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Presynaptic membrane potential affects transmitter release in an identified neuron in *Aplysia* by modulating the Ca^{2+} and K^+ currents

(ionic current/voltage clamp/synaptic transmission)

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ABSTRACT **We have examined the relationships between the modulation of transmitter release and of specific ionic currents by membrane potential in the cholinergic interneuron L10 of the abdominal ganglion of *Aplysia californica*. The presynaptic cell body was voltage-clamped under various pharmacological conditions and transmitter release from the terminals was assayed simultaneously by recording the synaptic potentials in the postsynaptic cell. When cell L10 was voltage-clamped from a holding potential of −60 mV in the presence of tetrodotoxin, graded transmitter release was evoked by depolarizing command pulses in the membrane voltage range (−35 mV to +10 mV) in which the Ca^{2+} current was also increasing. Depolarizing the holding potential of L10 results in increased transmitter output. Two ionic mechanisms contribute to this form of plasticity. First, depolarization inactivates some K^+ channels so that depolarizing command pulses recruit a smaller K^+ current. In unclamped cells the decreased K^+ conductance causes spike-broadening and increased influx of Ca^{2+} during each spike. Second, small depolarizations around resting potential (−55 mV to −35 mV) activate a steady-state Ca^{2+} current that also contributes to the modulation of transmitter release, because, even with most presynaptic K^+ currents blocked pharmacologically, depolarizing the holding potential still increases transmitter release. In contrast to the steady-state Ca^{2+} current, the transient inward Ca^{2+} current evoked by depolarizing clamp steps is relatively unchanged from various holding potentials.**

In some spike-generating neurons the presynaptic terminals controlling transmitter release are electrically sufficiently close to the cell body so that the release of transmitter from the terminals can be modified by injecting current into the cell body (1–4). Klein and Kandel (5) have recently found that changes in the Ca^{2+} current in the cell body of an *Aplysia* neuron parallel changes in transmitter release at the terminals. These two sets of observations suggested to us that we might be able to examine the relationships between transmitter release and specific ionic currents of the presynaptic membrane. Toward this end we have combined two separate techniques: (*i*) pharmacological separation and voltage-clamp analysis of the ionic currents in the cell body of the presynaptic neuron and (*ii*) assay of transmitter release obtained by means of intracellular recordings of the synaptic potential in the post-synaptic cells. We have found that this combined technique provides a powerful method for studying changes in specific ionic conductances associated with various presynaptic mechanisms for synaptic plasticity.

For these studies we have used the identified cholinergic neuron L10 of the abdominal ganglion of *Aplysia*. We eliminated impulse conduction by blocking Na^+ channels with tetrodotoxin (TTX) and found that graded depolarizing commands, applied to the membrane of the presynaptic cell body under voltage-clamp control, caused graded transmitter release from the terminals as determined by the graded changes in the synaptic potential in the postsynaptic cell. Although in many cases we probably lacked ideal voltage control of the terminals, this procedure nonetheless allowed sufficient control to study transmitter release while examining specific ionic currents in the soma of the presynaptic neuron. Voltage control can be further improved by axotomizing the presynaptic cell (6, 7) and by adding pharmacological agents that block each of the three known outward K^+ currents (8) and thereby also lengthen the effective space constant of the neuron.

By using these approaches, we have found that depolarizing the presynaptic membrane potential enhances transmitter release by two mechanisms: (*i*) by activation of a steady-state Ca^{2+} current and (*ii*) by decreasing the outward K^+ currents activated by a step depolarization (9, 10). This decrease in the K^+ currents increases the duration of the spike and, therefore, the transient Ca^{2+} current activated by the spike from depolarized levels of membrane potential.

In a subsequent paper (11) we use the same voltage-clamp approach to examine presynaptic inhibition at the synapses made by L10 and find that this action results from a direct modulation of the transient Ca^{2+} current. These findings indicate that the amount of Ca^{2+} coming into the presynaptic terminals with each action potential is not fixed but can be modulated by membrane voltage and by chemical transmitters. Moreover, the existence of several independent ways for regulating the Ca^{2+} current suggests that Ca^{2+} current modulation may be a fairly common mechanism for the presynaptic regulation of transmitter release.

