H) and artificial action potentials given in the presence of various drugs as indicated and recorded in a secondary branch of the excitor axon. *Top trace*, transmitter release in response to the natural and artificial action potentials shown in the middle trace. *Middle trace*, natural and artificial action potentials superimposed. *Arrow*, stimulus artifact for natural action potential elicited by stimulating the excitor axon in the meropodite. *Bottom trace*, current pulses injected into the secondary branch of the excitor axon to produce artificial action potentials. Dosages: TTX = 0.1 μM; 3,4-DAP = 1 mM; TEA = 10 mM. Scale: 1 ms, 0.5 mV (*top*), 50 mV (*middle*), 200 nA (*bottom*).

fibers removed from the subsynaptic muscle fiber) in response to artificial action potentials were only 20–40% of the amplitude of EPSPs recorded from the subsynaptic muscle fiber in response to artificial action potentials. We have also found that brief (1–2 ms), low-amplitude (10–20 mV) depolarizations of the secondary branch of the excitor axon produced measurable transmitter release in the subsynaptic muscle fiber. Thus it appears that, in the presence of TTX + aminopyridines + TEA, impalements in a secondary branch of the excitor axon are electrotonically near terminals innervating the subsynaptic muscle fiber, i.e., within <0.1 length constant.

Changes in Presynaptic Membrane Potential during Current Injection

Current clamp experiments were performed to characterize the electrical properties of the excitor axon. In these experiments, TTX was added to the saline to block g_{Na}.
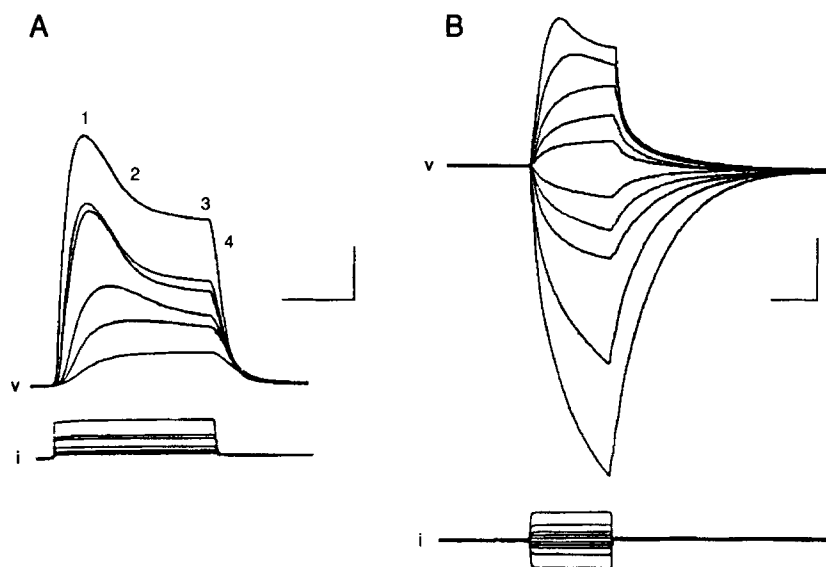

FIGURE 3. Membrane potential changes recorded in a secondary branch of the excitor axon in response to injected current pulses. Bathing solution = 0.1 μM TTX in Van H. Top traces, membrane potential recorded with electrode V_1 in Fig. 1. Bottom traces, current pulses injected through electrode i in Fig. 1. (A) Voltage response to depolarizing current pulses. Resting potential = −75 mV. 1, peak depolarization; 2, relaxation; 3, plateau; 4, final repolarization. (B) Voltage response to symmetrical depolarizing and hyperpolarizing current pulses. Scale: 2 ms, 50 mV, 200 nA.

When the excitor axon was depolarized by >60 mV from the resting potential with 2–30-ms duration constant current pulses, the membrane potential began to repolarize 0.5–2 ms after the start of the current pulse. This repolarization was observed only if the voltage recording electrode was in a secondary branch and not in the primary branch of the excitor axon.

The membrane potential change in a secondary branch during depolarizing current pulses had the following characteristics (Fig. 3 A): (a) an initial depolarization

to a peak followed by (*b*) a decrease in depolarization, referred to as the relaxation, (*c*) a plateau, and (*d*) a final repolarization after cessation of the current pulse. The time constant of the relaxation was taken to be 63% of the time from the onset of the relaxation to the beginning of the plateau. The rise time to peak depolarization is the time between the start of the initial depolarization and the peak depolarization.

As the strength of the current pulse was increased, the relaxation began earlier and had a faster time course. For example, in the experiment shown in Fig. 3 *A*, at a 60-mV peak depolarization, the relaxation began 2.5 ms after the start of the depolarization. At a 130-mV peak depolarization, the time of onset of the relaxation decreased to 0.9 ms. In other experiments (*n* > 20) values ranged from 2 ms for the time of onset of the relaxation at a 60-mV peak depolarization to 0.5 ms at a 130-mV peak depolarization. The decay time constant of the relaxation decreased from 1.9 ms at a 60-mV peak depolarization to 1.3 ms at 130 mV, but remained unchanged with any further increase in peak depolarization.

