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# Postsynaptic Calcium, but Not Cumulative Depolarization, Is Necessary for the Induction of Associative Plasticity in *Hermisenda*

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The neuronal modifications that underlie associative memory in *Hermisenda* have their origins in a synaptic interaction between the visual and vestibular systems, and can be mimicked by contiguous *in vitro* stimulation of these converging pathways. At the offset of vestibular stimulation (i.e., hair cell activity), the B photoreceptors are briefly released from synaptic inhibition resulting in a slight depolarization (2–4 mV). If contiguous pairings of light-induced depolarization and presynaptic vestibular activity occur in close temporal succession, this depolarization “accumulates” and has been hypothesized to culminate in a sustained rise in intracellular  $\text{Ca}^{2+}$  and a resultant  $\text{Ca}^{2+}$ -mediated phosphorylation of  $\text{K}^+$  channels as well as an associated increase in input resistance. Here we demonstrate that this cumulative depolarization is neither necessary nor sufficient for the biophysical modifications of the B cell membrane indicative of memory formation. Consistent with several recent reports of one-trial learning in *Hermisenda*, one pairing of light with mechanical stimulation of the vestibular hair cells resulted in a rise in neuronal input resistance across the B cell membrane that was attenuated by a prepairing iontophoretic injection of the  $\text{Ca}^{2+}$  chelator EGTA (25 mM), indicating that this potentiation was  $\text{Ca}^{2+}$  dependent. However, the use of a single pairing negates the possibility of an accumulation of depolarization across trials. In a subsequent experiment, B photoreceptors underwent a cumulative depolarization, and a coincident rise in input resistance, during multiple pairings of light and hair cell stimulation. However, if the B photoreceptor was voltage clamped at its initial resting potential before and after each pairing, thus eliminating the cumulative depolarization, the rise in resistance not only persisted, but was enhanced. Moreover, if unpaired light presentations were followed by a current-induced depolarization (to mimic cumulative depolarization), no increase in input resistance was detected. To assess directly the effect of a cumulative depolarization on the voltage-dependent  $\text{Ca}^{2+}$  current, an analysis of the inward current on the B cell soma membrane was conducted. It was determined that (1) the inward current may undergo a partial inactivation during sus-

tained depolarization, (2) the peak current was depressed during repetitive depolarizations, and (3) the peak current underwent a steady-state inactivation, such that it was reduced when elicited from holding potentials more positive than  $-60$  mV. The analysis of this current suggests that pairings of light and presynaptic activity would reduce voltage-dependent  $\text{Ca}^{2+}$  influx when those pairings are conducted at depolarized membrane potentials, such as during cumulative depolarization. In total, these results indicate that while the cumulative depolarization is a reliable correlate of *in vitro* conditioning, it does not contribute to the acquisition process per se, while contiguous pre- and postsynaptic activity, and the associated rise in postsynaptic  $\text{Ca}^{2+}$ , is critical to this process. These results suggest certain similarities to other activity-dependent models of learning-induced plasticity.

**[Key words: calcium currents, potassium currents, barium currents, associative learning, memory, long-term potentiation, *Hermisenda*]**

During associative learning in which light is paired with rotation, the B photoreceptors in the *Hermisenda*'s eye undergo an increase in excitability that is mediated by a reduction of neuronal  $\text{K}^+$  currents (Alkon et al., 1985, 1988; Farley and Auerbach, 1986). This increase in neuronal excitability is believed to contribute to a subsequent reduction of light-elicited phototactic behavior (Farley et al., 1983) as well as light-induced contraction of the animal's locomotor organ, the foot (Matzel et al., 1990b, 1992). These simple conditioned responses are indicative of memory induction and storage.

While memory-specific reductions of B cell  $\text{K}^+$  currents (Alkon et al., 1985; Crow, 1988; Matzel et al., 1990a) and  $\text{K}^+$  channel open time (Etchiberrigary et al., 1992) have been documented in a number of contexts, the nature of the synaptic events that lead to the induction of these biophysical correlates of memory are, nevertheless, unclear. During a typical conditioning trial, dark-adapted *Hermisenda* are exposed to a brief presentation of temporally contiguous light and rotation. The synaptic convergence of the two sensory events has been proposed to contribute to a rise in intracellular  $\text{Ca}^{2+}$  in the B cell that leads to a  $\text{Ca}^{2+}$ -mediated phosphorylation of  $\text{K}^+$  channels. At the offset of rotation-induced vestibular stimulation (i.e., hair cell activity), the hair cells undergo a transient period of hyperpolarization, releasing the B photoreceptors from the basal level of hair cell-induced inhibition that is typically exerted. In the absence of light, this release from inhibition results in a slight depolarization (2–4 mV) of the B cell (see Fig. 1). In contrast, during the presentation of light the B photoreceptors

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undergo a steady-state depolarization (i.e., generator potential) that is followed at offset by a slow repolarization, referred to as a long-lasting depolarization (LLD). During a typical conditioning trial, light and rotation coterminate, resulting in an enhancement of the LLD stemming from the sum of the slow repolarization and the simultaneous release from synaptic inhibition. If contiguous pairings of visual-vestibular activity occur in close temporal succession, this depolarization “accumulates” and has been hypothesized to culminate in a sustained rise in intracellular calcium and a resultant calcium-mediated phosphorylation of  $K^+$  channels (Alkon, 1980, 1984; Farley and Alkon, 1987).

Although a cumulative depolarization in the B photoreceptors is reliably observed during a training session, it is nevertheless unclear whether any elevation of  $Ca^{2+}$  that occurs during this depolarization is sufficient to account for the observed potentiation of neuronal excitability that occurs during memory formation. Two reports (Crow and Forrester, 1991; Matzel and Alkon, 1992) have suggested that a single discrete application of neurotransmitter paired with a brief period of  $Ca^{2+}$  elevation in the B cell may be sufficient to induce biophysical correlates of learning without any apparent sustained depolarization. Moreover, the modest depolarization that does occur after 5–10 pairings (typically asymptotic at 5–15 mV from a normal resting potential of –50 to –60 mV) is insufficient to activate voltage-dependent  $Ca^{2+}$  currents that are marginally activated at –40 mV (see Fig. 6 and Alkon et al., 1984). Thus, although an increase in intracellular  $Ca^{2+}$  is believed to contribute to the induction process (Alkon et al., 1988; Matzel and Alkon, 1992), it is less clear that an accumulation of depolarization across successive training trials critically contributes to this rise, and if so, whether such an accumulation is required for the observed potentiation. Given that the nature of the induction mechanism is critical to any formulation of ubiquitous concepts in the cellular analysis of learning (cf. Carew and Sahley, 1986), and since potential alternative mechanisms make differential predictions about psychological process underlying learning, we examined the role of cumulative depolarization and its putative contribution to *in vitro* correlates of conditioning in *Hermisenda*. These experiments took three forms. First, we examined the capacity of one *in vitro* pairing of light and vestibular stimulation to induce a rise in resistance in the B photoreceptor. Since a single pairing would exclude any accumulation of depolarization between trials, a rise in resistance would suggest that such an accumulation was not a prerequisite for this *in vitro* correlate of learning. In a second experiment, multiple pairings were administered such that a cumulative depolarization was observed in one group of cells, but blocked via application of a holding current in a second group. A rise in resistance in this latter group would indicate that cumulative depolarization was not necessary for the induction of potentiation. An additional group in this experiment received light-alone presentations that were followed by positive current injection, thus mimicking a cumulative depolarization, in order to determine if such a treatment was sufficient to induce these correlates of learning. Finally, we examined the activation, steady-state inactivation, and depression of a putative  $Ca^{2+}$  current in the B photoreceptor to determine if in fact this current could be enhanced when elicited from holding potentials more positive than the normal resting potential, a central assumption of the cumulative depolarization hypothesis. Although this latter approach does not take into account other potential sources of a sustained rise in  $Ca^{2+}$  that

might contribute to plasticity in this system (see Discussion), the activation of voltage-dependent currents by a progressive depolarization is one of the central tenets of the cumulative depolarization hypothesis (e.g., Farley and Alkon, 1985, 1987).

## Materials and Methods

**Animals.** *Hermisenda crassicornis* (0.7–1.2 gm) were obtained from Sea Life Supply Co., Sand City CA, and were confined in 12°C artificial sea water (ASW). Animals were maintained on a 12 hr:12 hr light/dark cycle, and were fed a portion of Hikari Gold fishfood 1 hr prior to the dark cycle twice weekly. The dim maintenance light was filtered through yellow acetate and had an intensity at the surface of the water of  $20 \mu W \times cm^{-2}$ . Experimental manipulations were conducted during the middle 8 hr of the light cycle.