METHODS

Abdominal ganglia were isolated from *Aplysia californica* weighing 100–300 gm. Cell L10, the presynaptic neuron used in these experiments, is a cholinergic interneuron involved in the neural circuit controlling heart rate and blood pressure (12). This cell exhibits a number of different firing states, and its normal resting potential can vary from −40 to −60 mV. In addition, inhibitory postsynaptic potentials (IPSPs) can cause long-lasting (0.5–1 sec) and large (10–15 mV) hyperpolarization of cell L10. Cell L10 produces different synaptic actions through its various branches (13–15). We focused on follower cells of the RB cluster [follower cells that receive an excitatory postsynaptic potential (EPSP)] and on cell L5 (an identified

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Abbreviations: TTX, tetrodotoxin; Et_4N^+, tetraethylammonium ion; 4-AP, 4-aminopyridine; PSP, postsynaptic potential; EPSP, excitatory PSP; IPSP, inhibitory PSP; EGTA, [ethylenebis(oxyethelenenitrilo)]-tetraacetic acid.

Table 1. Solutions

Sea-water solutions	Concentration, mM							
	NaCl	KCL	$CaCl_2$	$BaCl_2$	$CoCl_2$	$MgCl_2$	Tris	Et_4N^+
A. Normal	465	10	10	0	0	55	10	0
B. High divalent cation	265	10	60	0	0	140	10	0
C. Na^+-free	0	10	60	0	0	140	10	265
D. Ba^{2+}	465	10	0	10	0	55	10	0
E. High Ba^{2+}/high Mg^{2+}	265	10	0	60	0	140	10	0

[Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), tetraethylammonium ions (Et_4N^+), TTX, and 4-aminopyridine (4-AP) were added to stock solutions or titrated directly into the experimental chamber of known volume. EGTA stock solution was 1.0 M EGTA in distilled H_2O buffered to pH 7.6 with NaOH. All (Et_4N^+), 4-AP, Ba^{2+}, and Co^{2+} solutions were kept at 4°C until ready for use and were used within 24 hr of preparation.

follower cell that receives an IPSP). The cells of interest were exposed by desheathing the capsule of the ganglion. Cell L10 was impaled with two independent electrodes of low resistance (1–3 MΩ) for voltage-clamping (for details of the circuit, see ref. 10). Follower cells were recorded from with double-barreled microelectrodes, one barrel of which was used for current injection. All electrodes were filled with 2 M potassium citrate. To improve voltage-clamp control of cell L10, the axon in the pericardial nerve was cut short at its exit from the ganglion (6, 7). Further improvement in voltage-clamp control was achieved by the addition of TTX (30–60 μM) to the bath to block Na^+ currents. These procedures allowed us to record postsynaptic potentials (PSPs) in the follower cells that were, in most cases, graded with the size of the presynaptic depolarizing voltage-clamp steps.

All voltage-clamp experiments were performed at 15°C ± 1°C. Voltage-clamp holding potentials were varied from −30 to −90 mV. Depolarizing and hyperpolarizing pulses were 100–1000 msec in duration.

To compare current records from the relatively intact axotomized neuron connected to its synaptic terminals to the current records of a more ideal soma without processes, we isolated the soma of L10 by undercutting it (five preparations). Soma isolation was judged by the absence of axon spikes during long depolarizing pulses and by the absence of any synaptic activity onto or from the cell.

The solutions used are shown in Table 1.

The results presented here and in ref. 11 are based on over 60 successful voltage-clamp experiments.

RESULTS

Membrane Potential of the Presynaptic Cell Affects Transmitter Release. Intracellular stimulation of neuron L10 produces EPSPs in some follower cells (R15 and the RB cells) and IPSPs in other (L1–L6, LB_{VC}, LD_{HI}, etc.; see refs. 13, 15, and 16). As the membrane potential of L10 was progressively increased, its ability to release transmitter at its various branches was decreased (Fig. 1). Even a slight increase in membrane potential of 5 mV produced significant depression of transmitter release. With larger hyperpolarizing changes of 20–40 mV, transmitter release could be completely blocked (Fig. 1*A*). Conversely, depolarizing the cell's membrane potential increased transmitter release (Fig. 1*C*). The changes in membrane potential affected the duration and height of the spikes in L10. The spikes became narrower and shorter with hyperpolarization and broader and taller with depolarization (Fig. 1*B*). Both of these alterations induced by membrane potential are likely to alter transmitter release because they alter the Ca^{2+} current (refs. 5 and 17; Fig. 1*B*). However, as we shall see below, these alterations account for only part of the effects of membrane voltage in release. Some effect of membrane voltage is independent of changes in spike height and duration.