Hyperpolarizing and depolarizing current pulses of the same amplitude and duration did not produce symmetric voltage responses (Fig. 3 *B*). In contrast to the voltage response to a depolarizing current pulse, with hyperpolarizing current pulses, the peak and plateau responses were absent, the maximum recorded change was greater, the rise time to the peak hyperpolarization was longer, and the final repolarization was prolonged. Data suggesting possible ionic mechanisms for three aspects (relaxation, peak depolarization, and final repolarization) of the voltage response to depolarizing and hyperpolarizing current pulses are presented in the following paragraphs.

Ionic Mechanisms of the Relaxation

We considered four possibilities for the relaxation that occurred during depolarization of the presynaptic terminal. The relaxation could be due to: (*a*) membrane dielectric breakdown, (*b*) activation of g_K, giving rise to an outward current, (*c*) inactivation of g_{Na} or g_{Ca}, causing inactivation of an inward current, or (*d*) activation of a $g_{K(Ca)}$. These possibilities were examined using pharmacological agents as described below.

First, the relaxation is not likely to result from membrane dielectric breakdown, which has been associated with an increase in spontaneous miniature potential frequency or sudden bursts of spontaneous miniature potentials at the frog NMJ (Del Castillo and Katz, 1954). In these crayfish terminals, the spontaneous potential frequency did not change from its resting value of 0.5–1/s and no sudden bursts were observed, even when the terminal was depolarized or hyperpolarized up to 250 mV (*n* = 12). Furthermore, hyperpolarizations up to 250 mV produced no increase in g_m (Figs. 3 *B* and 5), as might have been expected if dielectric breakdown were occurring. In seven other preparations, hyperpolarizing potentials larger than 250 mV were accompanied by sudden increases in spontaneous miniature potential frequency and an increase in g_m, as indicated by an increase in the slope of the current–voltage (*I-V*) relationship. These changes might be due to dielectric breakdown at hyperpolarizing potentials greater than 250 mV.

Second, the relaxation is not likely to be due to an aminopyridine-sensitive g_K, since the relaxation was not decreased by the addition of 1 mM 3,4-DAP (Fig. 4 *A*),
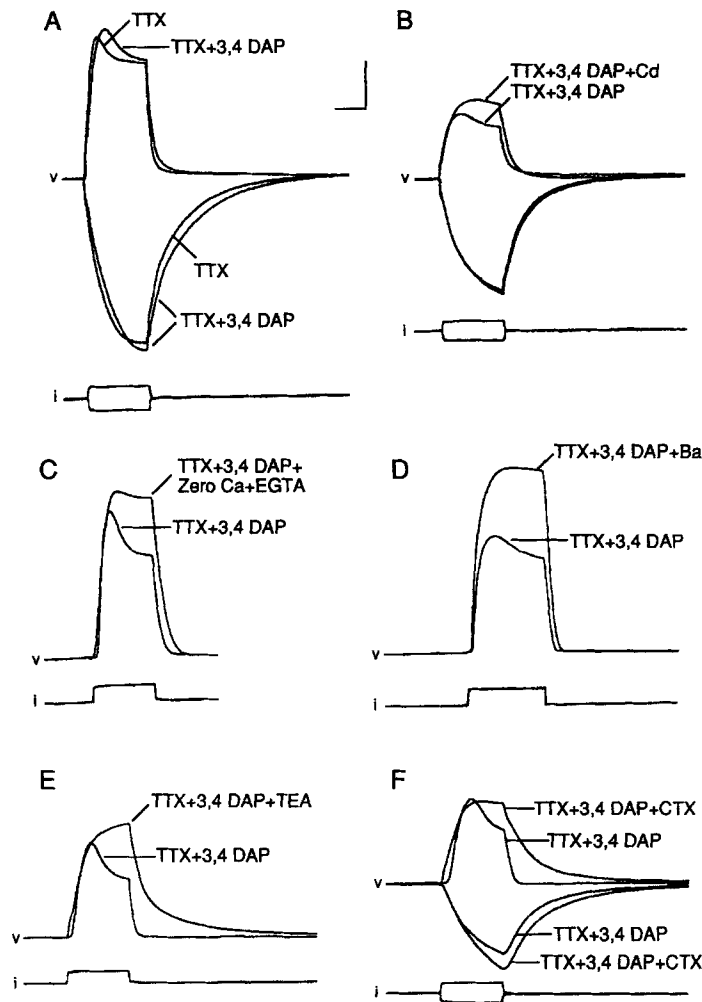
FIGURE 4. Effect of pharmacological agents on the relaxation. *Top traces,* membrane potential changes recorded in axon. *Bottom traces,* current pulses injected into axon. Effect of (*A*) 0.1 μM TTX, 1 mM 3,4-DAP; (*B*) 0.1 μM TTX, 1 mm 3,4-DAP, 0.5 mM cadmium; (*C*) 0.1 μM TTX, 1 mM 3,4-DAP, zero calcium + 1 mM EGTA; (*D*) 0.1 μM TTX, 1 mM 3,4-DAP, 13.5 mM barium; (*E*) 0.1 μM TTX, 1 mM 3,4-DAP, 15 mM TEA; (*F*) 0.1 μM TTX, 1 mM 3,4-DAP, 50 nM CTX. Each set of traces recorded from a different preparation. To preserve clarity, the reversibility of these drugs is not shown here. 1 μM apamin had no effect on the relaxation. Scale: 2 ms, 20 mV (*A, C, E*), 50 mV (*B, D, F*), 200 nA.