**Electrophysiology.** The nervous system of *Hermisenda* was surgically isolated and prepared for electrophysiological analysis as described previously (Alkon et al., 1985; Matzel et al., 1990a). Throughout a recording session, the nervous system was maintained in continually perfused artificial sea water [ASW: NaCl (430 mM),  $CaCl_2$  (10 mM),  $MgCl_2$  (50 mM), KCl (10 mM), buffered with Tris to a pH of 7.4] unless otherwise noted. For current-clamp recordings, the recording electrodes were pulled to a tip resistance of 20–30 M $\Omega$  in ASW when filled with 3.0 M K-acetate (KAc), and were connected by a chloridized silver wire to a high-impedance amplifier (Axoclamp 2A). Voltage responses were obtained on a storage oscilloscope and on a Brush Pen Recorder. To measure input resistance, 500 msec current pulses (–0.2, +0.2, +0.4, +0.6, +0.8 nA) were applied through the recording electrode using a balanced-bridge circuit. The voltage response was measured 300–400 msec after the onset of the current. Only cells that exhibited normal charging curves were accepted for analysis. An unfiltered white light ( $600 \mu W \times cm^{-2}$ ), focused through a fiber optic bundle onto the nervous system, was used to induce a light response in the B photoreceptor.

Iontophoretic injection of the  $Ca^{2+}$  chelator ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA; Sigma E-4378) was accomplished by filling the recording electrode with 25 mM EGTA (dissolved in KAc) and passing –0.4 nA, 500 msec current pulses at 1 sec intervals for 3 min.

Stimulation of the hair cells within the statocyst was accomplished by contacting the statocyst with a polished glass probe with a concave tip approximately 50  $\mu m$  in diameter. This probe extended from a piezoelectric crystal (Archer 273-073), attached to the output of an audio oscillator (Hewlett-Packard 200 AB), which when activated operated at 1600 Hz. Stimulation of the statocyst resulted in a circular movement of the statocyst within the cyst, estimated at several hundred rpm.

The preparation of the nervous system and recording electrodes differed for voltage-clamp analysis. Following removal of the nervous system, B photoreceptors were surgically axotomized via a razor cut of the cell's axon approximately 60  $\mu m$  from the soma, thus isolating the cell from action potentials and localized  $Na^+$  currents. A medial B cell was impaled with a single glass electrode pulled to a tip resistance of 8–10 M $\Omega$  (3.0 M KCl fill) measured in ASW. Capacitance was minimized by maintaining a minimal depth of the extracellular bath (<100  $\mu m$ ) and coating the electrode to within 50  $\mu m$  of the tip with Sylgard as necessary. Capacitance was further reduced by isolating the recording stage from ground. The recording electrode was connected to an amplifier (Axoclamp 2A) that was operated in the discontinuous single-electrode voltage-clamp mode. Typical sample rates from 10 to 15 kHz were obtained with this configuration. Currents were elicited with 600–1600 msec command steps (as indicated), and current magnitudes were measured at the midpoint of each command step. Leak current was assessed with equal-amplitude, opposite-polarity current steps. These leak values did not differ from extrapolated leak values obtained with small (–10 mV) voltage commands. As assessed on a storage oscilloscope, capacity transients in the current records settled in <15 msec and voltage steps occurred with a rise time (95% of peak) of <0.5 msec. Only cells with an initial resting potential of at least –35 mV were accepted for analysis, and holding currents for holding potentials of –60 mV were <–3.0 nA.

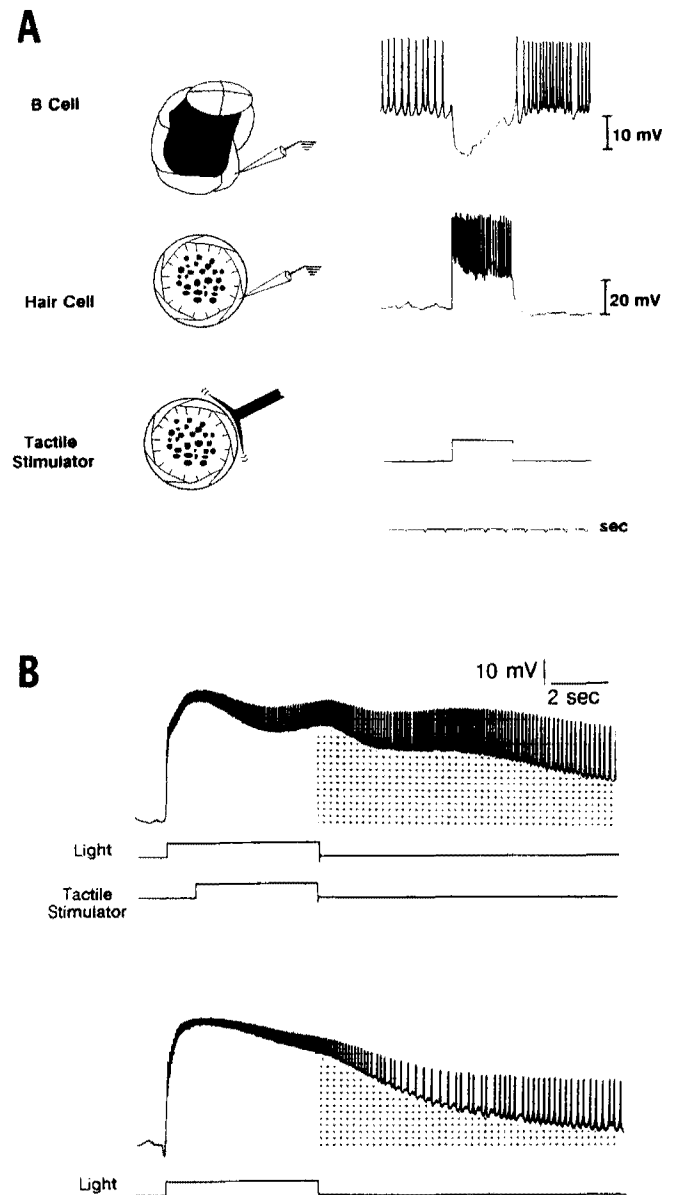
Membrane currents were first assessed in normal ASW, and characteristic transient and sustained outward currents were confirmed. An inward  $Ca^{2+}$  current was isolated as in previous reports (Alkon et al., 1984; Farley and Auerbach, 1986; Collin et al., 1988; Farley and Wu, 1989), using two different methods. By the first method, the properties of the inward current in the absence of any measurable outward currents

were determined by the addition of  $K^+$  channel blockers 4-aminopyridine (4-AP; 5–10 mM) and tetraethylammonium chloride (TEA; 100 mM) to the extracellular bath (osmolality was maintained by the reduction of NaCl to 320 mM). To block  $Ca^{2+}$ -dependent processes (e.g.,  $Ca^{2+}$ -dependent  $K^+$  currents), and to enhance the magnitude of the inward current (e.g., Tsien et al., 1988), external  $Ca^{2+}$  was replaced with  $Ba^{2+}$  (10–100 mM).  $Ba^{2+}$  has previously been shown to be carried selectively by  $Ca^{2+}$  channels in many cell types, including *Hermisenda* photoreceptors. In addition to this method, which would exclude the potential for any  $Ca^{2+}$ -dependent inactivation of the inward current, a second method was used to measure the inward  $Ca^{2+}$  current. With normal extracellular  $Ca^{2+}$ , and in the presence of TEA and 4-AP, external  $K^+$  was raised to 300 mM (with a corresponding reduction of external NaCl to 30 mM). Under these conditions, the reversal potential for  $K^+$  is shifted to 0 mV, thus allowing the measurement of  $I_{Ca}$  in the absence of any contamination by outward currents. Using this method it is also possible to measure a reversed  $Ca^{2+}$ -dependent  $K^+$  current ( $I_{K-Ca}$ ) expressed at the offset of depolarizing command steps (i.e., a tail current), thus providing a second index of  $Ca^{2+}$  activity. Previous results have demonstrated that the inward current carried under each of these conditions is characteristic of a  $Ca^{2+}$  current, including reduction or elimination by the  $Ca^{2+}$ -channel blockers  $Cd^{2+}$  and  $Co^{2+}$ , elimination of the current by removal of  $Ca^{2+}$  (or  $Ba^{2+}$ ) from the extracellular bath, and a null potential greater than +50 mV (Alkon et al., 1984; Farley and Auerbach, 1986; Collin et al., 1988; Farley and Wu, 1989). Some similar properties have been determined directly by the use of the  $Ca^{2+}$ -sensitive indicator Arsenazo III (Connor and Alkon, 1984).

