Post-tetanic potentiation at an identified synapse in *Aplysia* is correlated with a Ca^{2+} -activated K^+ current in the presynaptic neuron: Evidence for Ca^{2+} accumulation

(synaptic modulation/voltage clamp)

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ABSTRACT We have examined the presynaptic changes underlying post-tetanic potentiation (PTP) in *Aplysia* by using voltage-clamp techniques combined with specific pharmacological blocking agents. The amplitude and time course of PTP parallel a slow outward clamp current that we have identified as a Ca^{2+} -activated K^+ current. Because this current is proportional to intracellular Ca^{2+} concentration our findings provide evidence for the “residual Ca^{2+} hypothesis,” according to which PTP is caused by the accumulation of intracellular Ca^{2+} after tetanus. To obtain further evidence for this mechanism we injected EGTA intracellularly and found that it decreased the duration of both PTP and the Ca^{2+} -activated K^+ current.

Post-tetanic potentiation (PTP) is a well-described form of synaptic plasticity whereby high-frequency stimulation of a presynaptic pathway leads to an increase in the amount of transmitter released (1–12). Previous studies have shown that Ca^{2+} plays an important role in PTP and that the potentiation is not dependent on the shape of the presynaptic spike and is affected by agents that alter the intracellular buffering capability for Ca^{2+} . On the basis of these observations Katz and Miledi (9, 10) and Rahamimoff (11, 12) have proposed that facilitation and PTP may result from residual increases in intracellular Ca^{2+} due to Ca^{2+} entry during the tetanus and—perhaps additionally—due to its subsequent release from internal stores by Na^+ . In previous analyses, it has not been possible to test the “residual Ca^{2+} hypothesis” directly because the presynaptic terminal could not be impaled for intracellular recording. In addition, these analyses have been compromised because tetanic stimulation also gives rise to membrane potential changes in the presynaptic terminals that may have secondary effects on release, masking the actual time course of the facilitated release process. Recently, neurons have been described in *Aplysia* in which presynaptic release sites are accessible to intracellular electrodes and can be studied under voltage-clamp conditions, in which secondary changes in membrane potential can be controlled (13–15). One of these neurons, the identified cholinergic cell L10, makes connections to follower cells that undergo PTP. We have therefore analyzed the presynaptic ionic mechanisms underlying PTP at these synapses and have tested the idea that Ca^{2+} accumulation contributes to PTP.

To examine Ca^{2+} accumulation we have relied on measurement of the Ca^{2+} -activated K^+ current in the presynaptic cell L10. This outward current is activated by intracellular Ca^{2+} , and there is good evidence that this current provides an index of the amount and time course of increases in intracellular Ca^{2+} concentration (16–18). We here describe that PTP is paralleled

by an increase in the Ca^{2+} -activated K^+ current and thus provide evidence for an increase in intracellular Ca^{2+} during PTP. To test this model further we next blocked the accumulation of Ca^{2+} before and after PTP by injecting the calcium-chelating agent EGTA into the presynaptic neuron. As predicted by the Ca^{2+} accumulation model, we found that injected EGTA decreased the Ca^{2+} -activated K^+ current, the size of postsynaptic potentials (PSPs), and the duration of PTP.

MATERIALS AND METHODS

Experiments were performed on over 100 desheathed abdominal ganglia removed from *Aplysia californica* weighing 50–125 g. Cell L10 was impaled with two independent electrodes of low resistance (1–5 M Ω) for current and voltage clamping (13, 19). Follower cells were recorded from with a third electrode connected to a bridge circuit for intracellular recording and current passing. Experiments were performed in a seawater solution containing high concentrations of divalent cations to reduce spontaneous synaptic activity. The composition of this solution (ASW) was 284 mM NaCl, 10 mM KCl, 165 mM MgCl_2 , 22 mM CaCl_2 , 10 mM Tris-HCl, pH 7.4. To improve voltage control over transmitter release sites in voltage-clamp experiments, the preparation was treated with 30 μM tetrodotoxin (TTX). In some experiments K^+ channel-blocking agents tetraethylammonium (Et_4N^+) chloride and 4-aminopyridine (4-AP) were added to the bath. In other experiments Ca^{2+} was replaced by Ba^{2+} , or Co^{2+} or Ni^{2+} was added to the bath. When 0.5 M EGTA was injected it was first dissolved in KOH solution, resulting in a potassium EGTA solution of pH 7.5, which was pressure-injected from one barrel of a two-barrel electrode. The second barrel was filled with 2 M potassium citrate and was used as the voltage-sensing electrode in the voltage-clamp circuit.