which has been shown to block a g_K in other preparations (reviewed by Castle et al., 1989). This result was observed in >25 opener terminals and was also seen with 0.1–1 mM 4-AP. Although addition of 4-AP reduced g_m by ~20%, thus decreasing the slope of the *I-V* relationship (Fig. 5), the relaxation was not eliminated by 1 mM 3,4-DAP or by yet higher concentrations of 4-AP or 3,4-DAP (data not shown). Moreover, the time course of the relaxation was only slightly slower and delayed compared with the TTX control.

Third, the relaxation is not likely to result from inactivation of a TTX-insensitive g_{Na} during the depolarizing pulse, since removal of external sodium (with equimolar substitution with Tris) did not affect the relaxation (data not shown). The relaxation is not likely to result from the inactivation of g_{Ca} either, even though the addition of 0.5 mM cadmium, a blocker of voltage-dependent g_{Ca} (reviewed by Hagiwara and Byerly, 1981), reversibly blocked or reduced the relaxation (Fig. 4 *B*). That is, if the peak depolarization during the constant current pulse were due merely to the
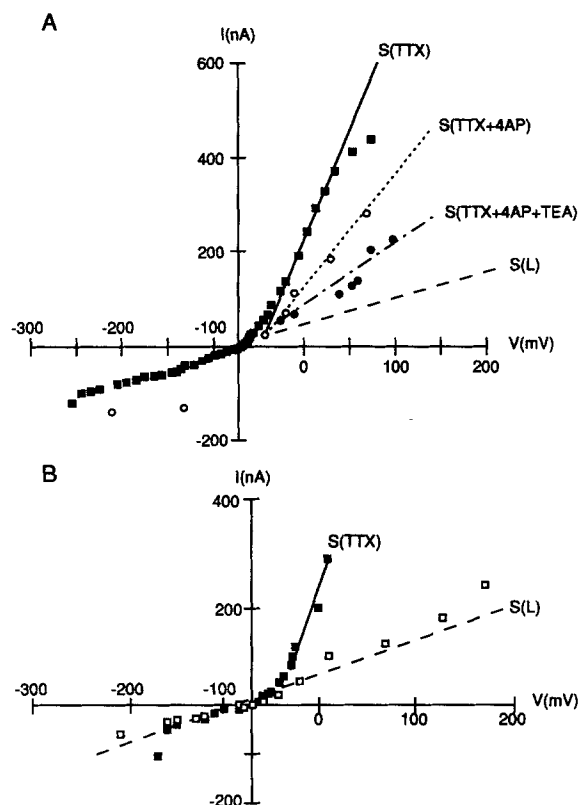

FIGURE 5. Steady-state *I-V* relationships of secondary branches of the excitor axon determined with 50-ms current pulses. (*A*) 0.1 μM TTX (*filled squares*), 0.1 μM TTX + 0.1 mM 4-AP (*open circles*), 0.1 μM TTX + 0.1 mM 4-AP + 10 mM TEA (*filled circles*). S(L) is the leakage slope (*dashed line*) projected from the hyperpolarizing quadrant. S(TTX) is the maximal limiting slope (*solid line*) in TTX; S(TTX + 4-AP) is the maximal limiting slope (*dotted line*) in TTX + 4-AP; and S(TTX + 4-AP + TEA) is the maximal limiting slope (*dashed-dotted line*) in TTX + 4-AP + TEA. The percentage decreases in the slope for the TTX + 4-AP solution and the TTX + 4-AP + TEA solution compared with TTX alone were 29 and 62%, respectively (see text). All measurements were performed on the same axon. Although there are two aberrant points at hyperpolarized potentials in this figure, such aberrant points were not present in other preparations. (*B*) 0.1 μM TTX (*filled squares*) and 0.1 μM TTX + 0.1 mM 4-AP + 50 nM CTX (*open squares*). S(TTX) is the maximal limiting slope (*solid line*) in TTX. S(L) is the leakage slope (*dashed line*) projected from the hyperpolarizing quadrant. The maximal limiting slope of the open squares and S(L) are very similar. Data in *B* were obtained from a different axon from that shown in *A*.

activation and inactivation of a g_{Ca}, then cadmium would have reduced the relaxation by reducing the magnitude of the peak voltage while leaving the plateau potential unchanged. However, in 0.5 mM cadmium, the peak depolarization remained unchanged or increased, while the plateau depolarization always increased (*n* = 6). A concentration of 0.5 mM cadmium was used because it was sufficient to block evoked release in this preparation and its effects were reversible; higher concentrations of cadmium also blocked evoked release, but its effects were not readily reversible.