Release of Transmitter from Terminals Can Be Controlled from the Cell Body in a Graded Manner. The ability to alter transmitter release from the terminals by injecting current into the cell body implies that at least some release sites are electrically close to the soma. This finding suggested to us that we might be able to voltage-clamp the cell body and control transmitter release from the terminals.

If the cell is axotomized and treated with TTX, graded depolarizing steps lead to graded release of transmitter (Fig. 2*A*). A depolarizing step of 13 mV from a holding potential of −38

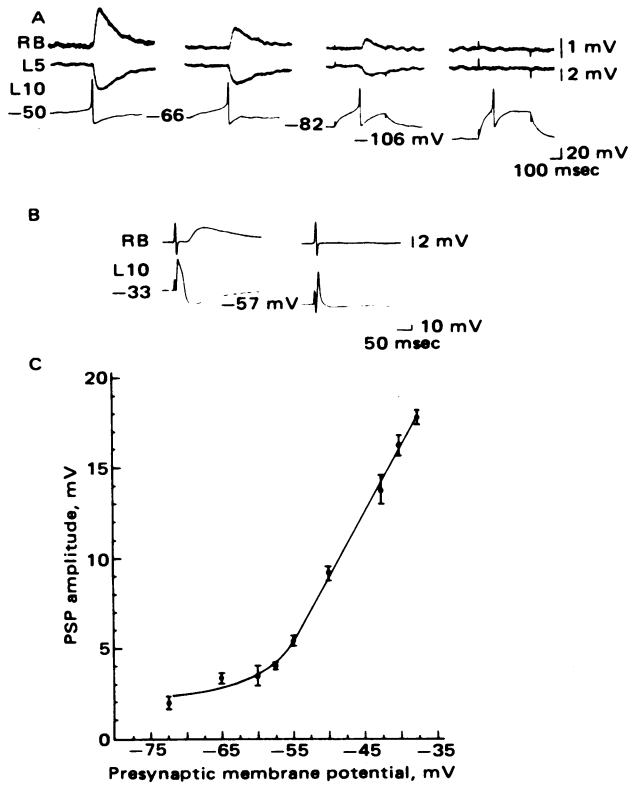

FIG. 1. Effect of presynaptic membrane potential on presynaptic spike and on transmitter release. (*A*) Spikes are elicited in interneuron L10 by intracellular injection of depolarizing current while the cell is maintained at various holding potentials (indicated at the lower left of each set of traces). (*B*) Effect of resting membrane potential on the spikes in L10. The spikes elicited by brief intracellular current injection are broader and larger from more depolarized membrane potentials than at more hyperpolarized levels. The duration of the spike parallels the effectiveness of synaptic transmission. (*C*) Evoked EPSP (RB cells) size is plotted as a function of presynaptic (L10) holding potential. Experiment as in *A*. Each point on the graph is the mean of 10 determinations (± SEM). [A solution containing a high concentration of divalent cations (solution B) was used in all these experiments].