**In vitro conditioning.** Following impalement of a medial B cell and placement of the vibrotactile probe on the statocyst, a synaptic interaction was confirmed. A minimum hyperpolarization in the B cell of 5 mV induced by statocyst stimulation was required in order to proceed with the experiment, and typical hyperpolarizations of 6–10 mV were observed. In experiment 1, following 6 min of darkness, each preparation received a 3 min iontophoretic injection of either 25 mM EGTA suspended in KAc, or KAc alone. One minute after the end of the injection period (10 min of darkness), the resistance of the B cell membrane was assessed by passage of current through the recording electrode. Each preparation then received a single trial of either a paired or unpaired presentation of 5 sec of light that either overlapped and coterminated with (paired), or was presented 30 sec after (unpaired), 4 sec of vibrotactile stimulation of the statocyst. One minute and 4 min after this treatment, the resistance of the B photoreceptor was estimated by passage of small positive and negative current pulses through the microelectrode. Thus, this experiment contained four groups: paired or unpaired, with or without EGTA.

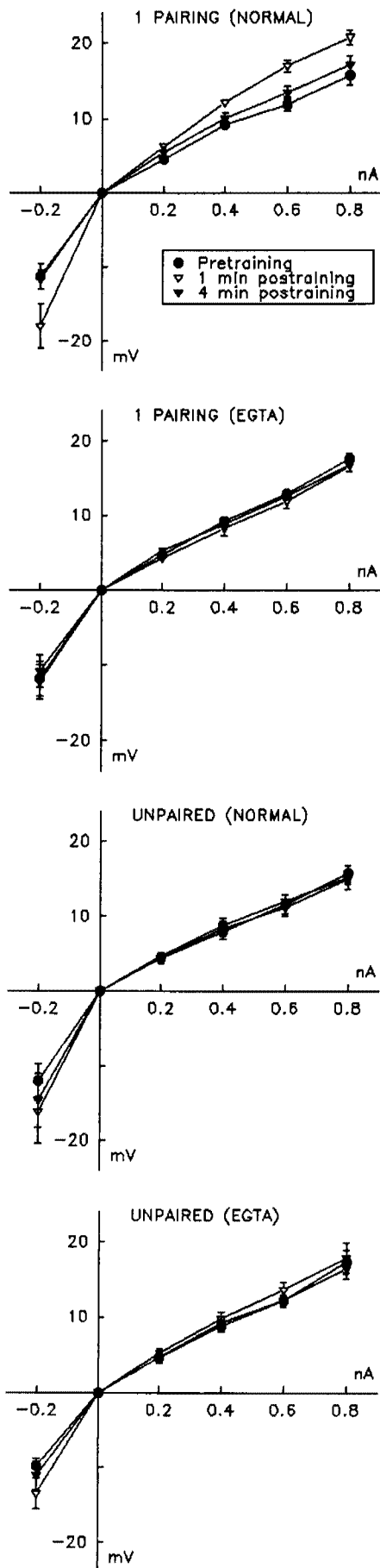
In experiment 2, nervous systems underwent *in vitro* conditioning as described above, but nine training trials were administered and no iontophoretic injections were employed. Also, the B photoreceptors were held, via current injection through the recording electrode, at an initial membrane potential of  $-60$  mV. All nervous systems then received one of four treatments. Two groups received nine pairings of the 5 sec light and 4 sec of vibrotactile stimulation of the statocyst in either a paired or unpaired manner. Paired trials were separated by 60 sec, and unpaired presentations were identical except for the addition of a 30 sec interstimulus interval. B photoreceptors from paired preparations underwent a normal cumulative depolarization (from the initial holding potential of  $-60$  mV) with successive trials. A third group received nine pairings, but the B cell was manually voltage clamped at the initial resting potential of  $-60$  mV before and after each of the nine pairings, thus permitting a transient depolarization during the pairing, but eliminating any potential activation of voltage-dependent  $Ca^{2+}$  currents via an accumulation of depolarization across trials. A final group received only light presentations, but sufficient current was injected at the offset of each light step to induce an “artificial” cumulative depolarization that paralleled that observed following normal pairings, but that did not require presynaptic activity. Thirty seconds following the end of the final training event, sufficient current was injected in each cell to return it to its initial potential of  $-60$  mV (if the cell had been permitted to depolarize during training). Before, 1 min, and 10 min after training, the resistance of the B cell was assessed, and 5 sec prior to each of these measurements, the B cell was momentarily released from any applied current in order to record the cell’s normal resting potential.

**Statistical analysis.** The voltage response of the B cell to  $+0.8$  nA current injections was used as the index of input resistance so that each subject contributed only one score to the analysis. This point was chosen



**Figure 1.** *A*, Stimulation of the vestibular organ, the statocyst, with a probe attached to a piezoelectric crystal, results in a rotation of the statoconia in the interior of the statocyst when current is applied to the piezoelectric crystal (*bottom*). During this mechanical stimulation a glass microelectrode recorded activity in a caudal hair cell and a medial B photoreceptor. Stimulation resulted in a  $\approx 20$  mV depolarization in the hair cell that was accompanied by a burst of action potentials, and a poststimulation hyperpolarization (*middle*). During the burst of activity in the hair cell, a fast,  $\approx 10$  mV hyperpolarization was observed in the B cell that was followed at offset by a brief period of depolarization ( $\approx 2$  mV) and an increase in impulse activity. *B*, Typical voltage responses of a medial B photoreceptor to light. The 5 sec light presentations coterminated with (*top trace*) or without (*bottom trace*) 4 sec of overlapping mechanical stimulation of the statocyst. Such a pairing of light and hair cell activity is analogous to *in vivo* conditioning of *Hermisenda* in which light is paired with rotation. Note the reduction in the light-induced generator potential, but an increase in postlight depolarization if light is paired with hair cell activity.

based on prior published data where pairing effects were most apparent in the positive region of the  $I-V$  relationship. Data from all groups were subject to two-factor analysis of variance (ANOVA), where factor 1 was the treatment condition and factor 2 was the time of the observation (i.e., before or after treatment). Planned comparisons of individual means



were conducted using Dunn's modification of the *t* statistic, which corrects for the possible error arising when making multiple comparisons.

## Results

### Hair cell–B cell interactions

Figure 1*A* illustrates a typical interaction between the vestibular hair cells and a B photoreceptor in the *Hermisenda* eye. Simultaneous intracellular recordings from a B photoreceptor and a caudal hair cell were made during vibrotactile stimulation of the statocyst. A depolarization and a burst of action potentials in the hair cell results in a synaptically mediated hyperpolarization and cessation of firing in the ipsilateral photoreceptors. Following the burst of activity in the hair cell, the hair cell hyperpolarizes and a slight depolarization can be observed in the photoreceptor (note spike rate increase). When paired with light, this afterdepolarization is enhanced, presumably as a consequence of the summation of this release from inhibition and the light-induced LLD, as depicted in Figure 1*B*.

### Effect of one in vitro conditioning trial on resistance, with and without EGTA

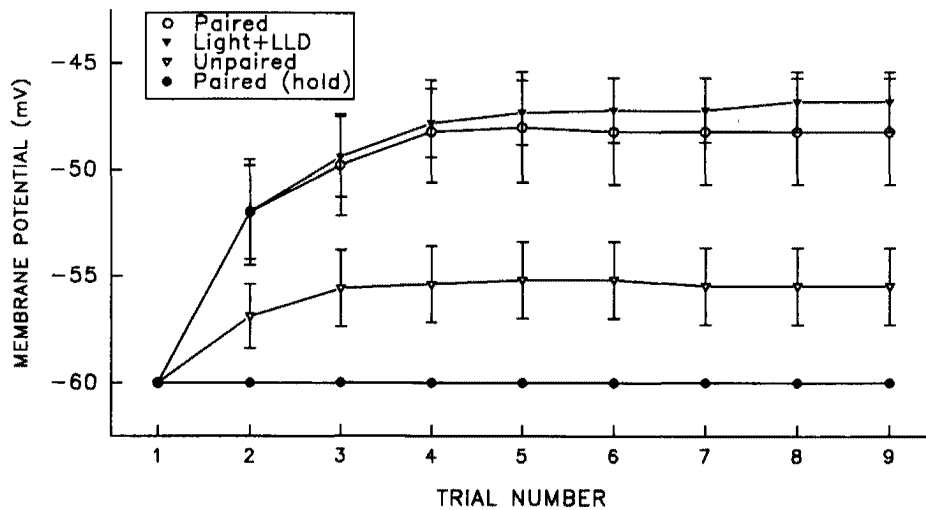
Figure 2 illustrates the resistance of the B cell membrane for all groups at each of the three test intervals. Prior to conditioning, the mean resistance of the paired and unpaired groups did not differ. The pairing of light and hair cell stimulation ( $n = 6$ ) resulted in an initial rise in resistance relative to the unpaired regimen ( $n = 6$ ) 1 min after training, and this initial rise dissipated during the 4 min subsequent to the pairing. As is evident from Figure 2, this transient rise in resistance was blocked by the prepairing injection of EGTA ( $n = 6$ ), such that paired cells preinjected with EGTA did not differ from their unpaired controls ( $n = 5$ ) at any time. These observations were confirmed by ANOVA, in which a significant effect of time of the resistance measurement was found [ $F(2, 36) = 5.02, p < 0.02$ ], as was an interaction of treatment condition with time [ $F(6, 36) = 5.25, p < 0.01$ ].