Several lines of evidence support the fourth possibility, that the relaxation results from a $g_{K(Ca)}$. Reduction or abolition of the relaxation by cadmium (Fig. 4 B) suggests that the relaxation is activated at least partly by a calcium current. Furthermore, removal of all the external calcium abolished or greatly decreased the relaxation (Fig. 4 C). As described below, the effects of barium, TEA, CTX, and apamin are in agreement with a hypothesis that the relaxation is produced by a high or intermediate conductance $g_{K(Ca)}$.

Barium has been shown to carry current through calcium channels (Augustine and Eckert, 1984) but does not, in general, activate $g_{K(Ca)}$ (Vergara and Latorre, 1983; Miller et al., 1987). In these crayfish terminals ($n = 5$), replacement of all the calcium (13.5 mM) in the bathing medium by barium reversibly blocked the relaxation (Fig. 4 D). The addition of 2 and 5 mM barium partially blocked the relaxation. The relaxation was also abolished by bath application of 10–20 mM TEA (Fig. 4 E), which has been shown to block high and intermediate conductance $g_{K(Ca)}$ from the external surface of the membrane (Blatz and Magleby, 1984; Vergara et al., 1984; Yellen, 1984; Farley and Rudy, 1988). This effect of TEA was observed in more than 20 preparations. Lower concentrations (2–5 mM) of TEA partially blocked the relaxation in most preparations.

The relaxation was reduced ($n = 3$) by bath application of 50 nM CTX (Fig. 4 F), which has been reported to block high (Miller et al., 1985) and intermediate (Farley and Rudy, 1988) conductance $g_{K(Ca)}$ at nanomolar concentrations. The relaxation was not affected by the addition of 1 μM apamin to the bath. In other preparations (Romey and Lazdunski, 1984; Cook and Haylett, 1985; Blatz and Magleby, 1986) an apamin-sensitive, low conductance $g_{K(Ca)}$ is associated with an after-hyperpolarization of the membrane following tetanic stimulation. When the crayfish excitor axon was tetanized with 100–1,000 stimuli at 50–100 Hz, the presynaptic terminals exhibited a prolonged post-tetanic after-hyperpolarization which lasted for several seconds (see also Wojtowicz and Atwood, 1985; Bittner, 1989). However, I-V relationships measured during the after-hyperpolarization showed no evidence of a change in g_m. Therefore, a low conductance $g_{K(Ca)}$ is probably not present in these terminals.

Our method for analyzing the contributions of g_K and $g_{K(Ca)}$ to the relaxation or repolarization phase may overestimate the contribution of $g_{K(Ca)}$. Our recorded variable was membrane voltage; hence, the effect of pharmacologically deleting a given current will vary inversely with g_m. Since $g_{K(Ca)}$ was always examined when g_K was blocked, g_m was always higher during the measurement of $g_{K(Ca)}$ than during the measurement of g_K (Fig. 4 A). Therefore the voltage effects of TEA and other $g_{K(Ca)}$ blockers were greater than they would have been had only g_{Na} been blocked.

Ionic Mechanisms of the Peak Depolarization

In addition to blocking the relaxation, barium and TEA increased the peak depolarization, as did zero calcium and 0.5 mM Cd. Pharmacological reduction of the g_K and the $g_{K(Ca)}$ ought to increase the magnitude of the peak depolarization (induced by outward current pulses) by reducing the membrane conductance. Ba, TEA, aminopyridines, and CTX would be expected to block potassium conductances. Moreover, Ba^{2+} can substitute for Ca^{2+} as an inward current carrier. Cadmium or zero Ca^{2+} + EGTA would prevent the influx of Ca^{2+} and the resulting increment of $g_{K(Ca)}$.

In Fig. 4 *D*, substitution of external calcium by barium (13.5 mM) produced a 20-mV increase in the peak depolarization. In three other experiments with solutions containing 13.5 mM barium, the peak was increased by 15–23 mV compared with solutions that contained only TTX + 3,4-DAP.

