CALCIUM-MEDIATED DECREASE OF A VOLTAGE-DEPENDENT POTASSIUM CURRENT

DANIEL L. ALKON AND JONATHAN J. SHOUKIMAS
Section on Neural Systems, Laboratory of Biophysics, Intramural Research Program, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ELIAHU HELDMAN
Department of Pharmacology, Israel Institute for Biological Research, Ness-Ziona, Israel

ABSTRACT Elevated intracellular Ca^{++} concentration reduces the amplitude of an early, voltage-dependent K^+ current (I_A) in the Type B photoreceptor of *Hermissenda crassicornis*. Internal Ca^{++} is increased by activating a voltage and light-dependent Ca^{++} current present in these cells or by direct iontophoresis of Ca^{++} ions. Substitution of Ba^{++} for Ca^{++} or elimination of Ca^{++} from the sea water bathing the cells abolishes the reduction in I_A during paired light and depolarizing voltage steps. The delayed K^+ current (I_B) in these cells is also reduced during paired light and voltage steps, but this decrease of I_B is not affected by removal of extracellular Ca^{++}. I_B (but not I_A), apparently much less dependent on intracellular Ca^{++} levels, is reduced by light alone. Ca^{++} iontophoresis also abolishes the light-dependent Na^+ current, which recovers with a time course of minutes.

INTRODUCTION

Using light as a conditioned stimulus and rotation as an aversive unconditioned stimulus, it was possible to produce a long-lasting behavioral change of the nudibranch mollusc *Hermissenda crassicornis* (Alkon, 1974*a*; Crow and Alkon, 1978). Following repeated pairing of light with rotation (i.e., maximal rotation preceded by light onset by ~ 1.0 s) the animal's movement toward a light source was markedly reduced. This behavioral change did not follow randomly associated light and rotation nor a number of other control training regimens (Crow and Alkon, 1978). Other features of vertebrate associative learning also obtained for this behavioral change include acquisition, stimulus specificity, and persistent retention (Crow and Alkon, 1978; Alkon, 1979; Crow and Offenbach, 1979; Farley and Alkon, 1980, 1981, 1982). More recently, such features as contingency (Farley and Kern, 1982) and extinction (Farley et al., in press) have also been demonstrated.

Extensive analysis of the synaptic interactions within and between the two sensory pathways that mediate this learning behavior (Alkon, 1974*b*; 1980) and correlation of neuronal with behavioral changes (Crow and Alkon, 1980; West et al., 1981; Farley and Alkon, 1981, 1982, West et al., 1982) implicated Type B photoreceptors as primary loci for encoding the learned information and actually causing the behavioral change. This interpretation was supported by the findings that injection of appropriate currents into specific hair cells and interneurons, when paired with illumination of the Type B photoreceptor, could simulate the effects of training (Farley and Alkon, 1982*b*) and changes intrinsic to the Type B soma membrane, only of conditional animals, could be identified after the Type B cell had been electrically isolated from all other cells in the *Hermissenda* nervous system (West et al., 1981; Alkon et al., 1982). More recently, membrane changes of Type B cells previously correlated with conditioning were demonstrated to be necessary and sufficient to cause retention of the associatively learned behavior (Farley et al., 1982). This was achieved by producing pairing-specific membrane changes in single Type B cells of living animals whose behavior was measured on subsequent days.

An early voltage-dependent K^+ current (I_A) of the Type B cells was decreased for days following training with paired but not randomized light and rotation (Alkon et al., 1982). This reduction of I_A, a current intrinsic to the soma membrane (Shoukimas and Alkon, 1980) could account for an enhanced steady-state response during and following a light step. The augmented Type B response causes increased inhibition of the medial Type A photoreceptor (Goh and Alkon, 1982). The Type A cell then causes less excitation of interneurons, which in turn cause less excitation of identified motorneurons responsible for actual movement in response to light (Goh and Alkon, 1982).

