Long-Lasting Synaptic Potentiation Induced by Depolarization Under Conditions That Eliminate Detectable $\text{Ca}^{2+}$ Signals

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Reyes FD, Walters ET. Long-lasting synaptic potentiation induced by depolarization under conditions that eliminate detectable $\text{Ca}^{2+}$ signals. J Neurophysiol 103: 1283–1294, 2010. First published December 30, 2009; doi:10.1152/jn.00704.2009. Activity-dependent alterations of synaptic transmission important for learning and memory are often induced by $\text{Ca}^{2+}$ signals generated by depolarization. While it is widely assumed that $\text{Ca}^{2+}$ is the essential transducer of depolarization into cellular plasticity, little effort has been made to test whether $\text{Ca}^{2+}$-independent responses to depolarization might also induce memory-like alterations. It was recently discovered that peripheral axons of nociceptive sensory neurons in Aplysia display long-lasting hyperexcitability triggered by conditioning depolarization in the absence of $\text{Ca}^{2+}$ entry (using nominally $\text{Ca}^{2+}$-free solutions containing EGTA, “0Ca/EGTA”) or the absence of detectable $\text{Ca}^{2+}$ transients (adding BAPTA-AM, “0Ca/EGTA/BAPTA-AM”). The current study reports that depolarization of central ganglia to $\sim 0$ mV for 2 min in these same solutions induced hyperexcitability lasting $>1$ h in sensory neuron processes near their synapses onto motor neurons. Furthermore, conditioning depolarization in these solutions produced a 2.5-fold increase in excitatory postsynaptic potential (EPSP) amplitude 1–3 h afterward despite a drop in motor neuron input resistance. Depolarization in 0 Ca/EGTA produced long-term potentiation (LTP) of the EPSP lasting $\geq 1$ days without changing postsynaptic input resistance. When re-exposed to extracellular $\text{Ca}^{2+}$ during synaptic tests, prior exposure to 0Ca/EGTA or to 0Ca/EGTA/BAPTA-AM decreased sensory neuron survival. However, differential effects on neuronal health are unlikely to explain the observed potentiation because conditioning depolarization in these solutions did not alter survival rates. These findings suggest that unrecognized $\text{Ca}^{2+}$-independent signals can transduce depolarization into long-lasting synaptic potentiation, perhaps contributing to persistent synaptic alterations following large, sustained depolarizations that occur during learning, neural injury, or seizures.

INTRODUCTION

The activity-dependent synaptic alterations thought to be important for learning, memory, and other types of neuronal plasticity are assumed—almost universally—to be induced by transient elevation of intracellular $\text{Ca}^{2+}$ levels. $\text{Ca}^{2+}$ is well suited for transducing electrical activity into cellular responses because the concentration of free intracellular $\text{Ca}^{2+}$ is kept extremely low, its entry into the cytoplasm is enhanced dramatically by depolarization, and it can directly activate many enzymes and other protein effectors (Burgoyne 2007; Case et al. 2007; Hille 2001), thereby initiating functional changes within depolarized neurons. $\text{Ca}^{2+}$ transients can also trigger exocytosis from depolarized neurons, releasing neuromodulators that alter synaptic strength (e.g., Kandel 2001). While the importance of $\text{Ca}^{2+}$ as a trigger for activity-dependent plasticity is clear (e.g., Abrams et al. 1991; Malenka 1991; Rao and Finkbeiner 2007; Xu and Kang 2005; Zucker 1999), this does not mean that $\text{Ca}^{2+}$-independent, depolarization-induced triggers of synaptic plasticity do not also exist and are not also important.

Two early and relatively neglected reports suggested that depolarization-induced, $\text{Ca}^{2+}$-independent synaptic potentiation might exist. Potentiation of hippocampal synapses lasting $>30$ min was induced by 3-min treatment with a solution having high $K^+$ and very low $\text{Ca}^{2+}$ concentrations (HiK/0Ca) following 4-min infusion of a nominally $\text{Ca}^{2+}$-free solution (May et al. 1987). However, this study did not use $\text{Ca}^{2+}$ chelators to further reduce $\text{Ca}^{2+}$ levels in the “$\text{Ca}^{2+}$-free solutions”. More convincing evidence came from the finding of persistent ($>1$ h) potentiation of the crayfish neuromuscular junction that was induced by 10 min of 20-Hz stimulation; this “long-term facilitation” was induced in a nominally $\text{Ca}^{2+}$-free solution containing the membrane-permeant $\text{Ca}^{2+}$ chelator, BAPTA-AM (Wojtowicz and Atwood 1988). Other studies have revealed nonsynaptic, memory-like plasticity involving $\text{Ca}^{2+}$-independent induction signals during depolarization (see DISCUSSION). Of particular relevance for the possibility of $\text{Ca}^{2+}$-independent, depolarization-induced synaptic potentiation was the demonstration that long-lasting hyperexcitability of Aplysia sensory neuron axons can be induced by intense 2-min depolarization (using HiK/0Ca solution) in the presence of the $\text{Ca}^{2+}$ chelators, EGTA and BAPTA-AM, at concentrations sufficient to eliminate $\text{Ca}^{2+}$ transients detectable with fura 2-AM imaging in the axons (and somata) during depolarization (Kunjilwar et al. 2009). Synaptic terminals of these and related sensory axons in Aplysia have been shown in a large and influential body of work to exhibit, short-, intermediate-, and long-term potentiation/facilitation induced both by activity-dependent signals (Antonov et al. 2001; Bailey et al. 2000; Eliot et al. 1994; Hawkins et al. 1983; Lin and Glanzman 1994; Schacher et al. 1997; Sutton and Carew 2000; Walters and Byrne 1983a, 1985), and by extrinsic modulators (reviewed by Byrne and Kandel 1996; Glanzman 2008; Kandel 2001; Reissner et al. 2006). Thus it was natural to investigate whether depolarization in the absence of detectable $\text{Ca}^{2+}$ signals leads to potentiation of these highly plastic synapses.