Ionic Mechanisms of the Final Repolarization

The time course of the final repolarization after cessation of depolarizing current pulses was greatly prolonged in the presence of TTX + 4AP + TEA or CTX and slightly prolonged in the presence of TTX + 4AP + cadmium, barium, or zero calcium (Fig. 4). In the presence of TTX + 4AP + TEA, CTX, cadmium, barium, or zero calcium, the time course of the final repolarization was similar for depolarizing and hyperpolarizing current pulses (Fig. 4 *F*). 3,4-DAP or 4-AP did not have a pronounced effect on the time course of the final repolarization (Fig. 4 *A*).

I-V Relationships of the Presynaptic Terminal

To quantify the contributions of $g_{K(Ca)}$ and g_K to g_m, we examined the steady-state *I-V* relationship of the presynaptic terminal of the excitor axon in the presence of agents that block these conductances (Fig. 5, *A* and *B*). In the presence of TTX alone, g_m increased greatly with increasing depolarization. Part of this increase in g_m was reduced when 4-AP was present in addition to TTX. g_m was further reduced, and the membrane *I-V* was nearly linear when $g_{K(Ca)}$ was blocked with TEA (Fig. 5 *A*) or CTX (Fig. 5 *B*).

A quantitative estimate of the contribution of $g_{K(Ca)}$ and g_K to g_m was obtained in the following way. *I-V* curves were generated with 50-ms depolarizing and hyperpolarizing current pulses in TTX, TTX + 4-AP, TTX + 4-AP + TEA (Fig. 5 *A*), and TTX + 4-AP + CTX (Fig. 5 *B*). At depolarized potentials, the *I-V* curves were nonlinear, the extent of nonlinearity varying with the blocking agent. At membrane potentials more hyperpolarized than the resting potential, the *I-V* curves in all the blocking agents overlapped and were linear. Since the *I-V* relationships obtained for hyperpolarizing potentials were linear, we assumed that no voltage-dependent channels were activated during hyperpolarizing current pulses in any of the blocking agents used. Extrapolation of this linear part of the *I-V* curve into the region corresponding to depolarizing potentials was taken to represent leakage currents (the "leakage *I-V*" curve) that were not voltage dependent. The slope of this leakage *I-V* curve is the leakage slope, $S(L)$, shown as a dashed line in Fig. 5, *A* and *B*. The maximum limiting slopes of the depolarizing portions of the *I-V* curves in each combination of blocking agents (S(TTX), S(TTX + 4-AP), S(TTX + 4-AP + TEA)) were then determined as illustrated in Fig. 5 *A*. S(L) was then subtracted from each maximum limiting slope to give the net slope due to the blocking agent. The fraction of g_m that was affected by each potassium channel blocking agent was determined by the ratios of the slope of each combination of blocking agents to the net slope in TTX (assuming that TTX by itself did not prevent any component of g_m from contributing to the increase in outward current). For example, the fraction of g_m blocked by 4-AP is given by the ratio [S(TTX) − S(TTX + 4-AP) − S(L)]/[S(TTX) − S(L)].

The addition of 10 mM TEA to the TTX + 4-AP solution produced a 62% block compared with a solution containing only TTX. Higher doses of TEA produced

greater block. An almost complete block was produced by 30 mM TEA. The addition of 50 nM CTX to the TTX + 4-AP solution blocked virtually all of the outward rectification and made the I-V relationship nearly linear over a 300-mV range (Fig. 5 B). Five other preparations gave similar results.

Functional Effects of g_K and $g_{K(Ca)}$

The data presented in the above paragraphs suggest that a high or intermediate conductance $g_{K(Ca)}$ is present in presynaptic terminals of the excitor axon and that both $g_{K(Ca)}$ and an aminopyridine-sensitive g_K affect the normal repolarization of the
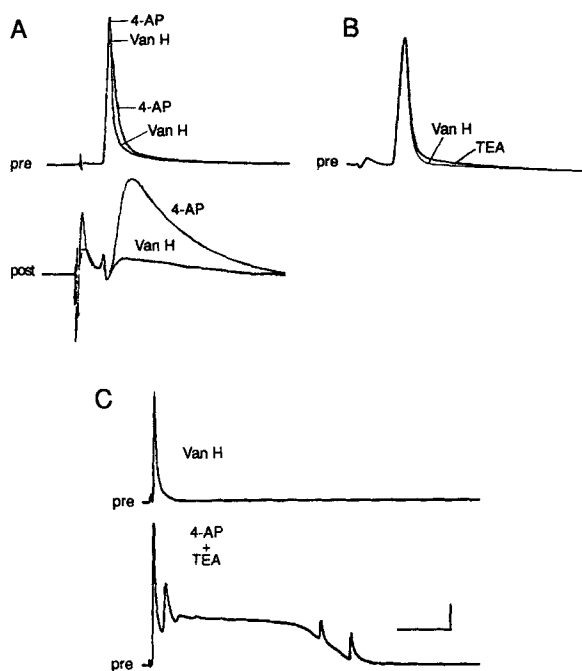