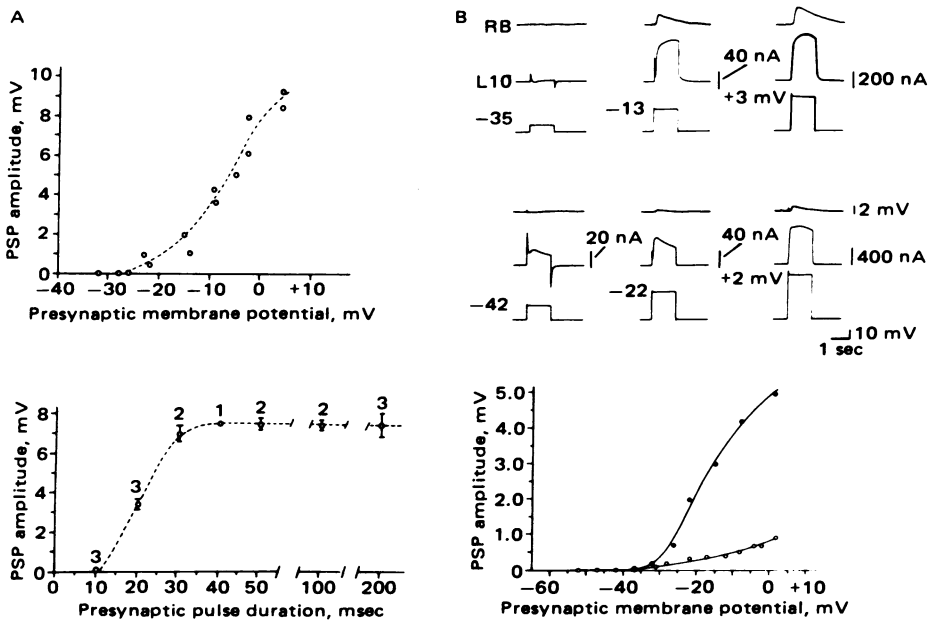

FIG. 2. (*A*) Voltage-clamp depolarizations of presynaptic neuron L10 elicit graded release of transmitter. (*Upper*) PSP amplitude (cell L5) is a graded function of size of presynaptic (cell L10) depolarization. Cell L10 was voltage-clamped at holding potential −38 mV in high-divalent cation sea water solution (solution B) containing TTX. Depolarizing pulses of 200 msec duration were stepped to various membrane potentials once every 30 sec and the size of the IPSP elicited was plotted against size of step depolarization. The range of membrane potentials in which the PSP size is increasing is identical to that in which voltage-dependent Ca^{2+} current is increasing (see Fig. 3*A*). (*Lower*) PSP amplitude (L5) is a graded function of duration of presynaptic (cell L10) depolarization. Depolarizing voltage-clamp steps from a holding potential of −38 to −3 mV were delivered once every 30 sec. Duration of this pulse was varied. From durations of 10 msec to about 40 msec the size of the PSP is a graded function of duration of the presynaptic depolarization. Number of PSPs averaged for each duration is given above each point.

(*B*) Synaptic potential is modulated by presynaptic membrane potential. (*Upper*) Presynaptic cell L10 is voltage-clamped at a holding potential of −45 mV (top set of traces) and −62 mV (bottom set of traces) in an artificial seawater solution (solution A) containing 30 μM TTX. Step depolarizations of 0.1 sec each from each holding potential (interpulse interval = 1 min) evoke EPSPs in the follower cell (top trace in each set of traces). The middle trace is the monitored current from L10 and the bottom trace of each set is the voltage record. From a holding potential of −45 mV the step depolarizations elicit PSPs. As the size of the depolarizations increases, clamp current becomes more outward and PSP size increases. From a holding potential of −62 mV, step depolarizations elicit smaller PSPs. Each clamp current is more outward than for steps from −45 mV (e.g., compared steps to +3 and +2 mV, noting change in current calibration). (*Lower*) Plot of results of experiment in *Upper*. The threshold of evoked release is similar at the two holding potentials: −45 mV (●) and −62 mV (○).

mV to about −25 mV produced a small inward current in the presynaptic neuron and a small EPSP in the postsynaptic cell (Fig. 2*A*). Further depolarizations of cell L10 in the voltage range that produces graded increases in the peak inward current (−30 mV to +10 mV) produced graded increases in transmitter release (see Fig. 3*A* below). Release is also a graded function of the duration of the depolarizing step (Fig. 2*A*). Klein and Kandel (5) found that the PSPs are a graded function of spike duration in *Aplysia* sensory neurons. Our clamp data showing PSPs graded with command pulse duration provide further evidence that K^+ currents, which contribute to spike duration, can play a role in modulation of transmitter release.

The sigmoid function relating transmitter release to presynaptic depolarization (Fig. 2*A*) is similar to that reported at the squid giant synapse (18, 19). However, in contrast with results in squid, hyperpolarizing the presynaptic cell decreases the size of the PSP elicited by step depolarization to the same level (Fig. 2*B*).