RESULTS

In unclamped cells, firing spikes in L10 with brief depolarizing current pulses at 0.2 Hz elicits stable PSPs in the follower cell. Tetanic stimulation of L10 (100 ± 4 spikes at 4 Hz) increases the size of PSPs up to 250% and produces PTP that can last 3–5 min (6). This PTP is accompanied by a hyperpolarization of the presynaptic cell that is 5–10 mV in amplitude and lasts for up to 5 min (Fig. 1A₁). This hyperpolarization is accompanied by an increased presynaptic membrane conductance of 10–20% (Fig. 1A₂). One effect of the hyperpolarization and increased membrane conductance is that the first few PSPs after the tet-

Abbreviations: PTP, post-tetanic potentiation; PSP, postsynaptic potential; TTX, tetrodotoxin; Et_4N^+ , tetraethylammonium; 4-AP, 4-aminopyridine; ASW, artificial seawater.

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FIG. 1. PTP of the L10-RB cell synapse. (*A*₁) For traces a and b L10 is impaled with two electrodes, one to pass current, the other to record the membrane potential. Electrodes were filled with 2 M potassium citrate. All experiments were performed at $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$. An RB follower cell is also monitored (trace a). L10 is stimulated at 0.2 Hz (L10 spikes trace b) to record a stable control PSP. A 25-sec tetanus of L10 spikes at 4 Hz causes L10 hyperpolarization and a long-lasting potentiation of transmitter release, which appears to build up slowly and then decrease. Trace c is a higher gain record of L10 membrane potential (trace b) to show presynaptic hyperpolarization caused by tetanic stimulation. (*A*₂) Changes in presynaptic membrane conductance. The membrane conductance is measured by means of a constant current hyperpolarizing pulse (5 nA) before and after the tetanus. Tetanic stimulation increases membrane conductance by 13.5%. V_m , membrane potential; I_m , membrane current. (*B*₁) L10 is voltage-clamped at a holding potential of -40 mV and depolarizing clamp steps to $+10$ mV at 0.2 Hz in TTX solution elicit stable PSPs in an RB follower cell (upper trace). A tetanus of 100 50-msec pulses elicits potentiation of transmitter release and a slow outward shift in current baseline (lower trace). At this gain the transient currents caused by step depolarizations are off scale. The voltage record is not shown. (*B*₂) Changes in presynaptic membrane conductance. The conductance increases 37.5% after the tetanus. The voltage step was from -40 to -60 mV for 750 msec.

anus are frequently smaller and PTP appears to build up slowly. A similar buildup for PTP elicited by evoked activity has been repeatedly described for other invertebrate and vertebrate synapses (1, 2, 4, 6, 8, 20). Indeed, this buildup has posed a problem for the residual Ca^{2+} hypothesis because clearly the peak facilitation does not seem to correlate with the peak of Ca^{2+} accumulation immediately after the tetanus. Because hyperpolarization of this presynaptic cell has recently been found to decrease transmitter release (21, 13), the buildup of PTP may reflect not the true kinetics of PTP but a secondary change due to the voltage dependence of transmitter release.

To control for membrane potential changes and to examine the ionic conductance changes in the presynaptic neuron, we voltage-clamped L10. As described earlier (13), when L10 is exposed to TTX it is possible to elicit graded transmitter release with graded voltage-clamp command depolarizations. To elicit control PSPs, command pulse depolarizations of 50-msec duration were delivered at 0.2 Hz from a holding potential of -30 to -50 mV to 0 or $+10$ mV. To produce PTP a tetanus was given consisting of 100 ± 4 pulse depolarizations at 4 Hz. After the tetanus PSPs were again elicited at the control rate of 0.2 Hz. Under these conditions PTP is still elicited (Fig. 1*B*₁). But now the initial buildup of the PTP is gone (Fig. 2). Preventing the hyperpolarization of L10 after the tetanus completely eliminates the normal buildup of PTP and reveals a new fast component of PTP. PSPs elicited immediately after the tetanus are at their largest and decrease with a double exponential time course.

Tetanic stimulation also causes a buildup of a slow outward current (Fig. 1*B*₁). This outward current is accompanied by an increased membrane conductance (Fig. 1*B*₂), and neither the current nor the membrane conductance increase is voltage sensitive between -110 and -30 mV (Fig. 3*A*). The slow outward

current lasts for 3–5 min. It decays with two time constants that correlate well with the membrane hyperpolarization observed in the unclamped state (Table 1) and with the decay of the PSP. These two time constants may correspond to what Magleby and Zengel (5) have called augmentation and potentiation.