FIGURE 6. Broadening of presynaptic action potentials by 4-AP and TEA. Excitor axon stimulated with extracellular hook electrodes in meropodite and action potentials recorded with intracellular electrode in the secondary branch of excitor axon in propodite. Postsynaptic potentials recorded from central muscle fibers (see Fig. 1). Note that TTX is not added to any preparation. (A) Top traces, action potentials recorded in Van H alone and Van H containing 0.1 mM 4-AP. Bottom traces, postsynaptic potentials in response to the action potentials in top trace. (B) Top trace, action potentials recorded in Van H alone and Van H containing 20 mM TEA. (C) Top trace, action potential in Van H alone. Bottom trace, action potential in Van H containing 0.1 mM 4-AP + 20 mM TEA. Same axon as in A. Spontaneous spikes occur during the prolonged plateau after the action potential. Scale: (A) 2 ms, 20 mV (pre), 0.5 mV (post); (B) 1 ms, 20 mV; (C) 8 ms, 20 mV for the top trace; 24 ms, 20 mV for the bottom trace.

presynaptic membrane. However, a comparison of the effects on the action potential of blocking g_K and $g_{K(Ca)}$, as described below, suggest that these two conductances are activated at different times and to different extents during a normal action potential.

When 0.2–1 mM 4-AP was used to block g_K, action potentials in the excitor axon were broadened by 2–4 ms and transmitter release was enhanced (Fig. 6 A; see also Wojtowicz and Atwood, 1984). The addition of TEA alone slightly increased the duration of a single action potential (n = 2) (Fig. 6 B). However, the addition of 20 mM TEA (which blocks $g_{K(Ca)}$) to a bathing solution that already contained 4-AP or 3,4-DAP resulted in an action potential with a prolonged depolarizing after potential

or plateau (Fig. 6 *C*). The plateau lasted for 120–150 ms and reached an amplitude of 30–50 mV. In the presence of both TEA and 4-AP, spontaneous action potentials of attenuated amplitude often occurred during the prolonged plateau phase of the initial action potential. These spontaneous action potentials were not affected by 0.5 mM cadmium in the bathing solution (data not shown) and could occur more or less frequently than shown in Fig. 6 *C*. The duration of the plateau was not affected by the number of spontaneous action potentials. Evoked transmitter release could not be accurately measured because of large muscle contractions which sometimes occurred in the presence of ≥ 20 mM TEA even in the absence of nerve stimulation.

Because the plateau and spontaneous spikes were not blocked by 0.5 mM cadmium, and because long plateau potentials were never observed when 0.1 μM TTX was added to solutions containing 4-AP and/or TEA, we conclude that the plateau is generated by a TTX-sensitive sodium current. This current has evidently not been completely inactivated at the potential of the plateau. From these and other data presented above, we also conclude that both g_K and $g_{K(Ca)}$ are present in presynaptic terminals of the excitor axon and that repolarization of the membrane is affected by both $g_{K(Ca)}$ and g_K.

DISCUSSION

Transmitter release from nerve terminals is controlled by presynaptic depolarization and a subsequent change in internal calcium concentration (Katz and Miledi, 1970; Llinas et al., 1981*a*, *b*; Augustine et al., 1985*a*, *b*). It has been difficult, however, to control or measure either variable in most presynaptic terminals. To a great extent, this difficulty has been overcome at the squid giant synapse because two or three electrodes can be inserted into the presynaptic terminal (Katz and Miledi, 1970; Charlton and Bittner, 1978*a*, *b*; Llinas et al., 1981*a*, *b*; Augustine et al., 1985*a*, *b*). However, an inability to measure and control the presynaptic membrane potential remains a problem at other synapses including those at the vertebrate NMJ, the vertebrate CNS, and the *Aplysia* abdominal ganglion. The ability to record membrane potential quite near the intact presynaptic terminals at the crayfish NMJ furthers our understanding of the factors controlling transmitter release at a synapse other than the squid giant synapse. The crayfish NMJ is of additional interest since it exhibits many important forms of synaptic plasticity such as facilitation, long-term facilitation, post-tetanic potentiation, and long-term potentiation (Atwood and Wojtowicz, 1986; Bittner, 1989).