Here we show that intermediate-term potentiation (ITP, lasting $\geq 1$ h) and long-term potentiation (LTP, lasting 1 day) of sensorimotor synapses in Aplysia are induced by conditioning depolarization in conditions that eliminate detectable $\text{Ca}^{2+}$ transients in sensory neuron processes and that this synaptic potentiation is associated with hyperexcitability of the central processes of the sensory neurons. These findings provide

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strong evidence for the existence of signals other than Ca\(^{2+}\) for transducing depolarization into long-lasting synaptic alterations.

**METHODS**

**Nerve-ganglion preparation and solutions**

*Aplysia californica* (90–250 g; from Alacrity Marine, Redondo Beach, CA or from the University of Miami—National Institutes of Health National Resource for *Aplysia*, Miami, FL) were kept in aerated artificial seawater (ASW, instant ocean) at 16°C on a 12-h light/dark cycle. After being anesthetized by injection of isotonic MgCl\(_2\) solution (383 mM), the pedal-pleural ganglia were excised and pinned in a chamber with the attached posterior pedal nerve, p9, threaded through a series of smaller wells (Fig. 1A) (Weragoda et al. 2004). The pleural and (in experiments on synaptic potentiation) the pedal ganglia were surgically desheathed in a 1:1 mixture of normal ASW [containing, in mM: 460 NaCl, 11 CaCl\(_2\), 10 KCl, 55 MgCl\(_2\), 10 Tris buffer (pH 7.6) and isotonic MgCl\(_2\)]. This anesthetizing solution was then washed out with ASW. Experiments were conducted at room temperature (20–22°C). In all experiments, the ganglia were bathed ≥30 min prior to treatment (Fig. 1, B and C) with one of two “0 Ca” solutions. 0 Ca/EGTA solution contained (in mM) 460 NaCl, 10 KCl, 1 EGTA, 66 MgCl\(_2\), and 10 HEPES. This solution had ~100 mM free Ca\(^{2+}\) as indicated by fura 2 imaging (see Kunjilwar et al. 2009). In many experiments, 10 mM BAPTA-AM was added to the HiDi solution (see following text) ≥15 min prior to beginning the pretests as well as to the 0 Ca/EGTA solution (i.e., 0 Ca/EGTA/BAPTA-AM). Thus the BAPTA-AM had ≥60–90 min to diffuse into the cells before HiK treatment. Conditioning depolarization (2 min) was produced by washing in “HiK” solutions that were identical to those containing elevated concentrations of divalent cations (HiDi): ASW containing 2.2 × normal [Ca\(^{2+}\)] \((24.2\) mM) and 2 × normal [Mg\(^{2+}\)] \((110\) mM) with NaCl adjusted to maintain normal osmolarity. This solution effectively blocks polyysynaptic components of connections between tail sensory neurons and tail motor neurons without altering the amplitude of the monosynaptic EPSP (Liao and Walters 2002). In long-term experiments, the excised ganglia and nerves were stored overnight at 16°C in ASW.

**Electrophysiological measurements**

Short-term (~15 min) and intermediate-term (1–3 h) hyperexcitability (STH and LTH) of sensory neuron processes within the pedal ganglion neuropil were examined by determining the threshold of action potentials evoked by constant current test pulses passed between a 1.5-mm-diam Pt-Ir electrode, insulated except for the tip, placed underneath the center of the ventral surface of the fully sheathed pedal ganglion and an identical electrode pressed against the dorsal surface, ~2–3 mm posterior to the root of the pleural-pedal connective. This is near the region occupied by the somata of identified tail motor neurons (Fig. 1A) (Walters et al. 1983) and includes the region where synapses between tail sensory and tail motor neurons are concentrated (Wainwright et al. 2002; Zhang et al. 2003). To identify tail sensory neurons, extracellular test stimuli were also applied peripherally to segments of the posterior pedal nerve (p9) ~2 cm from the pedal ganglion, using described methods (Kunjilwar et al. 2009; Weragoda et al. 2004). Sensory neuron spike thresholds were obtained with ascending series of 5-ms pulses delivered first to the nerve and then across the pedal ganglion with the evoked action potential monitored by intracellular electrodes in somata in the pleural ganglion (Figs. 1A and 2, A and B). Intracellular recordings from tail sensory neurons and tail motor neurons were made with glass capil-

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**FIG. 1.** Preparation and experimental sequences. A: nerve-ganglion preparation consisting of excised pedal and pleural ganglia with the attached tail nerve (p9). Extracellular test stimuli were applied to the nerve to identify tail sensory neurons and then across the pedal ganglion (“neuropil test”) to determine the thresholds of their central processes in the vicinity of sensorimotor synapses (monitored by intracellular recording of evoked spikes conducted to the sensory neuron soma). Monosynaptic excitatory postsynaptic potentials (EPSPs) were tested by stimulating a tail sensory neuron (SN) and recording its synaptic response in a tail motor neuron (MN) that was manually clamped to ~70 mV. MN input resistance \((R_{\text{in}})\) was tested by intracellular stimulation through a separate stimulating electrode. Neuropil test stimulation and MN recordings were performed in separate experiments. B: sequence of solution changes and microelectrode impalements into sensory neurons in experiments examining excitability of processes in the neuropil. The times indicated are relative to the offset of the 2-min high K\(^{+}\) (HiK) treatment. C: solution changes and SN and MN impalements in experiments examining synaptic potentiation. When present, BAPTA-AM was applied before and during the pretests, and was also included in the 0Ca/EGTA and high divalent cation (HiDi) solutions. In long-term experiments, the sequence was the same except that the posttests in HiDi were conducted 18–24 h after treatment.
lary microelectrodes (35–50 MΩ) filled with 3 mM potassium acetate. Neurons were impaled for pretests, the electrodes removed before the washes associated with treatment, and then the same neurons reimpaled for posttests. Motor neurons were impaled with separate electrodes for measuring membrane potential and passing current. EPSP recordings were made in HiDi solution (see preceding text), with the motor neuron manually clamped to −70 mV. In all cases, the short-latency (4–8 ms) EPSP associated with the first observed action potential in the sensory neuron was measured. This often occurred during impalement of the sensory neuron (Fig. 3A); otherwise during injection of 20-ms depolarizing currents into the sensory neuron soma (Fig. 3B), which also provided a measure of the action potential threshold in the soma. Motor neuron input resistance, $R_{\text{in}}$, was tested in HiDi solution with descending series of 1-s current pulses while manually clamped at −70 mV. $R_{\text{in}}$ was determined from the linear portion of the resulting V–I curve.