A slow outward current turned on by activity has been described in other cells (16, 22) and has been alternatively attributed to a Ca^{2+} -activated K^{+} current (17, 23) or a slow voltage-dependent K^{+} current (22). To identify this slow current in L10, we first determined its reversal potential. As the membrane potential of L10 was hyperpolarized, the outward current became smaller and reversed at -75.7 ± 1.9 mV ($n = 5$; Fig. 3*A*). In addition, the slow outward current was present in solutions in which the Na^{+} channel was blocked by TTX, and the delayed and early K^{+} channels were blocked by Et_4N^{+} and 4-AP (Fig. 3*A*). These findings suggested that the outward current is due to a Ca^{2+} -activated K^{+} current. Our ability to reverse the slow

Table 1. Time constants τ of the three parameters that change post-tetanicly

Parameter	τ , sec	
	Fast component (τ_f)	Slow component (τ_s)
Hyperpolarization	8.8 ± 2.2 ($n = 4$)*	69.0 ± 25.3 ($n = 4$)*
Ca^{2+} -activated K^{+} current	7.9 ± 1.8 ($n = 6$)†	79.8 ± 24.1 ($n = 6$)†
Postsynaptic potential	7.5 ± 2.6 ($n = 3$)†	99.9 ± 46.6 ($n = 3$)†

The time constant τ is defined as the time interval between the end of the tetanus and the e^{-1} value ($\approx 37\%$) of the maximal change in either the PSP amplitude or the slow outward current. The baseline amplitude or current was determined pretetanicly for 10 stimuli or 50 sec. Results are mean $\pm$ SD.

* ASW, unclamped cells.

† ASW, $30 \mu\text{M}$ TTX, clamped cells.

FIG. 2. Time course of PTP differs in unclamped and clamped presynaptic cells. PSPs were elicited in clamped ($\circ$) and unclamped ($\bullet$) L10 cells at a frequency of 0.2 Hz. The pretetanic control value is represented by the broken line (100%). A standard tetanus was given to elicit PTP. The mean values ($\pm$ SEM) of PSPs are presented ($n = 5$). The two curves were fitted by eye. L10 was clamped at either -40 or -30 mV and 50-msec pulses were delivered to 0 or $+10$ mV. The time course of PTP in unclamped L10 cells shows a buildup region that lasts up to 45 sec after the end of the tetanus. No such buildup of PTP was seen when the presynaptic cell was clamped. Therefore, the buildup of PTP results from the post-tetanic hyperpolarization of L10 and the voltage dependence of transmitter release.

outward current and its voltage independence suggest that tetanic stimulation causes a large increase in intracellular Ca^{2+} concentration, because it is only then that the Ca^{2+} -activated K^+ current behaves in a voltage-independent manner (24, 25).

To further test the identity of the slow outward current we examined the outward current produced by tetanic command pulses in solutions containing Co^{2+} or Ni^{2+} , which block Ca^{2+} inflow. These solutions blocked the outward current and the membrane conductance increase ($n = 4$; Fig. 3B). We next substituted Ba^{2+} for Ca^{2+} and delivered tetanic stimulation. Ba^{2+} flows through Ca^{2+} channels but does not activate the Ca^{2+} -activated K^+ current (26). Again, the slow outward current was blocked ($n = 3$; Fig. 4B). Finally, the slow outward current was also reduced by injecting EGTA into L10 ($n = 6$; Fig. 4A and B), a treatment that blocks Ca^{2+} -activated K^+ current (18, 23, 27). These several findings demonstrate that tetanic stimulation leads to an increase in intracellular Ca^{2+} concentration, which serves two purposes: (i) it turns on a Ca^{2+} -activated K^+ conductance and (ii) it enhances transmitter release. Both of these Ca^{2+} -activated processes decay with similar time constants and presumably reflect increases in the free Ca^{2+} concentration that are due to the Ca^{2+} inflow during the tetanus. Although release of Ca^{2+} from internal stores by Na^+ influx (12) may contribute a small amount to PTP, the experiments performed in TTX indicate that normal PTP can be obtained in the absence of Na^+

FIG. 3. Properties of slow outward current. (A) Reversal potential of slow outward current. Cell L10 was clamped at various membrane potentials and a tetanus of 100 clamp pulses at 4 Hz was delivered. The size of the peak baseline current shift caused by the tetanus is plotted against holding potential (15 min between runs). The experiment was repeated in the presence of various pharmacological agents (as indicated in the figure) designed to block voltage-dependent Na^+ and K^+ currents. The reversal potential V_{rev} of the slow outward current is about -76 mV. This slow outward current is not blocked by 10 mM 4-AP or 25 mM Et_4N^+ (TEA), suggesting that it is a Ca^{2+} -activated K^+ current. (B) Ba^{2+} and Co^{2+} block slow outward current. Control runs show that a tetanus in normal Ca^{2+} -containing ASW is accompanied by outward baseline current shift. Ba^{2+} substitution for Ca^{2+} eliminates the baseline shift (PSP not monitored). In another experiment 25 mM Co^{2+} added to the bath also blocked the slow outward current.

influx (Figs. 1B and 2 and ref. 8).