Comparisons of individual means were performed using Dunn's correction for multiple comparisons. One pairing of light and hair cell stimulation produced an increase in resistance 1 min after training relative to before training [ $t(36) = 6.18, p < 0.001$ ], with a return to baseline within 4 min ( $t = 1.8$ ). No significant differences between any of the remaining pre- and postmeasures were detected ( $t$  values  $< 1.11$ ).

Consistent with indirect evidence from earlier reports, this experiment indicates that a light-induced  $\text{Ca}^{2+}$  rise in the B cell, coincident with presynaptic activity, is critical to the induction of the neuronal potentiation observed during this *in vitro* analog

←

**Figure 2.** Following 10 min of darkness, two groups of nervous systems were exposed to one paired presentation ( $n$  values = 6) of light and vestibular stimulation, and two groups received the light and vestibular stimulation unpaired ( $n$  values = 5). Prior to this treatment, one group of paired and one group of unpaired nervous systems received a 3 min iontophoretic injection into the B cell of the  $\text{Ca}^{2+}$  chelator EGTA, while the remaining groups received a comparable injection of the vehicle (KAc). Current–voltage relationships before (30 sec), 1 min, and 4 min after training are depicted. Relative to before training, a rise in resistance was observed in the paired group not pretreated with EGTA, while no change in resistance was seen in any other group. Even in the paired, no-EGTA condition, the rise in resistance was transient, returning almost to baseline levels within 4 min. No differences in resting membrane potential were observed at any of the test intervals, with initial mean values between  $-51$  and  $-53$  mV across groups.



**Figure 3.** The mean membrane potential of each of the four treatment conditions during the conditioning session in experiment 2 (six or seven observations per group). The B cells in all groups were initially held at  $-60$  mV. Two groups (Groups Paired and Paired-Hold) received nine training trials as in the previous experiment. Trials were separated by 60 sec. Group Paired was allowed to depolarize normally at the offset of each trial and underwent a cumulative depolarization across trials, while Group Paired (hold) was manually voltage clamped at  $-60$  mV at all times except during the training trial, thus eliminating any posttrial depolarization (LLD) and an accumulation of depolarization across trials. Group Unpaired received nine unpaired presentations of light and rotation with a 30 sec interstimulus interval. Group Light+LLD received light presentations alone, but sufficient depolarizing current was applied at the offset of each light presentation such that the cell underwent a cumulative depolarization that mimicked that observed in a corresponding Paired animal. The membrane potential was recorded 5 sec prior to each light presentation.

of conditioning. However, since this experiment only involved a single pairing of visual-vestibular stimulation, it suggests that the observed potentiation does not depend on an accumulation of depolarization and/or  $\text{Ca}^{2+}$  across trials.

#### Effect of blocking cumulative depolarization on multitrial acquisition

While an accumulation of depolarization across trials was not necessary to induce an increase in input resistance correlated with learning, it is conceivable that it facilitates multitrial acquisition and/or retention. Unpublished work in our laboratory has indicated that while one pairing induces a rise in resistance which persists for only several minutes, the potentiation following nine trials persists at least 90 min, and the conditioned response established with 50 trials (*in vivo*) persists at least 24 hr (Lederhendler and Alkon, 1989; Matzel et al., 1990b). Here we set out to determine if the cumulative depolarization that results from multiple pairings augments the induction, and/or facilitates the maintenance of the memory. In addition, we asked whether a sustained depolarization, in the absence of presynaptic activity, was sufficient to induce the potentiation correlated with memory formation.

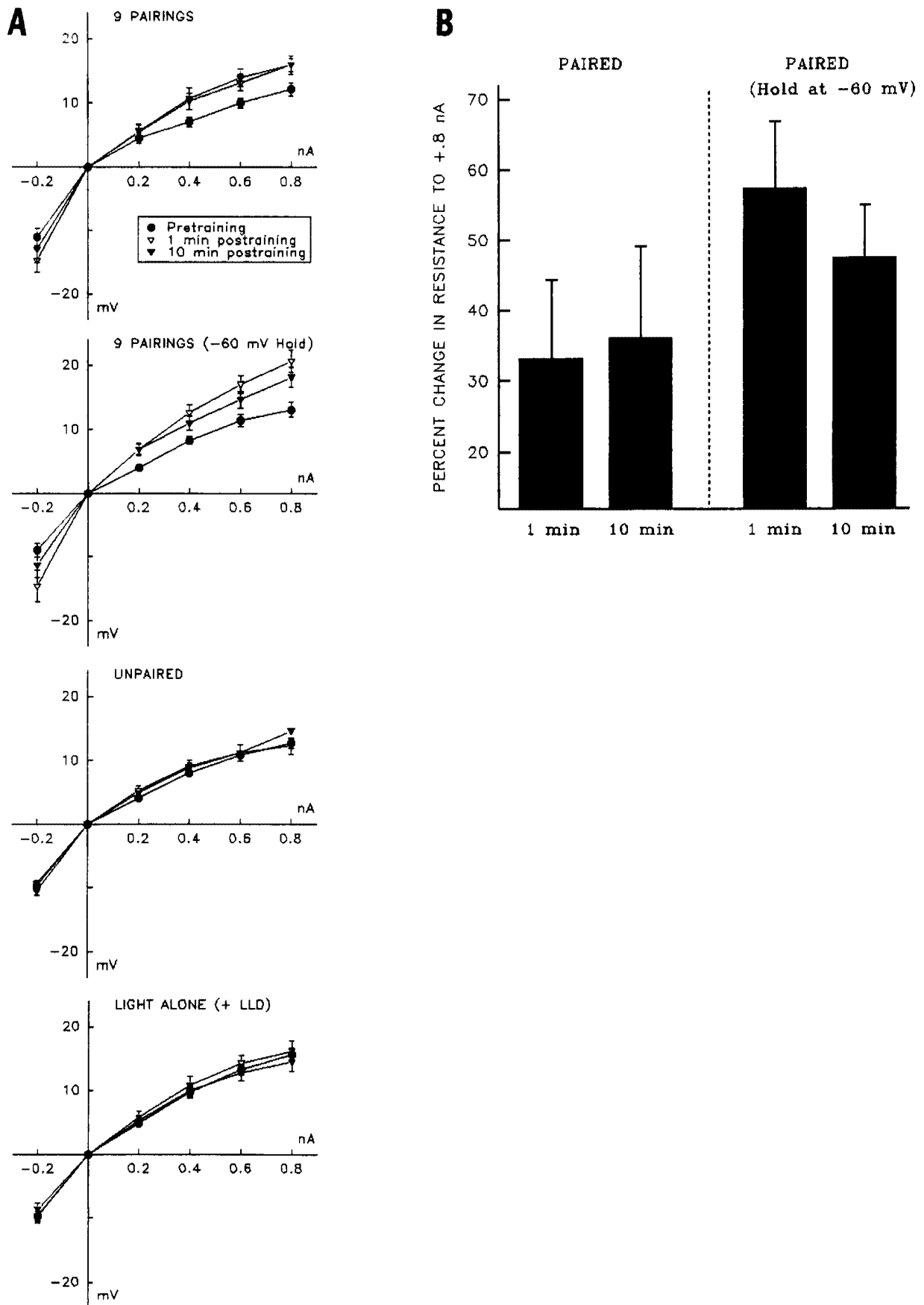
Figure 3 illustrates the average membrane potential of each of the groups in this experiment 5 sec prior to each of the nine light presentations. All groups were initially held, via current injection, at  $-60$  mV at the start of the conditioning session. Two groups, Groups Paired ( $n = 6$ ) and Unpaired ( $n = 6$ ), were allowed to depolarize from this holding potential throughout the session. Group Paired-Hold ( $n = 7$ ) was held at  $-60$  mV throughout training except during the actual pairing of light and vestibular stimulation, when the cells were allowed to depolarize normally. Group Light+LLD ( $n = 6$ ) received light presentations without presynaptic vestibular activity, but at the offset of the light, sufficient current was injected such that the cells in this group underwent a "cumulative depolarization" like that

seen in corresponding paired cells. As is evident in Figure 3, Group Paired underwent a progressive depolarization throughout training, which was mimicked in Group Light+LLD. A relatively minor depolarization was observed in Group Unpaired, and no depolarization was permitted in Group Paired-Hold. Based on a model of plasticity that relies solely on cumulative depolarization, these latter two groups should exhibit no increase in membrane resistance while the former two should.