Data analysis

Data are reported as means ± SE with $n$ indicating the number of preparations tested in each condition. Multiple sensory neurons (and in some cases, motor neurons) were usually tested in each preparation and averaged, with the median of the measurements per preparation used as a single data point. Even when the number of cells is also reported, the statistical tests were performed using each preparation, not each cell, as the statistical unit. Measurements were performed with blind procedures. Comparisons between treatments of unpaired preparations were made with unpaired $t$-tests. Comparisons of a single group before and after treatment were made with paired $t$-tests. Comparisons of two groups across multiple tests utilized two-way ANOVA with repeated measures. If there was a significant overall effect ($P < 0.05$), this was followed by Bonferroni post hoc tests. Differences in sensory neuron survival rates were assessed with Fisher’s exact tests. Statistically significant differences ($P < 0.05$) are indicated by asterisks in each figure (see figure legends for details of the statistical analyses).

RESULTS

Conditioning depolarization induces STH and ITH of sensory neuron processes near synaptic terminals in 0Ca/EGTA and 0Ca/EGTA/BAPTA-AM solutions

We first asked if depolarization-induced hyperexcitability could be triggered and maintained in the absence of Ca$^{2+}$ entry within compartments of tail sensory neurons located in the region of the pedal ganglion where these cells synapse onto tail motor neurons (Wainwright et al. 2002; Zhang et al. 2003). Intense 2-min depolarization of peripheral axons of these neurons in a solution (HiK/0Ca/EGTA) that eliminates the driving force for Ca$^{2+}$ entry into the cell is known to induce axonal STH, ITH, and LTH (Kunjilwar et al. 2009). To determine whether hyperexcitability occurs in sensory neuron processes near synaptic terminals after HiK treatment, we tested spike threshold by passing test currents across the pedal ganglion through electrodes placed below and above the tail motor neuron region while recording from sensory neuron somata in the pleural ganglion (Fig. 1A). Because the sensory axons and processes are primarily in the neuropil within the pedal ganglion (e.g., Wainwright et al. 2002), and the Ca$^{2+}$-free solutions block synaptic transmission (and therefore any indirect excitation of the sensory neurons), we assume that the sites of sensory action potential generation elicited by these test stimuli are within the neuropil. After bathing the pedal and pleural ganglia in 0Ca/EGTA for >60 min (Fig. 1B), we applied HiK/0Ca/EGTA solution to the ganglia for 2 min and found that the threshold for action potential generation in sensory neuron processes within the pedal ganglion decreased markedly. Examples of threshold responses in the neuropil (monitored in sensory neuron somata, see Fig. 1A) are shown in Fig. 2, A and B, before, 15 min, and 60 min after HiK or sham treatment. The 2-min depolarization produced by treatment with HiK/0Ca/EGTA significantly decreased the threshold for sensory neuron spike generation in the neuropil compared with the effects of sham treatment 15 min (STH) and 60 min (ITH) following treatment (Fig. 2C, see legend for statistics).

We next asked if STH and ITH of sensory neuron processes in the pedal ganglion neuropil could be induced and maintained by conditioning depolarization under conditions in which elevation of intracellular free Ca$^{2+}$ from both extra- and intracellular sources was minimized. Kunjilwar et al. (2009) showed that addition of the membrane-permeant Ca$^{2+}$ chelator, BAPTA-AM (10 μM), to 0Ca/EGTA solution prevented detectable Ca$^{2+}$ transients during HiK treatment of the axons of dissociated sensory neurons (although the same exposure to BAPTA-AM in ASW was not sufficient to effectively chelate the enormous amounts of Ca$^{2+}$ that enter the cell during prolonged depolarization in the presence of normal extracellular [Ca$^{2+}$]). Using 0Ca/EGTA/BAPTA-AM and HiK/0Ca/EGTA/BAPTA-AM solutions, we found that 2-min depolarization under conditions of extracellular and probable intracellular Ca$^{2+}$ chelation still produced significant STH and ITH of sensory neuron processes within the neuropil (Fig. 2D). To compare our results more closely to the effects on peripheral axons reported by Kunjilwar et al. (2009), test stimuli were applied every 15 min before and for 60 min after treatment. These results show that intense depolarization for 2 min under conditions associated with little or no Ca$^{2+}$ signaling produces STH and ITH of tail sensory neuron processes in the vicinity of their central synapses onto tail motor neurons. This hyperexcitability is similar in magnitude and time course to that seen in the sensory neurons’ peripheral axons (Kunjilwar et al. 2009).