An increased Type B light response was thought to

result from stimulus pairing when rotation-induced synaptic excitation enhanced a voltage-dependent light-induced Ca^{++} current (Alkon, 1979). Repeated pairs of light and rotation lead to cumulative membrane depolarization (Alkon, 1980*b*) and, in turn, further enhancement of the light-induced Ca^{++} current. Consistent with these findings was a recent observation that the Type B depolarizing response following a light-step is accompanied by a voltage-dependent rise of internal Ca^{++} as measured by differential absorption spectrophotometry of the Arzenazo-Ca^{++} complex (Connor and Alkon, 1982).

In the present study we hypothesized that elevated intracellular Ca^{++} due to the enhanced light-induced Ca^{++} current together with prolonged depolarization causes the long-term decrease of I_A observed for associatively trained animals. To test this hypothesis we examined the magnitude of I_A currents of isolated Type B somata during positive command steps under conditions that affect the levels of intracellular Ca^{++}. These conditions included pairing of command steps with light steps, zero external Ca^{++}, substitution of Ba^{++} for external Ca^{++}, and iontophoretic intracellular injection of Ca^{++}. The effects of all of these conditions were consistent with the conclusion that elevated intracellular Ca^{++} decreases the magnitude of I_A. The Ca^{++} mediated decrease observed here can persist for many minutes.

METHODS

The somata of Type B photoreceptors were isolated by axotomy from the excitable membrane of the axon as well as all synaptic interactions that occur only at terminal branches of the distal axon (Alkon, 1979). I_A was measured by depolarizing potential steps to -5 or 0 mV of 2–2.5 s duration from a holding potential of -60 mV. The slope of the voltage-independent, "leak" current was obtained from currents measured for small voltage pulses (± 20 mV maximum) from the holding potential. Extrapolated values of the leak current at -5 or 0 mV were used to correct the measured I_A values. A late potassium current, I_B, which inactivates only slightly (Shoukimas and Alkon, 1980) was also measured after correction for leak currents. I_B measurements were made with a second command pulse (10 mV more positive and of 800 ms duration) that immediately followed the first command pulse.

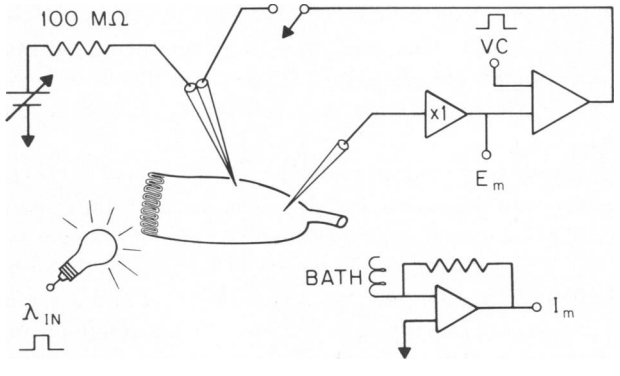

FIGURE 1 Schematic of the voltage-clamp apparatus. The circuit is a conventional, two microelectrode voltage clamp with current measured by a virtual ground amplifier. For iontophoresis, a double-barreled micropipette supplied clamp current from one barrel and iontophoretic current out of the other barrel via the DC voltage source and 100 MΩ dropping resistor. The light source is controlled by a negative feedback system to improve rise time.

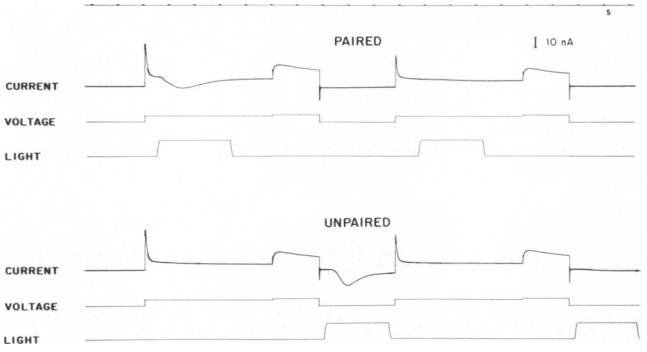

FIGURE 2 Positive command pulses paired (upper records) and unpaired (lower records) with light steps. Each command step to 0 mV is followed after 30 μs by a brief step to +10 mV. The interstimulus interval illustrated here was used for paired vs. unpaired comparisons. A shorter interval (1–1.5 s) was used for Table I comparisons.