Figure 4A plots the average current-voltage relationship of the four groups in this experiment. Following nine pairings of visual-vestibular stimulation, a rise in B cell resistance was observed in both Group Paired and Group Paired-Hold that persisted for at least 10 min. No rise in resistance was observed in Group Unpaired or in Group Light+LLD. ANOVA indicated a significant effect of time of observation (i.e., before or after treatment) [ $F(2, 42) = 19.88, p < 0.001$ ], as well as a treatment  $\times$  time interaction [ $F(6, 42) = 7.53, p < 0.001$ ].

Comparison of individual means indicated that the resistance of Group Paired increased following 9 pairings of light and hair cell stimulation [ $t(42) = 4.02, p < 0.01$ ], and that this increase persisted for at least 10 min [ $t(42) = 4.54, p < 0.01$ ]. Similarly, Group Paired-Hold exhibited an increase in resistance 1 min [ $t(42) = 7.12, p < 0.001$ ], and 10 min after the pairings [ $t(42) = 4.72, p < 0.01$ ]. Groups Unpaired and Light+LLD did not exhibit an increase in resistance at any time relative to their baseline levels ( $t$  values  $< 1.29$ ). These results indicate that cumulative depolarization is neither necessary nor sufficient for the induction or short-term maintenance of the neuronal potentiation indicative of memory formation.

Figure 4B compares the percentage change in resistance 1 and 9 min following normal pairings relative to pairings in which cumulative depolarization was prevented. The prevention of the cumulative depolarization did not attenuate the observed potentiation, and in fact, tended to facilitate it. ANOVA of the resistance values of the two paired groups in isolation revealed



*Figure 4.* *A*, The mean current-voltage relationship obtained from each of the four treatment conditions in experiment 2. A resistance increase was observed in each of the paired conditions, which persisted for at least 10 min postraining, but not in either Group Unpaired or Group Light+LLD. *B*, The mean percentage change in resistance in Groups Paired and Paired-Hold 1 and 10 min after conditioning. The percentage increase was significantly higher if the B cell was not permitted to depolarize following each training trial, suggesting that  $\text{Ca}^{2+}$  accumulation limited the rate or asymptote of learning.

a significant effect of time [i.e., before or after treatment;  $F(2, 22) = 32.08, p < 0.001$ ], as well as a tendency toward a treatment  $\times$  time interaction [ $F(2, 22) = 3.24, p < 0.06$ ]. Comparisons of individual means indicated that while Groups Paired and Paired-Hold did not differ before pairings [ $t(22) = 0.91$ ], Group Paired-Hold exhibited higher resistance 1 min after pairings [ $t(22) = 4.29, p < 0.01$ ], though the groups did not differ 10 min after pairings [ $t(22) = 1.54$ ].

Just prior to each measurement of resistance, the B cell was briefly released from all current in order to assess its normal resting potential. The mean resting potential of each group in the absence of applied current is presented in Table 1. The groups did not differ prior to training, but when the B cells were briefly released from all current just prior to the 1 min posttraining resistance measures, a depolarized resting potential was observed in each of the paired groups which returned to baseline levels by 10 min posttraining. ANOVA revealed a significant effect of time of observation [ $F(2, 74) = 37.70, p < 0.001$ ], as well as a time  $\times$  treatment interaction [ $F(6, 74) = 2.41, p < 0.05$ ]. Since this learning-induced depolarization could not have contributed to the rise in resistance, it is possible that some portion of the rise in resistance contributed to the depolarized resting potential, possibly via the reduction of a resting  $K^+$  conductance. This result indicates that while the cumulative depolarization is a reliable correlate of short-term *in vitro* conditioning (e.g., Farley and Alkon, 1987), it does not causally contribute to the induction process.

#### Measurement of $Ca^{2+}$ currents from depolarized holding potentials

The prevailing view of learning in *Hermissenda* has held that the cumulative depolarization provided for an enhancement of voltage-dependent  $Ca^{2+}$  influx during repeated pairings, and/or a sustained release of  $Ca^{2+}$  from intracellular stores. [With regard to this latter possibility, Connor and Alkon (1984) have demonstrated that the voltage-dependent  $Ca^{2+}$  channel blocker  $Cd^{2+}$  eliminates Arsenazo III binding during the light-induced generator potential, suggesting that the primary source of  $Ca^{2+}$  during the light response is through voltage-dependent channels.] The enhanced input resistance observed here in B cells that did not undergo cumulative depolarization during pairings of pre- and postsynaptic activity might be interpreted to suggest that the cumulative depolarization that occurs during closely spaced trials results in a steady-state inactivation of  $Ca^{2+}$  currents, or a depression of peak  $Ca^{2+}$  currents during repeated depolarization. Via either mechanism,  $Ca^{2+}$  influx might be reduced when repeated depolarizations occur in close temporal proximity or from steady-state levels of depolarization. To test these possibilities, a group of axotomized B photoreceptors were voltage clamped at a holding potential of  $-60$  mV in normal ASW. Figure 5A illustrates a typical outward current observed when the cell is stepped to 0 mV. When 10 mM  $Ba^{2+}$  was substituted for  $Ca^{2+}$  and  $K^+$  channel blockers were added to the ASW, the step to 0 mV revealed an inward current that has previously been shown to be conducted via  $Ca^{2+}$  channels (Alkon et al., 1984; Farley and Auerbach, 1986; Collin et al., 1988; Farley and Wu, 1988). Indeed, as illustrated in the right panel of Figure 5A, this inward current was largely attenuated by the addition of the  $Ca^{2+}$ -channel blocker  $Cd^{2+}$  (10 mM). The block of the inward current by  $Cd^{2+}$  also revealed a small, residual outward current, probably a remnant of the fast, transient  $K^+$  current ( $I_A$ ). To maximize the inward current relative to the

**Table 1.** The mean ( $\pm$ SE) resting membrane potential (mV) of each group in experiment 2 taken 5 sec prior to the measurement of input resistance

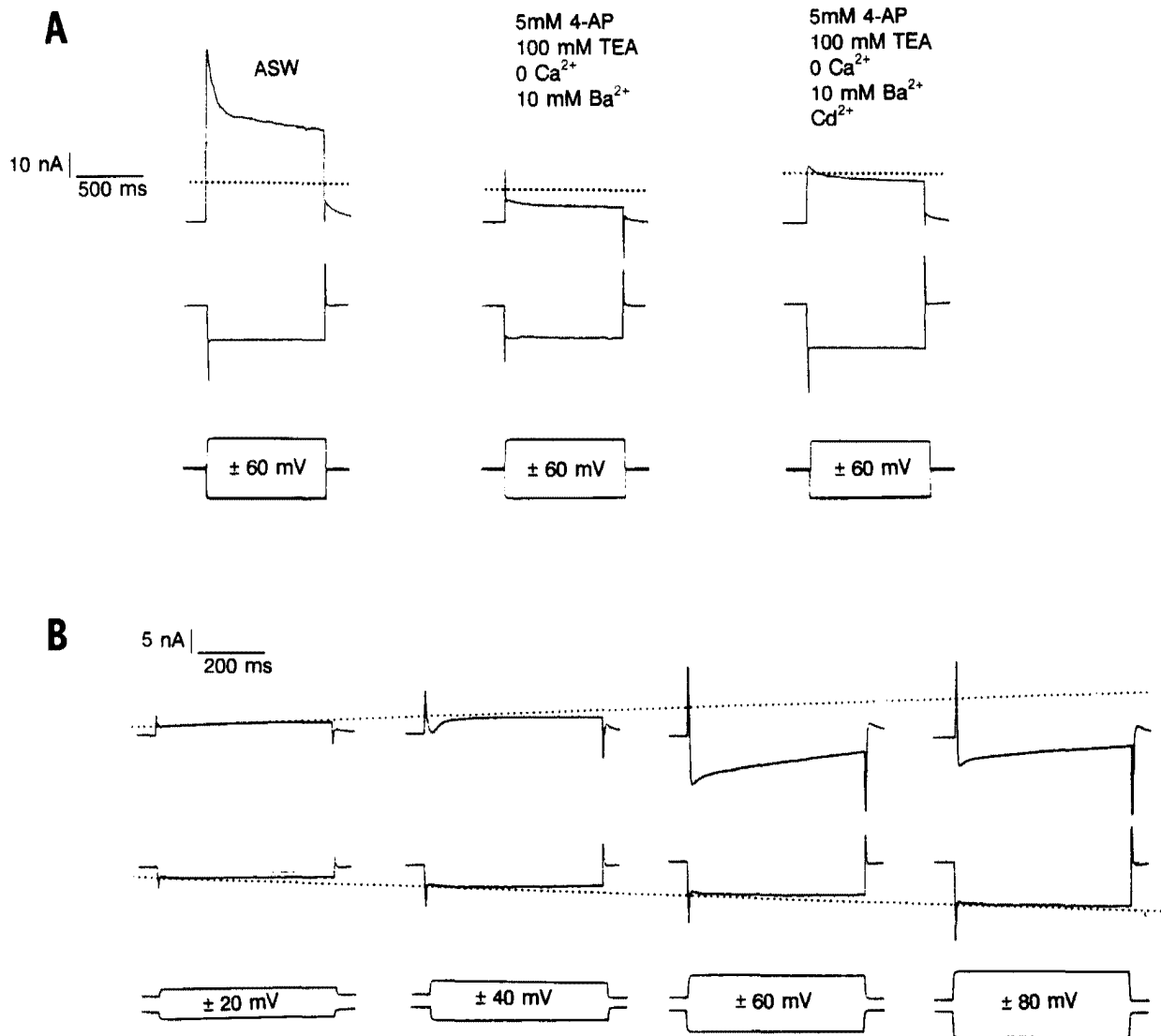
Group	Pretraining	Posttraining	
		1 min	10 min
Paired	55.6 $\pm$ 0.66	44.5 $\pm$ 3.01	56.8 $\pm$ 1.62
Paired (hold)	53.9 $\pm$ 1.93	44.8 $\pm$ 2.05	54.4 $\pm$ 1.38
Unpaired	55.0 $\pm$ 0.57	52.3 $\pm$ 2.71	57.8 $\pm$ 1.95
Light + LLD	54.6 $\pm$ 2.05	51.3 $\pm$ 2.05	57.1 $\pm$ 2.05