To obtain direct evidence that the Ca^{2+} -activated K^+ current parallels Ca^{2+} accumulation and that the intracellular accumulation of Ca^{2+} produced by tetanic stimulation is causally related to PTP we injected EGTA, a Ca^{2+} -buffering agent, into L10 and examined its effect on the size and time course of both the PTP and the Ca^{2+} -activated K^+ current. In each experiment two control runs of PTP separated by 10 min were performed prior to the injection of EGTA. Approximately 2.0 nl of 0.5 M EGTA in KOH at pH 7.5 (approximately 5–10% of the cell volume) was then pressure-injected into L10 and PTP runs were followed over the next several hours. Membrane input resistance after injection was unchanged or slightly increased. As previously reported (27, 28), EGTA injections decreased the

FIG. 4. Time course of PTP and slow outward current before and after EGTA injection. (A) Recordings of the PSP amplitude (RB trace) and the slow outward current (L10 trace) before (control) and after (3 min and 68 min) the end of 0.5 M EGTA injection into the presynaptic cell L10. Three minutes after the end of EGTA injection control PSPs and the absolute value of PTP are reduced; 68 min after injection control PSPs are abolished. However, during the tetanus a buildup of PSPs occurs and post-tetanic PSPs were recorded. EGTA injected into the cell body reduces the slow outward current. This effect was more rapid than the changes in PSP amplitude. (B) Amplitudes of post-tetanic PSPs are plotted as a function of post-tetanic Ca^{2+} -activated K^+ current for control and 3 min, 38 min, and 68 min after EGTA injection. Both post-tetanic PSP amplitude and K^+ currents are diminished in parallel by EGTA injection. Note that the baseline voltage-clamp current before tetanus has arbitrarily been assigned the value 0 for $I_{K_{Ca}}$, although at holding potential -40 mV a part of the resting leakage conductance is a Ca^{2+} -activated K^+ current (13). (C) The slow time constants of decay of PTP (●) and post-tetanic Ca^{2+} -activated K^+ (○) currents are plotted as a function of time after EGTA injection. Results are presented as mean $\pm$ SD with n above the error bar. While tetanus is still capable of enhancing transmitter release after EGTA injection, the duration of enhanced release is markedly reduced by EGTA injection. Values at time 0 represent control runs.

FIG. 5. Changes in transient currents and spike configuration. (A) Changes in spike configuration accompanying PTP. After the tetanus, spike width is increased, but the spike returns almost to control levels within 1 min while PSP is still enhanced. Spikes 1, 2, and 3 are from numbered areas of trace a in Fig. A₁. (B) Transient current changes accompanying PTP. L10 is voltage-clamped in $30 \mu M$ TTX, while an RB follower cell is monitored. Voltage-clamp steps from -40 to $+5$ mV at 0.2 Hz (bottom trace) elicit inward Ca^{2+} and outward K^+ currents (middle trace) and a stable RB cell PSP (top trace). After a tetanus of 100 pulses at 4 Hz the outward currents are inactivated and transmitter release is enhanced. Over the course of the next 2 min currents return to control levels while PTP is still seen. (C) Changes in transient Ca^{2+} current accompanying PTP. When cell L10 is voltage-clamped in a solution containing $30 \mu M$ TTX, 10 mM 4 -AP, and 25 mM Et_4N^+ (TEA), the transient Ca^{2+} current can be observed. This current is blocked by application of Co^{2+} or Ni^{2+} (not shown). L10 is voltage-clamped at a holding potential of -50 mV. Pretetanic voltage-clamp steps to 0 mV at 0.2 Hz (bottom trace) elicit inward Ca^{2+} current (middle trace) and a stable RB cell PSP (top trace). After tetanic stimulation of 100 pulses at 4 Hz, the inward Ca^{2+} current is reduced, while transmitter release is enhanced. Over the next 200 sec the inward current and transmitter release return to control levels. At the gain of current record in B and C, the slow outward current cannot be seen.

outward currents and increased the inward Ca^{2+} current produced by each depolarizing command. EGTA injection also reduced the slow outward current elicited by the tetanus (Fig. 4 A and B). In addition, EGTA affected the amplitude of PSPs and the decay time constants of PTP and the slow outward current. Within 5 min after injection of EGTA the control PSPs were reduced (Fig. 4A). The PSP still built up during and after the tetanus. However, the PSPs do not potentiate to the same degree as before EGTA injection (Fig. 4 A and B). With further time after injection, the size of potentiated PSPs were further reduced. In parallel with these changes in transmitter release, the Ca^{2+} -activated K^+ current was also reduced.