In both sets of experiments summarized in Fig. 2, we also tested the excitability of tail sensory neuron axons in nerve p9, −15 mm from the main chamber that received HiK treatment. No significant changes in axonal spike threshold were found after HiK treatment [the mean axonal thresholds in HiK-treated preparations in both 0Ca/EGTA ($n = 4$) and 0Ca/EGTA/BAPTA-AM ($n = 7$) across all the posttests remained within 95–100% of the baseline values], and no significant differences were seen between HiK-treated and sham-treated preparations in either study. This indicates that depolarization-induced, Ca$^{2+}$-independent STH and ITH are restricted to compartments of the sensory neuron that receive the conditioning depolarization, reminiscent of the site specificity of LTH found in peripheral axonal segments after localized treatment of nerve segments with HiK (Kunjilwar et al. 2009; Weragoda et al. 2004) or 5-HT (Weragoda and Walters 2007).

Conditioning depolarization induces synaptic ITP in 0Ca/EGTA and 0Ca/EGTA/BAPTA-AM solutions

To investigate whether depolarization-induced potentiation of sensorimotor synapses could be triggered in the absence of Ca$^{2+}$ entry, we applied HiK/0Ca/EGTA solution to the pedal
and pleural ganglia and measured monosynaptic EPSPs between tail sensory neurons and tail motor neurons before and 75–180 min after HiK or sham treatment (Fig. 1C). For 30 min prior to and 60 min after treatment, the main chamber was bathed in 0Ca/EGTA solution. EPSPs at these synapses can only be elicited in solutions containing sufficient Ca2+, but in normal Ca2+ concentrations, single action potentials in Aplysia sensory neurons often evoke polysynaptic as well as monosynaptic EPSPs (Walters and Cohen 1997). Therefore we tested the EPSPs in a solution (HiDi) containing elevated concentrations of the divalent cations, Ca2+ and Mg2+ that not only permits synaptic transmission but also greatly reduces the incidence polysynaptic EPSPs evoked by single spikes in the sensory neuron (Liao and Walters 2002). This solution was not washed in until the preparation had been in 0Ca/EGTA solution for 60 min after the 2 min HiK treatment (Fig. 1C), so no residual elevation of K+ would have been present when Ca2+ was reintroduced. Examples of EPSPs before and after HiK or sham treatment are shown in Fig. 3, A and B. In each case, the first EPSP observed during sampling of each sensory neuron was measured. This avoided possible posttetanic potentiation or depression of the synapses by bursts of spikes that were often elicited by impairment of the sensory neuron. As illustrated, membrane potential could change gradually or abruptly during impairment (but was stable afterwards), and the first spike and consequent EPSP were sometimes elicited before the electrode had completely penetrated the cell (Fig. 3A, left). In other cases, the first spike was evoked by a test pulse injected into the soma during the determination of soma spike threshold (Fig. 3B, left).

The 2-min depolarization by HiK/0Ca/EGTA treatment caused a 2.5-fold increase in mean EPSP amplitude 75–180 min following treatment (Fig. 3C), significantly increasing it compared with the effects of sham treatment. Interestingly, both HiK and sham treatments under these conditions tended to decrease input resistance (Rin) in the motor neuron (Fig. 3D). Rin decreased from pre- to posttest in 9 of 11 motor neurons tested in sham-treated preparations (from a mean of 42 to 37 MΩ) and in 8 of 9 motor neurons in HiK-treated preparations (from a mean of 33 to 24 MΩ). These decreases in Rin suggested that the effects on synaptic transmission might be even greater than indicated by the measured EPSP values (which would be reduced by decreased postsynaptic Rin – see DISCUSSION). No significant differences were found between the pretest values of EPSPs in HiK and sham-treated groups, and in sham-treated preparations no significant changes in EPSP values were found between the pre- and posttest. In addition, no significant differences were found in the resting membrane potentials (RMP) of sensory neuron somata (means for preand posttest values, respectively: sham, −38.2 and −40.3 mV, n = 24; HiK, −40.6 and 42.9 mV, n = 19).

To determine whether depolarization-induced potentiation of sensorimotor synapses could be triggered under conditions in which elevation of intracellular free Ca2+ from both extracellular and intracellular sources is minimized, we repeated the study just described but applied 0Ca/EGTA/BAPTA-AM and HiK/...
0Ca/EGTA/BAPTA-AM solutions to the pedal and pleural ganglia. In addition, BAPTA-AM was included in the HiDi solution used during synaptic testing, to provide additional time (60–90 min total) for diffusion across synaptic membranes prior to HiK treatment. Examples of EPSPs before and after HiK or sham treatment are shown in Fig. 4, A and B. Again, the first EPSP observed during sampling of each sensory neuron was measured. As illustrated here, the first EPSPs were often evoked by action potentials generated by impalement of the sensory neuron. No obvious effects of the BAPTA-AM were evident on synaptic transmission, consistent with the minor effect on Ca\(^{2+}\)/H11001 transients observed in sensory neuron axons when BAPTA-AM application was not combined with 0Ca/EGTA (Kunjilwar et al. 2009). Two-minute depolarization again significantly increased EPSP amplitude compared with the effects of sham treatment 60–180 min following treatment with the induction occurring this time in the presence of both intracellular and extracellular Ca\(^{2+}\) chelators (Fig. 4C). Both HiK and sham treatments under these conditions tended to decrease \(R_n\) in the motor neuron (Fig. 4D) with \(R_n\) decreasing from pretest to posttest in 5 of 6 motor neurons in the sham-treated preparations (from a mean of 17 to 11 M\(\Omega\)) and in 4 of 4 motor neurons in the HiK-treated preparations (from a mean of 32 to 12 M\(\Omega\)). In this synaptic potentiation study, we utilized one-tailed, rather than two-tailed, unpaired \(t\)-tests to assess statistical significance because the directions of change were predicted on the basis of the effects of HiK treatment found in the prior study using 0Ca/EGTA solutions (Fig. 3). As described in the following text, the very high mortality of the sensory neurons treated in 0Ca/EGTA/BAPTA-AM and tested in HiDi solution made it impractical to achieve sample sizes large enough for more conservative statistical tests. Sensory neuron somata surviving after prolonged 0Ca/EGTA/BAPTA-AM exposure were often relatively inexcitable and had reduced action potential amplitudes (see Fig. 4, A and B, right). However, no significant differences between HiK and sham groups were found.
in the RMPs of sensory neuron somata (means for pre- and posttest values, respectively: sham, –38.7 and –36.2 mV, n = 13; HiK, –35.6 and 33.8 mV, n = 7). No significant changes in EPSPs were found between the pre- and posttest in sham-treated preparations.