The B cell was briefly released from the normal holding potential of  $-60$  mV.

nonspecific leak conductance, the concentration of  $Ba^{2+}$  was increased to 100 mM (Tsien, 1983), and 4-AP concentration was increased to 10 mM. A family of inward currents elicited under these conditions is illustrated in Figure 5B. Under these conditions, a large ( $\approx 6$ –12 nA) inward current was elicited that typically peaked between 0 and +20 mV when stepped from a holding potential of  $-60$  mV. It should be noted from these current records that the inward current relaxed slightly ( $\approx 30\%$ ) during the 600 msec command step. This inactivation was further examined in Figure 6A, where the command step was extended to 1200 msec. Under these conditions, inactivation was still less than complete, but when a second command step was elicited 2.5 sec later, the peak current, measured 75 msec after onset of the command step, was markedly depressed, indicating that this current undergoes both a partial inactivation as well as a depression of the peak response during repetitive depolarization. This experiment was replicated four times, and the paired-pulse depression ranged from 30% to 40%. The average current magnitude for the first command step was compared to that of the second command step, and was found to be significantly depressed [ $F(1, 3) = 25.00, p < 0.001$ ].

Although the relaxation of the inward current observed here has been previously reported under conditions of  $Ba^{2+}$  substitution (e.g., Alkon et al., 1984, Fig. 3), it is typically not seen when elicited with physiological concentrations of  $Ca^{2+}$  in the bath (e.g., Collin et al., 1988; Farley and Wu, 1988). In fact,  $Ca^{2+}$  currents have often been found to be inactivated by  $Ca^{2+}$ , not voltage (Eckert and Tillotson, 1981; Higawara and Byerly, 1981), and such a mechanism might augment the depression of the inward current observed with repetitive depolarization in the absence of any apparent within-pulse inactivation. In Figure 6B are illustrated inward  $Ca^{2+}$  currents elicited in the presence of  $K^+$  channel blockers, normal external  $Ca^{2+}$ , and 300 mM external  $K^+$ . Under such conditions the reversal potential for  $K^+$  is shifted to 0 mV and the inward  $Ca^{2+}$  current can be measured with command steps to 0 mV with no contamination by an outward  $Ca^{2+}$ -dependent  $K^+$  current ( $I_{K-Ca}$ ). From a holding potential of  $-60$  mV an inward current was observed under these conditions that was reduced in magnitude relative to the inward  $Ba^{2+}$  current, and as previously described, this current exhibited no measurable steady-state inactivation. At the offset of the command pulse, an inward tail current was observed that reflects a reversed  $I_{K-Ca}$ . While no steady-state inactivation was observed, there was a small depression (range of 15–35%,  $n = 4$ ) of the inward  $Ca^{2+}$  current when a second command depolarization was elicited 2.5 sec later and a marked reduction (range, 50–75%) of the tail current ( $I_{K-Ca}$ ), providing further indication of a reduced  $Ca^{2+}$  influx. The inward current was fur-





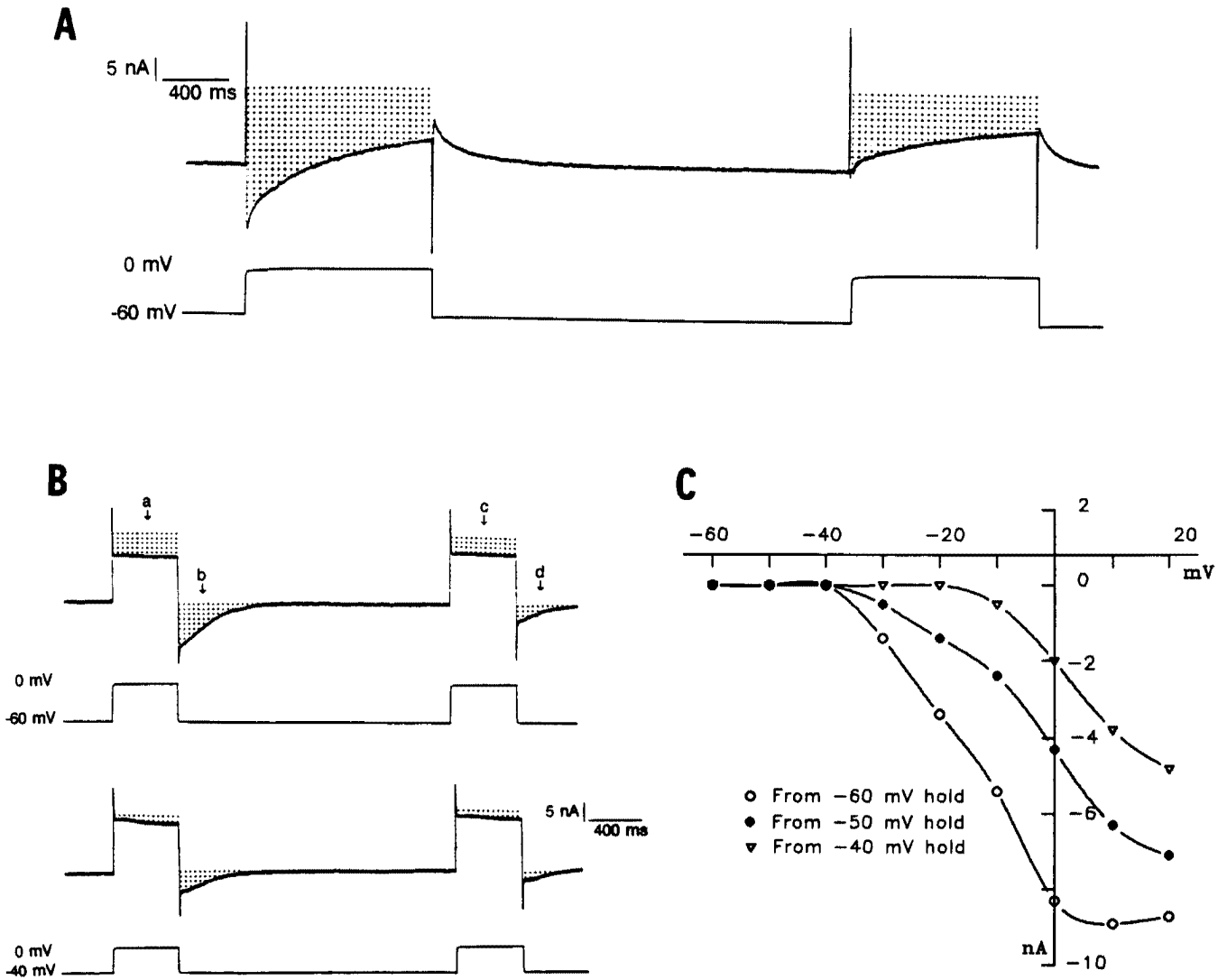
**Figure 5.** *A*, Single-electrode voltage-clamp recordings from an isolated B cell. A command step from  $-60$  to  $0$  mV induced a fast outward current ( $I_A$ ) and a slower outward plateau current in normal bathing solution (ASW). The broken line indicates extrapolated non-voltage-dependent leak current. Following the addition of  $100$  mM TEA,  $5$  mM  $4\text{-AP}$ ,  $10$  mM  $\text{Ba}^{2+}$ , and the removal of extracellular  $\text{Ca}^{2+}$ , the outward currents were abolished, leaving a small inward current, previously identified as being carried by  $\text{Ca}^{2+}$  channels (Alkon et al., 1984). This was confirmed by the addition of a  $10$  mM concentration of the  $\text{Ca}^{2+}$  channel blocker  $\text{Cd}^{2+}$ , which abolished the inward current, revealing a slight, transient outward current, presumably residual  $I_A$ . The middle records in each panel are the nonspecific leak current records elicited by equal-amplitude, opposite-polarity pulses. Voltage responses are depicted in the lower records. *B*, A family of inward currents elicited when the  $4\text{-AP}$  concentration was increased to  $10$  mM and  $\text{Ba}^{2+}$  was increased to  $100$  mM. Under these conditions, a relatively larger inward current is elicited, which peaked between  $0$  and  $+20$  mV, and which undergoes a partial inactivation during the depolarizing step. From the holding potential of  $-60$  mV, commands to progressively more negative potentials elicit a non-voltage-dependent leak current indicated by the lower broken line. The upper broken line represents the extrapolated leak current. The bottommost records are the voltage responses to the command depolarization.