Ca^{++} injection was accomplished by +0.5–1.0 nA of iontophoretic current for 1 min under voltage clamp through one barrel (containing 0.5 M KCl and 0.8 M $CaCl_2$) of a double-barreled microelectrode. The other barrel (containing 3 M KCl) was used for current injection necessary to maintain voltage clamp. Another separate microelectrode (containing 3 M KCl) monitored the potential of the cell (Fig. 1). Mg^{++} injection was accomplished with identical current parameters. The compositions of the experimental solutions are as follows (concentrations in millimoles): 430 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 TRIS HCl (pH 7.0) for Artificial Sea Water (ASW); 430 NaCl, 10 KCl, 10 $BaCl_2$, 50 $MgCl_2$, 10 TRIS HCl (pH 7.0) for Ba^{++} ASW; 436.7 NaCl, 9 KCl, 22.9 $MgCl_2$, 25.5 $MgSO_4$, 2.1 $NaHCO_3$ for Ca^{++}-free ASW.

RESULTS

Effect of Light Paired with Depolarization Steps

Five successive depolarization steps to -5 or 0 mV (2–2.5 s), each followed by a 10-mV more positive voltage step (800 ms) as illustrated in Fig. 2, were made with an

TABLE I
EFFECT OF LIGHT PAIRING UPON PEAK CURRENT DECAY

Ionic current	Superfusion solution	Mean decay curve differences*	$N \pm$ SD		Significant difference from zero (P = 0.01)
I_A	ASW	0.25	8	0.12	yes
I_A	0 Ca^{++}ASW	0.06	4	0.31	no
I_A	10 mM Ba^{++}ASW	0.02	4	0.08	no
I_B	ASW	1.17	7	0.89	yes
I_B	0 Ca^{++}ASW	1.01	4	0.67	yes

ASW, 0 Ca^{++}ASW, and 10 mM Ba^{++}ASW refer to the superfusion solutions whose compositions are shown in the text. Standard Z scores ($Z = \overline{X} - \mu/\sigma/\sqrt{N}$; for $\mu = 0$) were computed for each set of means and standard deviations. Decay areas are considered significantly different from zero for mean values that would occur <1% of the time by chance alone ($P < 0.01$).

*Combined data for voltage steps to -5 mV and 0 mV.

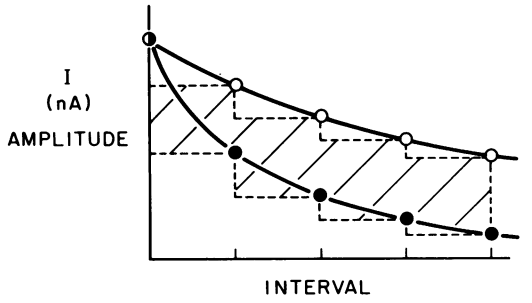

FIGURE 3 Diagram of difference quantitation technique. Technique for estimation of the area of difference curve is illustrated. (O) Control current amplitudes (voltage steps alone); (●) test current amplitudes (paired light and voltage steps). The rectangular approximation to the area between the curves drawn through the circles is shown by the slanted lines.

interstimulus interval of 1–1.5 s. The same five steps (together with the 800 ms steps) were then repeated with the same interstimulus interval but with a 2.0 s light step ($\sim 10^{3.5}$ erg/cm^2·s from a tungsten light source) following by 150 ms the onset of each of the five depolarization steps. Sequences of command steps alone, light paired with command steps, and command steps alone revealed no dependence on the order of the sequences used. Table I summarizes the effects of pairing repeated light and depolarization steps on the time course of decay of I_A and I_B amplitudes. To measure differences in decay for I_A we calculated the ratio of peak currents during each step to the peak current of the first of the five steps. For I_B decay rates we calculated the ratio of peak currents during the 800 ms pulses to the peak current of the steps preceding (by at least 40 s) the first of the five steps. For each of the five step pairs, the paired light-depolarization ratio values were subtracted from the ratio values for depolarization alone. The area under the difference curves so generated was then approximated by the sum of four difference points and used as a measure of divergence for the two decay rates (Fig. 3).