Net Outward Currents Are Decreased by Depolarization. With depolarized holding potentials, voltage-clamp currents elicited by step depolarization are relatively less outward than with hyperpolarized holding potentials (Fig. 2*B*; refs. 7–10). These differences in current presumably account for the difference in duration of the action potential at the two holding potentials in unclamped cells (Fig. 1*B*). However, the ability to modulate transmitter release with membrane potential changes under voltage clamp conditions (in which the duration of the presynaptic command is held constant) suggested that this type of plasticity is mediated by mechanisms in addition to K^+ current modulation—perhaps by a change in the transient Ca^{2+} current. To test this idea we selectively blocked the several outward K^+ currents, which are responsible for alterations in the shape of the spike, to see if the membrane potential was still capable of modulating the transmitter released by a depolarizing command and if this modulation is due to a direct action on the transient Ca^{2+} current.

Transient Ca^{2+} Current Is Not Increased by Depolarizaiton. Three pharmacologically separable K^+ conductances have been described in molluscan somata (8). Two of these are voltage-dependent; an early fast inactivating K^+ current sensitive to 4-AP (8–10) and a delayed K^+ current sensitive to Et_4N^+ (8, 10). A third K^+ conductance is dependent on intracellular Ca^{2+} concentration (20, 21) and can be blocked by agents that block Ca^{2+} current (e.g., Co^{2+} and EGTA) and by substituting Ba^{2+} for Ca^{2+}. Blocking the voltage-dependent K^+ channels individually did not change the effect of hyperpolarization on net currents; they still became more outward and we still could not observe the Ca^{2+} current directly. When high dosages of Et_4N^+ and 4-AP together were utilized to block both voltage-dependent K^+ channels, peak inward Ca^{2+} current was seen to decrease slightly when evoked from more depolarized holding potentials (Fig. 3*A*), perhaps due to steady-state inactivation or to changes in intracellular Ca^{2+} concentration.

With all K^+ channels blocked pharmacologically, the inward current through the Ca^{2+} channels, carried now by Ba^{2+} ions,

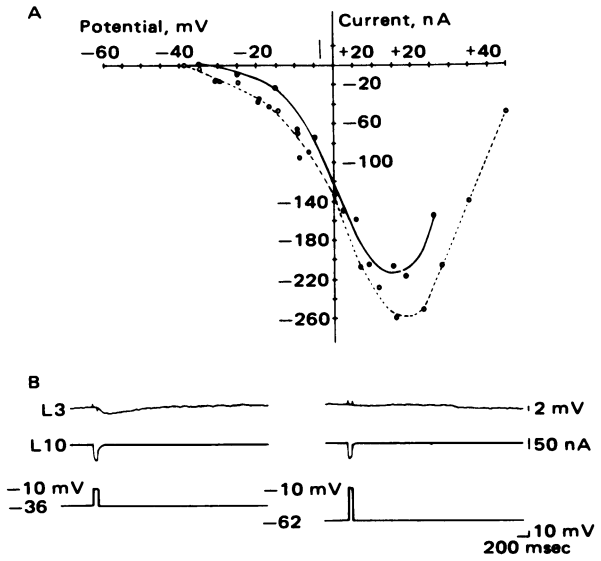

FIG. 3. (*A*) Transient calcium currents are independent of holding potential. The current/voltage relationship for Ca^{2+} current of the presynaptic neuron L10 from two holding potentials [−60 (○) and −40 (●) mV] in solution C/10 mM 4-AP is shown. Peak inward current is not decreased by hyperpolarization. (*B*) Amplitude of PSP is still modulated by holding potential after blockage of all K^+ currents. Presynaptic neuron voltage-clamped from two holding potentials in solution E containing 30 μm TTX, 25 mM Et_4N^+, and 10 mM 4-AP at a 100-msec step depolarization to −10 mV from holding potential −36 mV elicits large net inward current and large IPSP. A 100-msec step to −10 mV from −62 mV elicits larger peak transient inward current but only a small IPSP. Note the difference in the inward tail current at the two holding potentials.