ther reduced (range,  $20\text{--}50\%$ ) when elicited from a holding potential of  $-40$  mV. This latter observation was confirmed statistically [ $F(1, 3) = 10.37$ ,  $p < 0.05$ ].

Figure 6C illustrates the current–voltage relationship of a family of inward currents elicited in the presence of  $\text{K}^+$  channel blockers and  $30$  mM external  $\text{Ba}^{2+}$ . Under these conditions, a large ( $\approx 8$  nA peak), steady, inward current is elicited, with only marginal within-pulse inactivation (not shown). These  $I\text{-}V$  relationships were elicited from holding potentials of  $-60$ ,  $-50$ , or  $-40$  mV. The prepulse depolarization was initiated  $10$  sec prior to the  $600$  msec command steps, with a minimum of  $4$  sec between successive steps. From a holding potential of  $-60$  mV, an inward current was elicited by command steps to po-

tentials more positive than  $-40$  mV, which typically peaked between  $0$  and  $+20$  mV. From more positive holding potentials, the peak current was reduced, and the point of initial activation was shifted to more positive potentials. Figure 6C represents mean values obtained from eight cells, and the above observations were confirmed by ANOVA conducted on the current values elicited with command steps to  $0$  mV from  $-60$ ,  $-50$ , or  $-40$  mV holding potentials [ $F(2, 14) = 10.42$ ,  $p < 0.001$ ]. Comparisons of individual means indicated that the current elicited from  $-60$  mV was greater than that elicited from  $-40$  mV [ $t(14) = 4.50$ ,  $p < 0.05$ ] and differed marginally from the current elicited from  $-50$  mV [ $t(14) = 2.88$ ,  $p < 0.07$ ].

In total, this analysis of  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  currents indicates that



**Figure 6.** *A*, Inward current records elicited in ASW containing TEA (100 mM), 4-AP (10 mM),  $Ba^{2+}$  (100 mM), and 0  $Ca^{2+}$  as described above. During a 1600 msec depolarizing step, the inward current undergoes a partial inactivation, and a subsequent depression when a second depolarizing step of the same magnitude occurs 2.5 sec later. The shaded area indicates the extrapolated leak current. *B*, Here extracellular  $Ca^{2+}$  was maintained at physiological levels (10 mM) and no  $Ba^{2+}$  was added to the bath solution, while external  $K^+$  was elevated to 300 mM. Under these conditions, the reversal potential for  $K^+$  shifts to 0 mV and the inward  $Ca^{2+}$  current can be measured in isolation with command steps to 0 mV. During an initial depolarizing step (*a*) an inward  $Ca^{2+}$  current is observed as well as a  $Ca^{2+}$ -dependent tail current (*b*), previously determined to be a reversed  $Ca^{2+}$ -dependent  $K^+$  current. When a second depolarization was elicited 2.5 sec later, a small reduction of the  $Ca^{2+}$  current was directly evident (*c*) and a large reduction of the tail current occurred (*d*). Both the  $Ca^{2+}$  current and the  $Ca^{2+}$ -dependent tail current were depressed when elicited from depolarized potentials. The shaded areas at points *a* and *c* represent extrapolated leak. *C*, A current-voltage plot of the inward current elicited from a range of depolarized holding potentials in the presence of 30 mM  $Ba^{2+}$  and 0  $Ca^{2+}$ . Under these conditions, no steady-state inactivation of the inward current is observed. Holding potentials of -50 mV and -40 mV (for 10 sec) resulted in smaller peak currents, as well as induced a shift of activation curve to more positive potentials. This depression of the inward current suggests that cumulative depolarization would result in a reduced  $Ca^{2+}$  influx, thus diminishing the effectiveness of multiple conditioning trials. These plots represent the mean of eight cells.

these currents may undergo a moderate steady-state inactivation, and a relatively larger depression during repetitive depolarization, or when elicited from depolarized membrane potentials. Given the depression of the current observed when elicited from depolarized holding potentials, we can conclude that voltage-dependent  $Ca^{2+}$  currents would not be augmented, and in fact may be substantially reduced when elicited from relatively positive holding potentials during periods of cumulative depolarization. This conclusion must be tempered, however, given that the current elicited from -50 mV in physiological ASW was reduced, but not eliminated. Given that this is the approximate level of cumulative depolarization observed during pair-

ings of light and vestibular stimulation in experiment 2, it is impossible to state with certainty that the reduction of the  $Ca^{2+}$  current was solely responsible for the retardation of acquisition. Nevertheless, it is clear from this data that the inward current is not potentiated when elicited from depolarized potentials. Even so, it is possible that an enhanced sensitivity to light, or a corresponding increase in the level of depolarization during the light response, might compensate for any reduction in the overall magnitude of the  $Ca^{2+}$  current during a period of cumulative depolarization. This is unlikely, though, given that the light-induced generator potential (Farley and Alkon, 1987) and the underlying light-induced  $Na^+$  current (Talk et al., 1993) is

moderately reduced when elicited from depolarized membrane potentials.

## Discussion

Several experiments explored the viability of cumulative depolarization (1) to induce an increase in input resistance, a neurophysiological correlate of memory formation, and (2) to enhance the voltage-dependent inward  $\text{Ca}^{2+}$  current during periods of sustained depolarization. In each of two experiments we employed an *in vitro* conditioning preparation in which light was paired with vibrotactile stimulation of the statocyst. This procedure is a close approximation of conditioning of the intact animal in which light is paired with rotation, and is preferable to procedures in which only a single hair cell has been stimulated with current injection, since the latter procedure does not recruit the input from other hair cells during, or after, the offset of the stimulation. In experiment 1, we found that a single pairing of light and vestibular stimulation resulted in an increase in resistance in the B cell soma membrane. This rise in resistance was dependent on presynaptic stimulation occurring in conjunction with a rise in postsynaptic  $\text{Ca}^{2+}$ , in that injection of the  $\text{Ca}^{2+}$  chelator EGTA prior to the pairing blocked the increase in resistance, as did the unpaired presentation of the pre- and postsynaptic activity.

Since only a single pairing was administered in experiment 1, it was not possible for an accumulation of depolarization to occur across trials, indicating that such an "accumulation" was not a necessary prerequisite for learning. However, this experiment does not eliminate the possibility that a single pairing results in a persistent increase in  $\text{Ca}^{2+}$  above baseline levels that is sufficient to induce an increase in resistance. Such an elevation of intracellular  $\text{Ca}^{2+}$  might arise from two sources. First, a small, but sustained, depolarization might activate a voltage-dependent  $\text{Ca}^{2+}$  influx. This, however, is unlikely given that the voltage-dependent  $\text{Ca}^{2+}$  currents described for the B photoreceptors are not activated at membrane potentials more negative than  $-40$  mV (Figs. 5, 6; Alkon et al., 1984; Farley and Wu, 1988), and reaches half-maximal activation at  $\approx -10$  mV (see Fig. 6C). A second possibility is that  $\text{Ca}^{2+}$  might undergo a sustained release from intracellular stores, either in response to light (but see Connor and Alkon, 1984) or as a result of presynaptic stimulation (Oka et al., 1992). These potential sources of  $\text{Ca}^{2+}$  are, however, independent of the cumulative depolarization mechanism that has been previously proposed (e.g., Farley and Alkon, 1985, 1988). According to this proposal, cumulative depolarization of the membrane during repetitive pairings culminates in a voltage-dependent rise in  $\text{Ca}^{2+}$  that augments subsequent pairings of pre- and postsynaptic activity, or that in itself is sufficient to initiate a molecular cascade that results in the phosphorylation of membrane proteins associated with  $\text{K}^{+}$  channels. While intracellular sources of  $\text{Ca}^{2+}$  may contribute to the *in vitro* potentiation that we have observed, it is not clear how these sources of  $\text{Ca}^{2+}$  would be enhanced by an accumulating depolarization, particularly in that such a depolarization appears to depress voltage-dependent  $\text{Ca}^{2+}$  influx.