The time courses were significantly different in ASW (i.e., I_A and I_B decreased more rapidly for light-depolarization vs. depolarization alone) for both I_A and I_B (cf. Table I, Figs. 4, 5). The difference statistics given in Table I largely reflect the maximum suppression of peaks I_A and I_B. This maximum suppression of peak I_A was consistently greater for command pulses paired with light vs. command pulses alone for the ASW condition. The same was true for peak I_B for the ASW and the 0 Ca^{++} conditions. Removal of extracellular Ca^{++} or substitution of Ba^{++} for extracellular Ca^{++} abolished the difference in time courses for I_A (Table I). Removal of extracellular Ca^{++} did not affect the significant difference, however, for I_B decay rates (Table I). Substitution of Ba^{++} for extracellular Ca^{++} entirely eliminated I_B, thus not permitting I_B decay rate measurements. Ba^{++} substitution, however, did not affect I_A. The fact that there was no longer a significant difference between light paired with command pulses and command pulses alone in the presence of Ba^{++} further implicated a role for elevated intracellular Ca^{++} (as a consequence of a light and voltage-dependent Ca^{++} conductance) in depressing I_A.

Effect of Light Unpaired with Depolarization

When repeated light and command steps were alternated using a slightly longer interstimulus interval (Fig. 2), I_A did not decrease more rapidly than for the same command steps alone, while pairing light with command steps for the same cells did increase I_A depression (for three out of three cells). I_A was similarly unaffected when a sequence of five repeated light steps preceded a sequence of five command steps (four out of four cells). In contrast, the decay rate of I_B was transiently affected by these unpaired presentations of light steps.

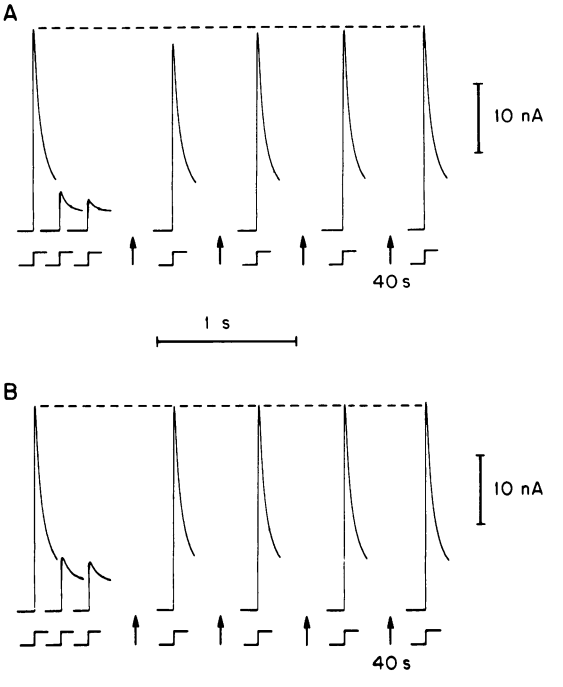

FIGURE 4 Rates of I_A decrease and recovery during and following repetitive command depolarizations. Current responses to only three of the first five depolarizing steps used are shown. The steps following arrows were given at 40 s intervals after the five depolarizing steps used for quantitation of differences described in Table 1. These five command depolarizations (2.2 s) to 0 mV occurred with a cycle time of 4.0 s. Each 2.2 s step was followed by a second command (800 ms) to +10 mV. (*A*) Command depolarizations paired with light. A light step (2.0 s) was presented 150 ms after the onset of each command depolarization. Light intensity; $10^{3.5}$ erg/cm^{-2} s. (*B*) Command depolarizations alone. Arrows indicate 40 s intervals. Lower rectangular traces under *A* and *B* indicate onset of 60 mV command steps. Note that I_A (peak-inward currents) decreases to lower values in *A* than in *B*, and peak I_A takes minutes to return to original values in *A* but not in *B*. The first three currents included in *A* and *B* are the first, second, and fifth currents elicited by the five successive command steps.