**LTP of sensorimotor synapses that persists for at least a day is induced by depolarization in 0Ca/EGTA solution**

To see if depolarization-induced synaptic potentiation lasting 1 day can be induced in the absence of Ca\(^{2+}\) entry, we tested monosynaptic EPSPs in HiDi solution (pretest), incubated the preparation in 0Ca/EGTA solution for ≥30 min prior to 2 min HiK or sham treatment and for ≥1 h afterward, kept the preparations overnight in ASW, and tested the EPSPs in HiDi solution the next day (posttest). Examples of EPSPs before and 1 day after HiK or sham treatment are shown in Fig. 5, A and B. Elimination of Ca\(^{2+}\) entry at the time of depolarization failed to prevent synaptic LTP assessed 18–24 h later as revealed by significantly greater EPSP amplitudes after HiK treatment than sham treatment (Fig. 5C). In contrast to the decrease in motor neuron \(R_{in}\) observed in the ITP studies, neither the HiK- nor sham-treated preparations showed any significant change in \(R_{in}\) when tested the day after treatment (Fig. 5D). No significant changes in EPSP or \(R_{in}\) values were found between the pre- and posttest in sham-treated preparations. Only a minority of sensory neurons survived into the 1-d posttest; 17% (7 of 42 cells) in the sham-treated group, and 25% (16 of 65) in the HiK-treated group. These survival rates did not differ significantly from each other. Because of the high mortality rates associated both with long-term testing and with synaptic testing following exposure to 0Ca/EGTA/BAPTA-AM (see following text), we did not investigate possible long-term synaptic effects of HiK treatment conducted in 0Ca/EGTA/BAPTA-AM.
Sensory neuron mortality is increased by re-exposure to extracellular Ca\(^{2+}\) following prolonged conditions that reduce Ca\(^{2+}\) signaling.

The prolonged exposure of neurons to very low Ca\(^{2+}\) levels (HiDi solution)—may produce cellular stresses that potentially interact with the depolarizing treatment, which in principle might contribute to differences in EPSP amplitude between HiK- and sham-treated preparations. To begin to address this question, we compared the survival rates of sensory neurons that had been tested and treated under each of our major experimental conditions. Even under optimal conditions, prolonged or repeated intracellular impalement with a sharp microelectrode will kill some of these sensory neurons (unpublished observations). We found that 43% of sensory neurons bathed in 0Ca/EGTA solution for 90 min (between the 1st impalement in the synaptic pretests and the 2nd impalement in posttests, both conducted in HiDi solution) survived through their synaptic posttests, and no significant difference was found between the survival rates of HiK-treated and sham-treated neurons (Fig. 6A, left). In contrast, only 19% of sensory neurons bathed in 0Ca/EGTA/BAPTA-AM (between impalements and synaptic tests conducted in HiDi solution) survived through their posttests, while, again, no significant difference was found between the survival rates of HiK- and sham-treated neurons (Fig. 6A, right). The percentage of sensory neurons surviving through their posttests was significantly lower in the 0Ca/EGTA/BAPTA-AM groups (HiK and sham, combined) than the groups impaled, tested and treated identically without the addition of BAPTA-AM (Fig. 6A). This indicates that prolonged intracellular chelation of Ca\(^{2+}\) increases the death of sensory neurons in these experiments but that the conditioning HiK treatment has no major effect on survival of sensory neurons bathed for 90 min in either 0Ca/EGTA/BAPTA-AM or just 0Ca/EGTA.

In the experiments that examined sensory action potential thresholds in the neuropil (Fig. 2), sensory neurons remained in
0Ca/EGTA or 0Ca/EGTA/BAPTA-AM solution continuously for the duration of the experiment (see Fig. 1B). They were impaled twice in these “Ca2+-free” conditions, once for the two pretests, and again for the two to four posttests, but were never exposed to HiDi solution (or other solutions containing high levels of Ca2+) because synaptic tests were not conducted. Again no significant effect of HiK treatment on survival was found under either condition (Fig. 6B), although the survival rate of HiK-treated sensory neurons in 0Ca/EGTA/BAPTA-AM appeared somewhat lower. Comparing the survival rates of sensory neurons treated in 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM but tested in HiDi (Fig. 6A) versus, respectively, neurons treated and tested in either 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM (Fig. 6B), the sensory neurons impaled and tested in HiDi displayed significantly less survival than those impaled and tested in the “0Ca2+-” solutions (i.e., comparing Fig. 6A, left, to B, left, and A, right, to B, right). These differences indicate that prolonged exposure to 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM by itself causes relatively little cell death in the time frame of these experiments; however, re-exposure to high extracellular Ca2+ levels following prolonged Ca2+ depletion (perhaps in combination with reimplantation of the neuron) is associated with substantial neuronal death in our synaptic experiments.