While a cumulative depolarization may not have contributed to the short-term plasticity described in this experiments, the increase in resistance that was observed following a single pairing dissipated within minutes of training, raising the possibility that an accumulation of depolarization (and/or  $\text{Ca}^{2+}$ ) across trials is necessary for the maintenance of the potentiation. In

experiment 2, the possibility was examined that the cumulative depolarization that accompanied multiple pairings of light and vestibular stimulation would facilitate the transition from the induction to the maintenance of the potentiation associated with memory formation. When nine pairings of light and vestibular stimulation occurred at 1 min intervals, a cumulative depolarization was observed across trials that was accompanied by an increase in resistance that persisted for at least 10 min. Neither the cumulative depolarization nor the increase in resistance occurred when light and vestibular stimulation were separated by a 30 sec interstimulus interval (i.e., unpaired). Likewise, when nine light-alone presentations were followed with positive current injection intended to mimic cumulative depolarization, no increase in resistance was observed, indicating that the cumulative depolarization was not sufficient to induce an increase in resistance in the absence of presynaptic activity.

A final group in experiment 2 received nine pairings of light and vestibular stimulation, but the B cell was held at its initial resting potential of  $-60$  mV before and after each of the pairings, thus eliminating any contribution of a cumulative depolarization. Surprisingly, the increase in resistance resulting from pairings not only persisted, but was enhanced following this treatment relative to pairings that were accompanied by a cumulative depolarization. When these B cells were briefly released from holding current after the ninth pairing, a depolarized resting potential was observed that was comparable to those B cells that underwent cumulative depolarization during training. Two conclusions can be drawn from this result. First, cumulative depolarization is not necessary for the induction or maintenance of the neuronal potentiation associated with memory formation, and in fact, limits the acquisition of this potentiation. Second, since a depolarized membrane potential was observed when the cell was released from holding current, it can be assumed that at least some of the cumulative depolarization typically observed during learning results from the reduction of a resting conductance, rather than causing that reduction as has been commonly assumed.

Based on the results of experiment 1, it is clear that transient depolarization acts in a  $\text{Ca}^{2+}$ -dependent manner to contribute to *in vitro* potentiation in this system, and based on experiment 2, it can be concluded that a cumulative depolarization does not contribute to the process. It is reasonable, then, to ask whether the cumulative depolarization might augment voltage-dependent  $\text{Ca}^{2+}$  influx as previously proposed. Under voltage clamp, it was clear that repetitive as well as sustained depolarization would depress voltage-dependent  $\text{Ca}^{2+}$  currents. When the voltage-dependent inward current was maximized by the replacement of external  $\text{Ca}^{2+}$  with 100 mM  $\text{Ba}^{2+}$ , the inward current underwent a modest inactivation during sustained command depolarization, and a marked depression during a second depolarization 2–3 sec after the first. In the presence of normal extracellular  $\text{Ca}^{2+}$ , no inactivation of the inward current during a command depolarization was detected. However, a depression of the current was observed with repetitive depolarization. Moreover, the  $\text{Ca}^{2+}$ -dependent "tail" current ( $I_{\text{K-Ca}}$ ) was similarly depressed. It is not clear at present whether the differential inactivation observed with high extracellular  $\text{Ba}^{2+}$  reflects the initial activation of a distinct current or simply the manifestation of a kinetic property of a single current not detected at submaximal levels of activation. That this inactivation is largely abolished with lower concentrations of  $\text{Ba}^{2+}$  suggests that this is not a distinct current.

Both in normal ASW and following  $Ba^{2+}$  substitution for extracellular  $Ca^{2+}$ , depolarizing steps from more positive resting potentials were less effective in the activation of the inward current. From a holding potential of  $-60$  mV, step commands to positive potentials elicited an inward current through  $Ca^{2+}$  channels that peaked between 0 and  $+20$  mV. From more positive holding potentials (i.e.,  $-50$  and  $-40$  mV), a shift in the activation curve to more positive levels was observed, as well as a reduction of the current magnitude. This observation suggests that as depolarization begins to accumulate with training, subsequent trials will recruit progressively smaller  $Ca^{2+}$  currents, thus minimizing the effectiveness of each successive pairing. At present, it is not entirely clear by what mechanism the current magnitude is reduced when elicited from the more positive potentials. With normal extracellular  $Ca^{2+}$  or  $10$ – $30$  mM  $Ba^{2+}$ , no steady-state inactivation of currents are typically observed, even with prolonged depolarizing steps (up to 20 sec; data not shown). Nevertheless, in the absence of any steady-state inactivation, depression of the  $Ca^{2+}$  current can still be observed during repeated depolarizing steps (e.g., Fig. 6B). That this depression could be observed in the  $Ca^{2+}$ -current tail as well as the  $Ca^{2+}$  current itself suggests that it could not arise via contamination by a slowly developing outward current. In the absence of steady-state inactivation, it must be assumed that the depression of the inward current that we observe when elicited from depolarized holding potentials must arise from a similar long-lasting depression of the current, which is apparently mediated by the modification of the voltage-dependent gating mechanism.

In total, we conclude that the accumulation of depolarization is neither necessary nor sufficient to induce biophysical modifications previously correlated with memory formation in *Hermisenda* B photoreceptors. Rather, transient depolarization, when paired with presynaptic activity, acts in a  $Ca^{2+}$ -dependent manner to induce the potentiation of excitability indicative of memory formation. Several mechanisms have begun to emerge that might account for this potentiation, including the activation of protein kinase C, which results from a light-induced  $Ca^{2+}$  influx in conjunction with the generation of synergistic cofactors that presumably arise as a result of transmitter release onto the synaptic terminals (Farley and Auerbach, 1986; Crow and Forrester, 1991; Matzel and Alkon, 1992). Both GABA and/or 5-HT have been hypothesized to contribute to this process (Crow and Forrester, 1991; Rogers et al., 1992), each of which may stimulate a common GTP-binding protein (e.g., Sasaki and Sato, 1987; Rogers et al., 1992), thus stimulating the generation of lipid-derived intermediaries (e.g., diacylglycerol or arachidonic acid) that reduce the kinase's requirement for  $Ca^{2+}$ . This mechanism does not require complicated interactions in the neural network (i.e., a release from synaptic inhibition and a light-induced LLD) and bears strong resemblance to activity-dependent mechanisms proposed to underlie learning in other systems such as *Aplysia* (Hawkins et al., 1983; Kandel et al., 1986; Byrne et al., 1991) and some forms of long-term potentiation (Jaffe and Johnston, 1990; Kullman et al., 1992). The activity-dependent facilitation observed here differs from that observed in *Aplysia* only in that at least one putative modulatory transmitter (i.e., GABA) does not potentiate, and in fact opposes, an increase in excitability when applied and/or released in isolation. This latter observation suggests that activity-dependent facilitation need not necessarily represent the enhancement of a sensitization process as it often does during associative learning in *Aply-*

*sia* (e.g., Ocorr and Byrne, 1985; Braha et al., 1990; Mercer et al., 1991).

It has been commonly observed that the acquisition of a memory is facilitated when the repetition of training events is distributed relative to when those events are temporally massed; that is, longer intertrial intervals typically reduce the number of training trials required for asymptotic learning (e.g., Kaplan, 1984; Fanselow and Tighe, 1988; Talk et al., 1993). The results presented here suggest a mechanism whereby this "trial-spacing effect" may be realized. Specifically, as  $Ca^{2+}$  accumulates during narrowly spaced trials, subsequent trials will be less effective. If intracellular  $Ca^{2+}$  were allowed to dissipate between trials, the requirement for  $Ca^{2+}$  influx on each subsequent pairing would be satisfied. This scenario raises questions concerning a conclusion of Farley (1987), who suggested that unsignaled unconditioned stimuli (USs; i.e., vestibular stimulation) during a training session reduce learning by hyperpolarizing the B cell membrane and reversing the cumulative depolarization, thus potentially accounting for contingency sensitivity in *Hermisenda*. Since we have found that the cumulative depolarization does not contribute to acquisition (and in fact may limit it), unsignaled US presentations must exert their debilitating influence via some other means.

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