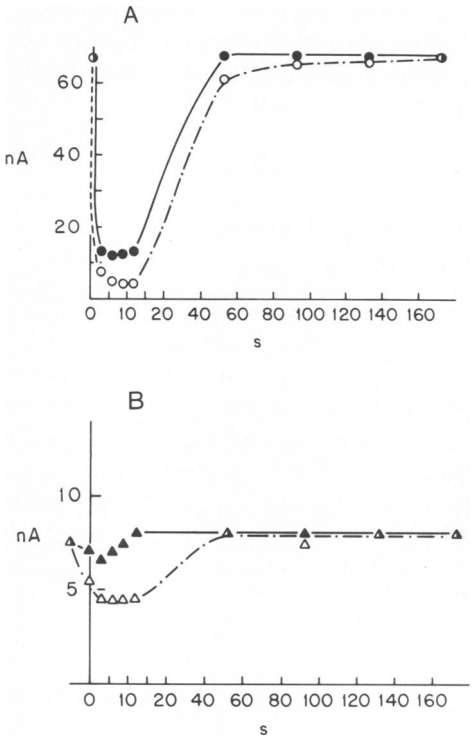

FIGURE 5 Rates of I_A and I_B decrease and recovery following repetitive command depolarizations. Same cell and conditions as in Fig. 4. (*A*) I_A values from Fig. 4. (●, positive pulse; ○, positive pulse plus light.) (*B*) I_B values for the same command depolarizations with and without light steps. (▲, positive pulse; △, positive pulse plus light.) Note that I_A decreases to lower values and takes minutes longer to recover for light paired with depolarizing commands (○) vs. depolarizing commands alone. I_B also decreases to lower values for light paired with depolarization but this decrease does not persist.

Persistence of I_A Decrement

Following five steps of light depolarization (to ≤0 mV), the magnitude of I_A did not return to its previous maximum value (e.g., during the first of the five steps) even after 40 s or more (Figs. 4, 5). This was not the case for I_A following five steps of depolarization alone (Fig. 4, 5). The decrement of I_B following five steps of light-depolarization also did not show this persistence (Fig. 5). With progressively more positive holding potentials, the persistence of I_A decrement following five light-depolarization steps increased (Fig. 8) even though the steps were to the same final potential for all holding potentials (for five out of five cells).

Injection of Ca^{++}

Iontophoretic injection of Ca^{++} into the cells caused persistent and substantial decreases (−38.2% ± 16.2 SD) of I_A for five out of five cells studied (Fig. 6). Command pulses to measure I_A and I_B were given at 60–90 s intervals. I_B was only slightly decreased following these injections but I_{Na^+} induced by light (cf. Alkon, 1979) was almost eliminated (Fig. 7). The changes of I_A and I_{Na^+} usually reversed