**DISCUSSION**

**Sensorimotor synapses exhibit Ca2+-independent, depolarization-induced potentiation**

The present results provide strong evidence that conditioning depolarization can trigger long-lasting potentiation of plastic synapses without using Ca2+ as the major induction signal. Synaptic potentiation lasting 1–3 h (IPT) was induced under conditions that prevent Ca2+ influx (0Ca/EGTA solution) and also under the conditions (0Ca/EGTA/BAPTA-AM solution) that we showed, using fura 2-AM imaging, to prevent both Ca2+ influx and detectable Ca2+ transients in the processes and somata of dissociated Aplysia sensory neurons (Kunjilwar et al. 2009). The lengthy exposure to 0Ca/EGTA (>30 min) and to the membrane permeant Ca2+ chelator, BAPTA-AM (60–90 min), prior to HiK treatment in the present study suggests that Ca2+ would have been effectively chelated in extra-and intracellular compartments in the desheathed ganglia preparations utilized, and thus that neuronal Ca2+-transients would have been effectively blocked, as they were in dissociated neurons (Kunjilwar et al. 2009). As discussed in the following text, effects of BAPTA-AM on neuronal survival also indicate that intracellular Ca2+ was effectively chelated in the present experiments, although we cannot rule out spatially or temporally restricted Ca2+-transients that would be below the limits of detection in our fura 2-AM experiments (Kunjilwar et al. 2009).

Depolarization to ~0 mV for ~2 min under these conditions induced long-lasting hyperexcitability (STH and ITH) of sensory neuron processes within the pedal ganglion (Fig. 2) in the region of the potentiated synapses (Wainwright et al. 2002; Zhang et al. 2003). In magnitude and time course, this central hyperexcitability is quite similar to that found after HiK treatment of peripheral sensory axons under the same conditions (Kunjilwar et al. 2009). Hyperexcitability of sensory processes close to their synaptic terminals onto motor neurons suggests that Ca2+-independent, depolarization-induced synaptic potentiation is correlated with some presynaptic alterations. Additional evidence for alterations of the presynaptic cell came from observations of modest hyperexcitability in sensory neuron somata after HiK treatment, but the effects of local and distant depolarization on soma properties, which are complex and of uncertain relevance to the synaptic alterations, will be described elsewhere (R. Crook, F. Reyes, K. Kunjilwar, Q. Yang, E. Walters, unpublished observations). The ITP induced by conditioning depolarization in 0Ca/EGTA and in 0Ca/EGTA/BAPTA-AM cannot be explained by an increase in postsynaptic input resistance, which instead decreased significantly, as monitored in motor neuron somata following HiK treatment and, to a lesser extent, following sham treatment (Figs. 3 and 4). As a crude estimate of the change in synaptic transmission corrected for the observed decreases in postsynaptic R\textsubscript{in}, we used Ohm’s Law and the measured EPSP and R\textsubscript{in} values to calculate an apparent excitatory postsynaptic current (EPSC) (see also Cleary et al. 1998) for each synaptic connection before and after HiK treatment.
HiK treatment in 0Ca/EGTA and in 0Ca/EGTA/BAPTA-AM produced a five- to sevenfold increase in the mean estimated EPSC versus the 2.5-fold increase in the mean measured EPSP for the same connections during ITP. Although the actual increases in synaptic currents are unknown, this simplified calculation indicates that the large increases in EPSP amplitude could substantially underestimate the robust increases in synaptic strength that were produced by 2-min depolarization in the absence of detectable Ca\(^{2+}\) signals.

The sites (or site) responsible for these synaptic alterations remain to be determined. Potential postsynaptic contributions to depolarization-induced ITP/LTP do not include increases in input resistance of the motor neuron (Figs. 3D, 4D, and 5D), or increases in its soma excitability (unpublished observations). However, other postsynaptic alterations, such as increased insertion of glutamate receptors (e.g., Glanzman 2008), might be critical for the observed synaptic potentiation. It will be important to investigate possible roles of the motor neuron, and see if depolarization also fails to evoke detectable Ca\(^{2+}\) transients in motor neuron processes under these conditions. Interestingly, ITP induced in 0Ca/BAPTA-AM at the crayfish neuromuscular junction depends on pre synaptic mechanisms (Wojtowicz and Atwood 1988). Together with suggestive findings from mammalian hippocampal synapses (May et al. 1987) as well as the crustacean neuromuscular junction (Wojtowicz and Atwood 1988), our results indicate that Ca\(^{2+}\)-independent, depolarization-induced ITP mechanisms exist and involve, at least in part, presynaptic alterations. In addition to ITP, we found that depolarization in 0Ca/EGTA produced LTP that lasted \(\geq 1\) day. Addressing important questions about the possible dependence of Ca\(^{2+}\)-independent depolarization-induced ITP and LTP on protein synthesis and gene transcription will be challenging because the combined stresses of Ca\(^{2+}\) depletion, Ca\(^{2+}\) re-exposure, pharmacological inhibition of translation or transcription, and prolonged maintenance of ganglia ex vivo substantially decrease neuronal survival (see also Kunjilwar et al. 2009).