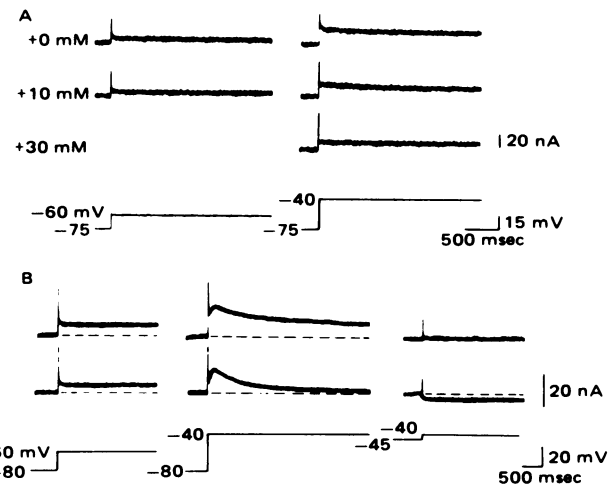

FIG. 4. Steady-state Ca^{2+} and Ca^{2+}-dependent K^+ currents in presynaptic neuron L10. (*A*) EGTA titration. Presynaptic neuron L10 was voltage-clamped in artificial seawater (solution A) containing 30 μM TTX, 100 mM Et_4N^+, and 0, 10, or 30 mM EGTA to reduce extracellular Ca^{2+} concentration. Voltage steps from −75 to −60 mV reveal little rectification and the leakage current is not affected by external Ca^{2+} concentration. By contrast, voltage steps from −75 to −40 mV in 0 EGTA reveal a delayed (outward) rectification that is decreased by EGTA, indicating that it is due to a Ca^{2+}-dependent K^+ current. (*B*) Ba^{2+} substitution. Presynaptic neuron L10 voltage-clamped in artificial seawater (solution A) containing 30 μM TTX, and 50 mM Et_4N^+ (upper curves) and solution D containing 30 μM TTX and 50 mM Et_4N^+ (middle curves). The leakage current elicited by a step from −80 mV to −60 mV is only slightly affected by substitution of Ba^{2+} for Ca^{2+}. But the steady-state current elicited by steps from −80 mV to −40 mV is more inward in presence of Ba^{2+}. The steady-state activation of net inward current can be observed directly with smaller leakage step from −45 to −40 mV.

can be observed directly. This inward current was not increased by changing the holding potentials from −60 to −40 mV (Fig. 3*B*). These results indicate that differences in net currents observed with steps from different holding potentials are due to changes in K^+ conductances and not to changes in the transient Ca^{2+} current (22).

Even though the transient divalent cation current was not changing, the membrane potential could still modulate transmitter release (Fig. 3*B*). Depolarization still increases transmitter release even though peak transient inward current is smaller and occurs later from depolarized than from hyperpolarized levels.

Depolarization Activates a Steady-State Ca^{2+} Current. Although the membrane potential modulation of transmitter release does not result from the direct modulation of the transient Ca^{2+} current, it could result from changes in the steady-state activation properties of the Ca^{2+} channels. Steady-state activation—the contribution of Ca^{2+} current to the resting leakage current—could be greater at depolarized levels leading to: (*i*) an increased intracellular level of Ca^{2+} at more depolarized holding potentials; (*ii*) saturation of intracellular Ca^{2+} buffering systems competing with the release process; or (*iii*) changes in intracellular screening of charged membrane proteins near the active release site (23, 24). As a result, a constant influx of Ca^{2+} could interact with the transient Ca^{2+} influx brought into the terminals with each action potential to provide an adequate concentration of Ca^{2+} for transmitter release. Such steady-state activation of Ca^{2+} channels has been described in molluscan somata (25, 26).

We therefore examined the resting membrane conductances at various holding potentials and found that part of the normal resting membrane conductance at depolarized holding potentials is a Ca^{2+}-dependent K^+ conductance (Fig. 4*A*).

More direct evidence for a steady-state Ca^{2+} channel was obtained by substituting Ba^{2+} for extracellular Ca^{2+} (Fig. 4*B*). In the presence of Ba^{2+} (which flows through Ca^{2+} channels without activating K^+ conductance), we directly observed the existence of a voltage-dependent steady-state inward current flowing through the Ca^{2+} channels at depolarized holding potentials (Fig. 4*B*).