Proximity of Recording Sites to Synaptic Terminals

To determine whether our current injection and recording sites in the secondary branch are electronically close to the nearest synaptic terminals, we used natural and artificial action potentials having amplitudes corresponding to the steep region of the input–output curve relating transmitter release to presynaptic membrane potential (Fig. 1 of Sivaramakrishnan et al., 1991), where small changes in membrane potential produce large changes in transmitter release. Compared with the EPSP amplitudes in response to natural action potentials, the 10–50% reduction in EPSP amplitude in response to artificial action potentials generated in TTX and in TTX + 4-AP (Fig. 2)

suggests that our recording site is, at worst, within half a space constant from the nearest terminal. When $g_{K(Ca)}$ is also blocked by adding TEA to a solution containing TTX + 4-AP, the 2–5% reduction in the EPSP amplitude with artificial action potentials compared with the EPSP amplitude with natural action potentials suggests that our current injection and recording sites in the secondary branch are within a small fraction (≤ 0.1) of a space constant from the nearest terminals. The observation that significant transmitter release can be evoked with brief, low amplitude (20 mV) pulses of 1–2 ms duration injected into the secondary or tertiary branches of the excitor axon supports this conclusion (see also Fig. 1 A of Sivaramakrishnan et al., 1991). These conclusions are in agreement with calculations made by Wojtowicz and Atwood (1984) on the electrotonic distance between the secondary branch and the presynaptic terminals.

The electrotonic distance of our recording electrode from terminal release sites is affected by the amplitude of our presynaptic depolarizations when $g_{K(Ca)}$ is *not* blocked. As the depolarization is increased in the secondary branch, and therefore in the terminal, $g_{K(Ca)}$ increases in the terminal. This increased $g_{K(Ca)}$ should decrease the membrane resistance (r_m) in the terminal region. A decrease in r_m should decrease the space constant and therefore reduce our ability to measure voltage changes accurately in the terminal. At sufficiently large depolarizations our recording site might no longer be within a space constant of the terminal and the slope of the resulting I-V curve would be expected to decrease, as we have observed (see Fig. 5 A). With $g_{K(Ca)}$ largely blocked, the I-V curve recorded in the secondary branch should more faithfully represent the I-V relationship of the terminal region.

We see no evidence of regenerative calcium currents in our recordings in the secondary branch, though Kawai and Niwa (1977) have reported the presence of calcium action potentials in the inhibitor axon of the lobster claw. Calcium action potentials in the terminals of the excitor axon of the crayfish walking leg might be too small to record in the secondary branch (Wojtowicz and Atwood, 1984). However, our data suggest that calcium action potentials may not be present at all in the excitor axon because recordings from tertiary branches of the excitor axon show no evidence of calcium action potentials, and transmitter release is a graded function of presynaptic depolarization (Fig. 1 of Sivaramakrishnan et al., 1991). This difference between the excitor axon in the crayfish and inhibitor axon in the lobster may reflect a difference in the relative magnitudes of inward calcium current relative to total outward current in the two axons.

Effect of $g_{K(Ca)}$ on Presynaptic Voltage Changes

The relaxation in membrane potential that occurs during depolarization of the terminals of the excitor axon is likely to be due to a high or intermediate conductance $g_{K(Ca)}$, since it is greatly reduced by the removal of external calcium and by the addition of CTX or TEA to the bathing medium (Fig. 4). CTX has been reported to specifically block high conductance $g_{K(Ca)}$ in rat brain synaptosomes (Farley and Rudy, 1988), mammalian NMJs (Anderson et al., 1987), and rat myotubes (Miller et al., 1985; Smith et al., 1986; Anderson et al., 1988), and intermediate conductance $g_{K(Ca)}$ in rat synaptosomes (Farley and Rudy, 1988). In rat synaptosomes, low concentrations (2–5 mM) of TEA are sufficient to block the high conductance $g_{K(Ca)}$, while high concentrations (50 mM) of TEA are needed to block

the intermediate conductance $g_{K(Ca)}$ (Farley and Rudy, 1988). In the crayfish NMJ, the high concentrations of TEA (15–20 mM) required to block the relaxation implies that it is due to an intermediate rather than a high conductance $g_{K(Ca)}$. CTX does, however, block low conductance $g_{K(Ca)}$ in *Aplysia* (Hermann and Erxleben, 1987), as well as potassium conductances not activated by calcium (Sands et al., 1989). A low conductance $g_{K(Ca)}$ is probably not present in the crayfish presynaptic terminal, since apamin, which has been shown to block low conductance $g_{K(Ca)}$ in other preparations (reviewed by Castle et al., 1989) has no effect on the relaxation. The block of the relaxation by barium supports, but does not confirm, the hypothesis that the relaxation is due to a $g_{K(Ca)}$, since barium blocks other potassium channels (Eaton and Brodwick, 1980; Armstrong et al., 1982) in addition to $g_{K(Ca)}$ (Miller et al., 1987). Our observation that removal of external calcium (Fig. 4 C) reduces the relaxation strongly suggests that the relaxation is due to a $g_{K(Ca)}$.

The kinetic and steady-state properties of the relaxation response to a constant current pulse are voltage dependent, since the potassium conductances that generate them are voltage dependent. Moreover, since the length constant would vary while the potential varied, the distribution of potentials along the axon would not be uniform or constant. For these reasons, we do not place great emphasis on our quantitative measures for the kinetic and steady-state parameters. However, our values do indicate order of magnitude values and trends for any change in these parameters.