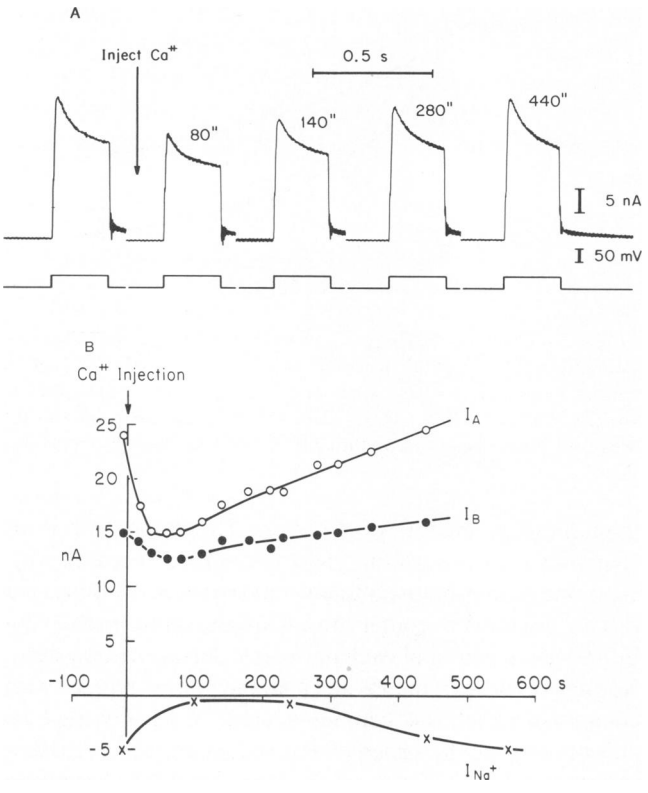

FIGURE 6 (*A*) Response of B photoreceptor to potential steps to −5 mV before and after Ca^{++} injection. First measurement of postinjection membrane current occurred at 80 s. I_A is determined from peak value while I_B is determined from current amplitude at the end of the step (approximately 250 ms). I_A is 95% inactivated at this time (Shoukimas and Alkon, in preparation). Numbers over current records are times in seconds after Ca^{++} injection. (*B*) Effects of iontophoretic injection of Ca^{++} on Type B currents. I_A (in response to a command depolarization to −5 mV) and the light-induced inward Na^+ current decrease markedly following Ca^{++} injection. Both currents take several minutes to return to original values. I_B is affected much less by the Ca^{++} injection.

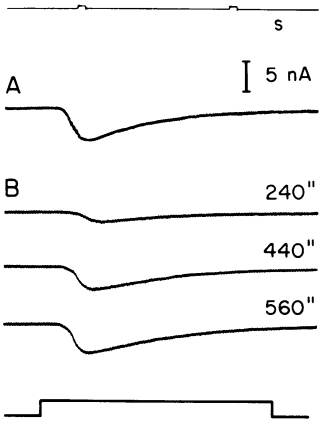

FIGURE 7 Effect of iontophoretic injection of Ca^{++} on light-induced inward Na^+ current. (*A*) Before injection. (*B*) After injection. Note that I_{Na^+} is markedly reduced and shows a prolonged rise to peak following injection. Numbers indicate seconds following injection. These records are for the same cell as in Fig. 6.

within several minutes after Ca^{++} injection (Figs. 6, 7).

Iontophoretic injection of Mg^{++} (see Methods) caused little or no decrease of I_A or I_B for five out of five cells studied. I_{Na^+} induced by light was, however, markedly reduced or eliminated. The I_{Na^+} effect was only partially reversible.

DISCUSSION

The results can be explained as follows. An inward, light-induced Ca^{++} current only occurs when light is paired with the positive command steps. This voltage-dependent current does not occur at the cell's resting potential (cf. Alkon, 1979). When Ca^{++} enters the cell it intensifies and prolongs the decrease of I_A, which occurs during repetitive positive command steps alone. Peak I_A decreases with repetitive depolarization because the interpulse interval is too short to allow inactivation to recover to resting levels. Several of the early outward molluscan K^+ currents show voltage-dependent inactivation (Connor and Stevens, 1971; Neher, 1971). However, this is the first early outward K^+ current whose inactivation appears to be modulated by calcium. Each of the tests in this paper gave results consistent with this hypothesis.

I_B, unlike I_A, decreases during a light step for a holding potential of -60 mV (Alkon et al., 1982). Leonard and Lisman (1981) have made a similar observation for the delayed K^+ current in *Limulus* ventral photoreceptor. The light-dependent decrease in I_B is consistent with the observations presented here that unpaired light steps affect I_B (but not I_A) rates of decay. These findings together with the absence of the voltage-dependent, light-induced Ca^{++} current at -60 mV (Alkon, 1979) and the small effect of Ca^{++} iontophoresis upon I_B reported here suggest that free, intracellular calcium levels do not greatly affect I_B. Barium is more permeable than calcium ions in calcium channels of *Helix* neurons (Akaike et al., 1978). Barium is also known to be a potent blocker of K^+ channels, both internally (Eaton and Brodwick, 1980) and externally (Schwindt and Crill, 1980). That barium replacement for calcium eliminates the light dependent effect on I_A and also abolished I_B suggests that barium enters the photoreceptor but does not substitute for calcium at whatever site influences the gating of I_A. External block of I_B is not ruled out, but it is likely that barium can block from the inside. This differential block of early and late K^+ currents by barium is much greater than the difference between 4-aminopyridine or tetraethylammonium ion potency upon early and late K^+ conductances (Thompson, 1977; Shoukimas and Alkon, in preparation). This makes barium an extremely useful tool for pharmacological separation of these currents.