Cell mortality but not synaptic potentiation appears to be increased by re-exposure to Ca\(^{2+}\) after Ca\(^{2+}\) depletion

In a prior study, we were impressed at how well Aplysia sensory neurons tolerate extreme reduction of extra- and intracellular free Ca\(^{2+}\) levels (Kunjilwar et al. 2009), which should disturb Ca\(^{2+}\)-dependent membrane repair and other cellular processes (Fishman and Bittner 2003; McNeil and Terasaki 2001). Except for the long-term (1 day) experiments, which typically have higher mortality rates, these earlier experiments were performed in the continuous presence of 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM. In the present study, experiments on neuropil excitability also showed relatively high rates of sensory neuron survival for \(\geq 60\) min after HiK or sham treatment in the continuous presence of 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM (Fig. 6B). It is, however, impossible to test conventional chemical synaptic transmission without sufficient extracellular Ca\(^{2+}\). Transmission at Aplysia sensorimotor synapses requires far more extracellular Ca\(^{2+}\) than was present in these solutions. For example, a solution containing 1% of normal [Ca\(^{2+}\)], a much higher concentration than in the solutions used here, blocks detectable transmission at Aplysia sensorimotor synapses (Weragoda et al. 2004). Moreover because we needed to largely eliminate polysynaptic components of the tested synaptic connections, we performed our synaptic tests in HiDi solution containing approximately twice the normal extracellular concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) (Liao and Walters 2002). Thus following 90 min in 0Ca/EGTA or 0Ca/EGTA/BAPTA-AM, the neurons were abruptly exposed to high levels of extracellular Ca\(^{2+}\) in the HiDi solution. Similarly, in long-term experiments the neurons were abruptly exposed to high levels of Ca\(^{2+}\) in the ASW used to maintain the ganglia overnight. In all of these experiments, the survival rates of sensory neurons were significantly lower than those observed when sensory neurons were reimplanted for posttests in 0Ca/EGTA or in 0Ca/EGTA/BAPTA-AM solutions that had been continuously present (see Fig. 6). Thus it seems likely that re-exposure to high levels of extracellular Ca\(^{2+}\), perhaps in combination with reimplantation by a microelectrode, severely reduces survival of the sensory neurons. The mechanisms are unknown, but one possibility is that store-operated Ca\(^{2+}\) currents, known to be activated by depletion of intracellular Ca\(^{2+}\) stores in Aplysia neurons (Kachoei et al. 2006), combine with other Ca\(^{2+}\) currents and leakage of Ca\(^{2+}\) at the site of impalement to bring Ca\(^{2+}\) concentrations to toxic levels after high extracellular Ca\(^{2+}\) levels are restored. Our experimental design did not allow us to measure effects of interactions among Ca\(^{2+}\) depletion, Ca\(^{2+}\) re-exposure, and reimplantation on motor neuron survival. However, the relative decreases in motor neuron input resistance found in HiDi solution after depolarization in 0Ca/EGTA and 0Ca/EGTA/BAPTA-AM are consistent with the possibility that these interactions produce considerable cellular stress in motor neurons as well.

Two important implications for Ca\(^{2+}\)-independent, depolarization-induced plasticity come from our survival observations in the sensory neurons. First, these observations provide independent evidence that BAPTA-AM penetrated the sensory neurons, presumably by chelating intracellular Ca\(^{2+}\). This adds, in the ex vivo ganglion preparation, to evidence for the effectiveness of BAPTA-AM treatment found by imaging free Ca\(^{2+}\) in dissociated sensory neurons (Kunjilwar et al. 2009) and strengthens our conclusion that depolarization-induced synaptic potentiation occurs in the absence of effective Ca\(^{2+}\) signaling. Second, we found no statistically significant effects of conditioning HiK treatment on neuronal survival, although in two of the studies shown in Fig. 6 there appeared to be a trend for lower survival in the HiK groups. Under the most severe conditions (synaptic tests in HiDi following 0Ca/EGTA/BAPTA-AM exposure), any Ca\(^{2+}\)-independent depolarization effects on mortality showed no indication of adding to the Ca\(^{2+}\)-dependent mortality in these cells (Fig. 6A, right). Thus 2-min depolarization did not protect against toxic effects of Ca\(^{2+}\) depletion and repletion. This argues against the possibility that better health of the HiK-treated preparations partially accounted for the differential effect on EPSP amplitudes measured after depolarization in the 0Ca/EGTA/BAPTA-AM solution.

Whether the depolarization effects and Ca\(^{2+}\) re-exposure effects are related bears on the general question of whether some of the effects of conditioning depolarization induced under conditions of little or no Ca\(^{2+}\) signaling involve a
subsequent Ca\(^{2+}\)‐dependent step to complete the induction process. Because of the requirement for Ca\(^{2+}\) influx in the tests of conventional synaptic transmission that we conducted, a late Ca\(^{2+}\)‐dependent step cannot be excluded as a contributor to the observed synaptic potentiation. However, other Ca\(^{2+}\)‐independent, depolarization‐induced effects in the sensory neurons—hyperexcitability of peripheral axons and central processes—are expressed in the absence of re‐exposure to Ca\(^{2+}\). Furthermore, by itself re‐exposure to Ca\(^{2+}\) after Ca\(^{2+}\) depletion induced little or no potentiation of sensorimotor synapses, as shown by the lack of significant EPSP potentiation compared with pretest values in sham‐treated preparations (Figs. 3 and 4). Thus although Ca\(^{2+}\) influx through store‐operated channels has been suggested to contribute to LTP induction in mammalian hippocampus (Baba et al. 2003; Mellentin et al. 2007), mechanisms activated by Ca\(^{2+}\) depletion and re‐exposure cause relatively little potentiation of Aplysia sensorimotor synapses under our experimental conditions. Also unlikely as a major induction signal is delayed Ca\(^{2+}\) influx via exchange for Na\(^+\) that might have accumulated during intense depolarization (e.g., Misler and Hurlbut 1983) because the HiK solutions we used for conditioning depolarization had virtually all Na\(^+\) replaced by K\(^+\).