The initial repolarization phase immediately after the peak of the action potential is prolonged by 4-AP (see Fig. 6 A). In contrast, only the late component of the action potential is prolonged by TEA (see Fig. 6 B). These results suggest that the aminopyridine-sensitive conductance activates faster than the TEA-sensitive conductance. The time course of the final repolarization is also unaffected by cadmium, which may indicate that an inward calcium current is not absolutely necessary to activate $g_{K(Ca)}$ in these terminals. As pointed out above, $g_{K(Ca)}$ requires both internal calcium and depolarization for its activation. Even without an additional calcium influx, the resting calcium concentration may be sufficient to support activation of $g_{K(Ca)}$ by depolarization. Another possibility is that 0.5 mM cadmium is insufficient to block all the presynaptic calcium channels. Thus, some calcium could enter the terminal during a depolarizing pulse and partially activate $g_{K(Ca)}$. Alternatively, the late repolarization phase could be due to a component of g_K that is insensitive to aminopyridines but is blocked by TEA.

The increase in maximum depolarization of the membrane when barium or TEA are present in addition to TTX + 3,4-DAP (Fig. 4, D and E) could reflect an increased membrane resistance as a result of the blocking of other potassium conductances in addition to g_K and $g_{K(Ca)}$ which we have not identified, or it could reflect a larger inward (calcium) current.

Our data suggest that external application of TEA blocks $g_{K(Ca)}$ while 4-AP or 3,4 DAP blocks the remaining outward rectification. First, in the absence of TEA, no additional block of g_K is observed when 4-AP is increased 10-fold from 0.1 to 1 mM. Hence, $g_{K(Ca)}$ appears to be insensitive to 4-AP at the concentrations used in this study. Second, in the absence of TTX, TEA or 4-AP alone is not capable of generating an action potential with a prolonged plateau phase. Conversely, the two drugs in combination do produce a plateau phase (Fig. 6). This result implies that

these drugs have some effects that do not overlap. Third, in the accompanying paper (Sivaramakrishnan et al., 1991), we demonstrate that a prepulse modulates the magnitude of the voltage response to a test pulse by activating $g_{K(Ca)}$. This $g_{K(Ca)}$ is insensitive to 4-AP but is blocked by the addition of TEA and disappears in 0 mM Ca + 2 mM EGTA. Fourth, Augustine (1990) has demonstrated in squid presynaptic terminals two separable components of outward potassium rectification, one sensitive to aminopyridines and the other to external TEA. These results parallel our observations in the crayfish NMJ which suggest that $g_{K(Ca)}$ is insensitive to 4-AP and is blocked by TEA.

Functional Significance of $g_{K(Ca)}$

The repolarization of the membrane during a natural presynaptic action potential is affected by both g_K and $g_{K(Ca)}$. For example, 4-AP (which blocks g_K) and TEA (which blocks $g_{K(Ca)}$) both slightly prolong the action potential. Furthermore, TEA and 4-AP in combination (Fig. 6 B) markedly prolong the depolarizing after potential (plateau phase). Moreover, since the relaxation has a time constant of < 2 ms when g_K is blocked by 4-AP (see Fig. 4 A), the $g_{K(Ca)}$ activation must be fast enough to affect repolarization. Thus, both these potassium conductances, acting in concert, restrict the duration of the action potential in the presynaptic terminal.

The above results suggest several physiological roles for g_K and $g_{K(Ca)}$ in these nerve terminals. The crayfish NMJ, unlike its vertebrate counterpart, does not rely on all-or-none transmission and exhibits different forms of synaptic plasticity at physiological calcium concentrations. In this respect it is similar to many vertebrate CNS synapses. The EPSP does not normally generate a postsynaptic action potential in the muscle fiber. Instead, muscle contraction is proportional to EPSP amplitude, which, in turn, is sensitive to various components of facilitation and depression (Bittner, 1968; reviewed by Atwood and Wojtowicz, 1986, and Bittner, 1989). Presynaptic terminals of the crayfish excitor axon are exquisitely sensitive to frequency and pattern of stimulation (Bittner, 1968; Bittner and Segundo, 1989); $g_{K(Ca)}$ and g_K may confer on the terminal part of its frequency sensitivity. Furthermore, the synapses studied in this paper were all located on central muscle fibers of the opener muscle and were so chosen because they exhibit greater facilitation than synapses made by the excitor axon on the more proximal or distal regions of the opener muscle (Bittner and Sewell, 1976). These central synapses are characterized by low release rates and high facilitation, whereas the proximal and distal excitor terminals release much more transmitter but facilitate less than the central synapses. It is conceivable that this difference in release and facilitation between different terminals on the opener muscle arises because of a difference in the duration of the presynaptic action potential in these different regions, possibly because of a difference in the density of presynaptic calcium-activated potassium channels between central and proximal/ distal synapses.

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