At present we do not know whether calcium reduces the number of I_A channels opened by depolarization or whether the actual inactivation vs. potential relation is altered. In the first case the number of open A channels would be reduced through direct block by internal calcium, elevated by means of the flux through the light and voltage-dependent calcium channels. If the concentration of internal calcium rises with repeated, paired light and depolarization, then one would expect to see cumulative, increased block of the A channels on the basis of the law of mass action alone. The second would be more like the shift in conductance versus voltage relations seen with charge screening. The effect of calcium upon sarcoplasmic reticulum (SR) K^+ channels (Miller, 1978) supports the first of these two hypotheses. In the SR preparation the magnitude of the conductance is clearly reduced and we suggest by analogy that the A conductance is also blocked by Ca^{++} ions.

The effects we report here of direct calcium injection into Type B photoreceptors on the light-induced Na^+ current are similar to the effect calcium has upon the light response in *Limulus* photoreceptors (Fein and Lisman, 1974; Lisman and Brown, 1975; Fein and Charlton, 1977) and support the hypothesis that Ca^{++} is intimately associated with adaptation. It is interesting that Mg^{++} ions also cause the same effect, possibly indicating a relatively nonspecific binding site. In contrast, we have found that Ca^{++} and Mg^{++} do not have similar effects upon I_A. This difference in the effect of Mg^{++} injection on I_A and I_{Na^+} suggests that different mechanisms underlie the effect of Ca^{++} injection on the two currents. The two currents may also have different sensitivity to small elevations of intracellular Ca^{++}, particularly in combination with repetitive membrane depolarizations. These differences may also help explain the long-term reduction of I_A but not I_{Na^+} during retention of associatively learned behavior (Alkon et al., 1982; West et al., 1982).

The Ca^{++}-dependence and persistence of the I_A decrease demonstrated here provide important insight as to how the previously observed decreases in I_A (and behavioral changes) arise during acquisition and retention of *Hermissenda's* associative learning. Depression of I_A during and following a single pairing of light with rotation may be small and comparable to that produced in the present study by light paired with positive command pulses from a holding potential close to the cell's resting membrane potential (Figs. 4, 5). With repeated pairing of light and

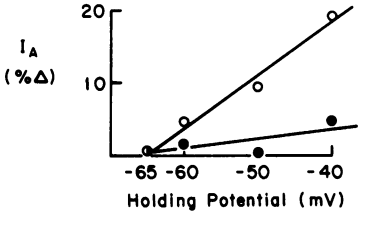

FIGURE 8 Effect of holding potential on persistence of I_A decrement. The percent change of I_A was measured 40 s after the last of five command depolarizations alone (●) and paired with light (○). Command steps were to the same potential (0 mV for 2.2 step and +10 mV for the 800 ms step) but from different holding potentials. Note that the more positive the holding potential, the larger the percent change 40 s after the five step series.

rotation the membrane potential of the Type B cell undergoes cumulative depolarization (Alkon, 1980; Crow and Alkon, 1980). Depression of I_A during and following this cumulative depolarization could become much greater and comparable to that produced in the present study by light paired with command pulses from a holding potential 15–20 mV more positive than the cell's resting potential (Fig. 8).

Intracellular Ca^{++} accumulating in the cell (cf. Connor and Alkon, 1982) could be responsible for progressively larger and more prolonged decreases of I_A, ultimately leading to a decrease which persists for many days (Alkon et al., 1982). This elevation of Ca^{++}, however, itself, may be relatively transient while its effects perhaps via a biochemical transformation may be long-lasting. A promising example of such a transformation depends on activation of a Ca^{++}-calmodulin-dependent protein kinase. Iontophoretic injection of this enzyme into the Type B cell under voltage clamp was found to reduce I_A more than I_B and did not decrease the light-induced Na^+ current (Acosta-Urquidi et al., 1982).

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