Most important, none of these potential Ca\(^{2+}\)‐dependent induction mechanisms could have operated until Ca\(^{2+}\) signaling was restored 60 min after depolarizing treatment. Therefore mechanisms must exist in or near these synapses that can sense intense, 2‐min depolarization in the probable absence of Ca\(^{2+}\) signaling and that induce an influential trace of this depolarization in the sensory neurons and their synapses—a trace that in our experiments persisted for ≥60 min. This Ca\(^{2+}\)‐independent depolarization signal by itself induces hyperexcitability of the sensory neuron and by itself or in series with subsequent Ca\(^{2+}\) signals is sufficient to trigger long‐lasting synaptic potentiation. We do not know whether the Ca\(^{2+}\)‐independent sensor(s) of depolarization is located in the sensory neuron, motor neuron, or in other cells that release signals that alter the sensory neuron and sensorimotor synapse. If the depolarization sensor is extrinsic to the sensory neuron, during the phases of our experiments that Ca\(^{2+}\) is effectively absent, this voltage sensor would have to communicate with the sensory neuron without using Ca\(^{2+}\) signaling, perhaps by voltage‐dependent, Ca\(^{2+}\)‐independent exocytosis of a neuromodulator (e.g., Bernath 1992; Zhang and Zhou 2002).

Possible significance of Ca\(^{2+}\)‐independent, depolarization‐induced synaptic potentiation

Nearly all investigations into the induction of activity‐ or depolarization‐dependent synaptic plasticity have focused on Ca\(^{2+}\) as a necessary and sufficient molecular trigger (e.g., Abrams et al. 1991; Malenka 1991; Rao and Finkbeiner 2007; Xu and Kang 2005; Zucker 1999). Few investigators have considered the possibility that Ca\(^{2+}\)‐independent triggers may be activated by depolarization to act in parallel with Ca\(^{2+}\) signals to induce some forms of synaptic potentiation. Interestingly, the three studies that have supported this possibility thus far examined synapses in three different phyla: Chordata (May et al. 1987), Arthropoda (Wojtowicz and Atwood 1988), and Mollusca (this study), suggesting that Ca\(^{2+}\)‐independent, depolarization‐induced synaptic potentiation is widespread. There is growing evidence for diverse mechanisms that can couple depolarization to cellular signaling pathways in a Ca\(^{2+}\)‐independent manner. These include nonconducting functions of voltage‐gated channels (Kaczmarek 2006), such as the depolarization‐dependent, conduction‐independent activation of a p38 MAP kinase by a voltage‐gated K\(^+\) channel (Hegele et al. 2006). Voltage sensitivity has been implicated in various membrane‐associated proteins, including a G‐protein‐coupled receptor (Ben‐Chaim et al. 2006), a phosphatidylinositol phosphatase (Iwasaki et al. 2008), and a proton permeation protein (Okamura 2007). Moreover, depolarization‐dependent, Ca\(^{2+}\)‐independent transcriptional regulation of a hypoxia‐inducible factor occurs in some cancer cells (Lan et al. 2007). Thus there may exist numerous Ca\(^{2+}\)‐independent transducers of depolarization, some of which might contribute to depolarization‐induced synaptic potentiation. An interesting question is where (or whether) Ca\(^{2+}\)‐independent signals of depolarization converge with Ca\(^{2+}\)‐dependent pathways to engage mechanisms of synaptic potentiation. Given the similarities between our results and those of Wojtowicz and Atwood (1988), it is interesting that intermediate‐ and long‐term synaptic potentiation/facilitation at both the crayfish neuromuscular junction (Dixon and Atwood 1989) and Aplysia sensorimotor synapses (e.g., Bergold et al. 1992; Schacher et al. 1988) involve activation of a cAMP‐PKA pathway. This pathway and others are potential sites of convergence of Ca\(^{2+}\)‐independent and Ca\(^{2+}\)‐dependent signals.

Another unanswered question is whether Ca\(^{2+}\)‐independent, depolarization‐induced synaptic potentiation might have functions distinct from the well‐known Ca\(^{2+}\)‐dependent forms of potentiation. An initial clue may come from the patterns of conditioning depolarization used thus far to induce this potentiation; we used 2‐min continuous depolarization to ~0 mV caused by HiK treatment; May et al. (1987) used 3–4 min HiK treatment (40–80 mM) that probably depolarized the cells into a range between ~30 and ~10 mV, and Wojtowicz and Atwood (1988) used a 10 min train of high‐frequency (20 Hz), intracellular pulses of 3‐ to 5‐ms duration that in TTX depolarized the motor neuron terminal sufficiently to evoke substantial transmitter release. Thus each induction protocol involved intense depolarization of presynaptic terminals that was much more prolonged than the transient depolarizations produced in experimental models of learning and memory, such as LTP (e.g., Glanzman 2008; Malenka 1991). Conditions of intense and prolonged local depolarization may occur in a variety of normal as well as clinically important contexts. In nociceptive Aplysia sensory neurons, intense depolarization occurs during strong pinching stimulation of their receptive fields with each pinch inducing high‐frequency activation and afterdischarge that can last many seconds (Clatworthy and Walters 1993; Illich and Walters 1997; Walters et al. 1983, 2004). In addition to both peripheral depolarization caused directly by membrane damage and central depolarization occurring during lasting trains of action potentials, resting depolarization of the sensory neuron soma lasting many minutes follows bursts of high‐frequency action potentials (Walters and Byrne 1983b, 1985). Although the amplitude observed in the soma of this sustained postburst depolarization is modest (~10 mV), it might be larger in the presynaptic terminals. More generally, large sustained depolarizations in synaptic regions might occur in the mammalian brain during some
forms of learning (Destexhe et al. 2003) and are likely during certain pathological states, including spinal cord injury (Park et al. 2004), traumatic brain injury (e.g., Shaw 2002), and epileptic seizures (e.g., de Curtis and Avanzini 2001). The potential involvement of Ca\(^{2+}\)-independent, depolarization-induced synaptic potentiation in these normal and abnormal neural states encourages a search for its underlying mechanisms, which today remain largely unknown.

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