These results support the residual Ca^{2+} model, but they are complicated by the fact that the control PSP is, of course, also

reduced by EGTA injection. To obtain a clearer indication of the effect of EGTA on PTP, independent of size changes in the control PSP, we examined the time course of PTP. As illustrated in Fig. 4C, we found that EGTA caused a progressive reduction in the time constants of PTP that paralleled the reduction in the slow outward current. Within 5 min after EGTA injection the fast time constants of both PTP and the slow outward current were blocked, and both the PTP and outward current could be fitted with a single slow exponential function. With further time after the injection this slow time constant also decreased progressively (Fig. 4C). One to 2 hr after the injection, tetanic stimulation (Fig. 4A and B) led to PTP that lasted only seconds. To obtain still further and independent evidence for Ca^{2+} accumulation during PTP, we have injected cell L10 with the Ca^{2+} -sensitive dye arsenazo III and measured alterations in the level of free Ca^{2+} (unpublished results obtained in collaboration with J. Connor). Tetanic stimulation of L10 causes a large increase in the free Ca^{2+} level as indicated by an increase in arsenazo III absorbance measured differentially ($A_{660} - A_{690}$). After the tetanus, the absorbance signal declines, as free Ca^{2+} levels return to baseline, with a time course similar to the time courses we report here for PTP and the Ca^{2+} -activated K^+ currents.

Our results thus provide direct evidence for the suggestion of Katz and Miledi and Rahamimoff that intracellular accumulation of Ca^{2+} during the tetanus contributes importantly to PTP, and they also provide evidence for the suggestion of Meech (23) that both PTP and post-tetanic hyperpolarization are due to Ca^{2+} accumulation. As a corollary, our finding indicates that intracellular Ca^{2+} metabolism—e.g., the kinetics and activity of Ca^{2+} -buffering systems—contribute to modulation of transmitter release.

Tetanic stimulation could also enhance transmitter release by increasing Ca^{2+} influx through changes in spike configuration (Fig. 5A). Indeed, we found that tetanic stimulation in TTX caused decreases in the outward currents that could qualitatively account for the post-tetanic spike-broadening observed in unclamped cells (Fig. 5A and B). After the tetanus, the clamp currents gradually returned to control levels as inactivation wore off. Thus our results do not exclude contributing mechanisms, such as change in spike duration, to the full expression of PTP. Indeed, these changes do occur and could contribute to PTP. But our demonstration that PTP is present in TTX and can be obtained by depolarizing commands of constant duration suggests that Na^+ inflow or changes in spike configuration are not necessary for the expression of PTP.

Pharmacological blockage of voltage-sensitive K^+ channels leaves residual clamp currents, which consist of an inward Ca^{2+} and very small slow outward Ca^{2+} -activated K^+ current (Fig. 5C). PTP can still be elicited with voltage-clamp commands in solutions containing TTX, Et_4N^+ , and 4-AP, indicating that changes in transient K^+ as well as in Na^+ currents are not essential for PTP. Also, this treatment allows observation of the transient Ca^{2+} current in isolation. The Ca^{2+} current also inactivates slightly after the tetanus (Fig. 5C), indicating that the transient Ca^{2+} current is actually decreased, not increased. Similar inactivation of the divalent cation inward current after tetanus was seen in Ba^{2+} solutions. These results suggest that, in the presence of a high intracellular concentration of Ca^{2+}

(Ca^{2+} accumulation), a reduced transient Ca^{2+} current can still elicit increased transmitter release, and the results emphasize the level-setting role of steady-state intracellular Ca^{2+} in setting the level of transmitter release. One possible interpretation for this result is that, once the intracellular buffering and binding sites are saturated by an increase in intracellular Ca^{2+} , most of the additional Ca^{2+} carried into the terminal by each action potential is used primarily for release.

These results, together with those of Klein and Kandel (15) and Shapiro *et al.* (13, 14), reveal that five types of short-term synaptic plasticity all involve modulation of Ca^{2+} activity in the terminals: (i) voltage-dependent release of transmitter; (ii) presynaptic inhibition; (iii) presynaptic facilitation; (iv) homosynaptic depression; and now (v) PTP. Despite the common dependence on Ca^{2+} modulation, the details of the mechanisms have been shown to differ in each case.

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