Using Isolated Cell Bodies to Control for Imperfect Clamping. By using isolated soma preparations, we found that hyperpolarization increases K^+ currents and does not decrease transient Ca^{2+} current, and Ca^{2+} and Ca^{2+}-dependent K^+ currents contribute to the resting conductance of L10 at depolarized membrane potentials.

DISCUSSION

The ability to analyze simultaneously the Ca^{2+} current of the presynaptic cell body and transmitter release from the terminals has allowed us to explore the effects of membrane potential on transmitter release. Under conditions of voltage-clamp in which we could control reliably the amplitude of the depolarization, we have confirmed the presynaptic effect of membrane potential on transmitter release. In addition, we have found that the voltage dependence of transmitter release is paralleled by, and appears to result from, reinforcing changes in Ca^{2+} and K^+ currents. Finally, the method described here can be extended to analyze directly the alternations in Ca^{2+} current underlying various plastic changes in transmitter release, such as homosynaptic depression and facilitation, posttetanic facilitation, and presynaptic inhibition and facilitation. It is important to realize that in this type of analysis much of the total voltage-

clamp current is recorded across soma membrane that parallels but may not be directly involved in transmitter release. However, control experiments demonstrate that the currents recorded in isolated somata, where adequate voltage clamp control can be achieved, resemble those recorded in the soma of cells with release sites still intact.

Our results show that depolarization affects transmitter release in two ways. (*i*) It decreases the voltage-dependent K^+ currents activated by a depolarized step and thereby increases the height and particularly the duration of the action potential. This, in turn, leads to an increased Ca^{2+} inflow and therefore more transmitter release. (*ii*) Depolarization also increases (and hyperpolarization decreases) a steady-state Ca^{2+} inward current without greatly altering the transient Ca^{2+} current activated by a depolarized command. We propose that the interaction of the modulated steady-state Ca^{2+} current and the constant transient Ca^{2+} current contribute to the control of transmitter release by the holding potential.

The fact that the membrane potential can affect the steady-state Ca^{2+} activation has been reported (25, 26). This effect might work in one of two ways. (*i*) The Ca^{2+} channels might exhibit overlapping voltage-dependent activation and inactivation curves (26, 27). (*ii*) The Ca^{2+} channels might not be activated and inactivated by voltage alone but also by the intracellular concentration of Ca^{2+} (28, 29). A combined mechanism that might explain these unusual properties is the control of Ca^{2+} channel inactivation (or activation) by both membrane potential and the binding of Ca^{2+} to an intracellular site (28). It has also been proposed that cyclic nucleotide binding may open (or close) voltage-sensitive channels (5, 27, 30).

The role of steady-state Ca^{2+} current in the membrane potential modulation of transmitter release receives support from the observation of Nicholls and Wallace (31) that spontaneous quantal release of transmitter is increased at depolarized potentials in leech neurons. However, our results indicate that an additional mechanism operates normally in the effect of membrane potential on transmitter release in cell L10: a decrease of the K^+ currents with depolarization. In an unclamped cell, the decrease in the K^+ currents accounts for the increase in the duration of the spike. In this way the transient Ca^{2+} current can also be modulated, albeit indirectly. In fact, our findings suggest that, despite a transient Ca^{2+} current relatively unaffected by changes in holding potential, powerful control over synaptic transmission—ranging from total block to enhanced effectiveness—can be achieved by variations in the steady-state Ca^{2+} current.

These results also lead to three further conclusions. (*i*) These data support the idea (1), that EPSPs and IPSPs have a dual function: in addition to controlling the probability of firing action potentials, they set the level for transmitter release. (*ii*) These results provide a possible mechanism for long-term regulation of synaptic output by nonsynaptic actions that affect resting potential, be they metabolic (32) or hormonal (33, 34). (*iii*) These results and those described in ref. 11 suggest that synaptic transmission can be modified by three independent control mechanisms, each acting on the Ca^{2+} current: (*i*) an indirect action by means of the opposing K^+ current, (*ii*) a direct action in the steady-state Ca^{2+} current, and (*iii*) a direct action on the transient Ca^{2+} current activated by the spike.

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