Regulation of Short-Term Associative Memory by Calcium-Dependent Protein Kinase

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Neural and behavioral correlates of an associative memory in *Hermissenda* were examined during induction and/or formation of the memory. *Hermissenda* received either light (conditioned stimulus or CS) and rotation (unconditioned stimulus or US) paired (i.e., Pavlovian conditioning), light and rotation unpaired (pseudoconditioning), or no exposure to light and rotation. Following 9 pairings in a 6 min session, conditioned animals exhibited a contraction of the foot in response to a test CS presented 2 min after the last conditioning trial, whereas pseudoconditioned and untreated animals exhibited a foot extension to the same CS. In addition, both an associative and a nonassociative reduction in light-induced locomotion was observed. To examine neural correlates of this learning within minutes of acquisition, the isolated nervous system of the *Hermissenda* (containing the visual and vestibular organs) was trained with stimulus conditions identical to those used for the intact animal. Prior isolation and preparation of the nervous system permitted immediate intracellular recording following the final conditioning trial. Relative to pseudoconditioned and untreated animals, the B photoreceptors in conditioned nervous systems were found to have elevated input resistance (inversely related to K+ channel conductance and positively related to excitability) and exhibited increased steady-state depolarization in response to the light CS, as well as a prolonged depolarization after the CS offset. These neural correlates of the associative memory were attenuated if the protein kinase inhibitor H7 was present in the extracellular bath during conditioning, demonstrating in the reduced preparation that antagonism of protein kinase activity blocks the induction of membrane alterations of identified neurons that correlate with memory storage. In a final experiment, *in vitro* conditioning was conducted in which discrete light steps were presented paired or unpaired with depolarizing current injected across the B cell soma membrane (to simulate rotation-induced depolarization). A pairing-specific, cumulative depolarization (presumably Ca2+-induced) of the B cell was observed that was accompanied by, and outlasted by, an increase in membrane resistance that persisted for at least 10 min. The increase in resistance (but not the cumulative depolarization) was blocked by H7 and sphingosine, which inhibit the activity and activation, respectively, of protein kinase C (PKC) and calmodulin-dependent protein kinase (CaM kinase). In contrast, HA1004, an inhibitor of the cAMP-dependent protein kinase, did not block the induction of elevated resistance. These results suggest that regulation of ion channel conductance by PKC and/or CaM kinase is an important early stage in the induction of associative memory in *Hermissenda*.

Increasing evidence suggests that neuronal excitability may be modulated by protein phosphorylation of ionic channels (e.g., Levitan, 1985; Naito et al., 1988). It has been suggested that persistent changes in protein phosphorylation that outlast the initiating signal may be an important initial stage in the acquisition and storage of memories (Neary et al., 1981; Schwartz and Greenberg, 1987; Lisman and Goldring, 1988; Naito et al., 1988). This combined process has been proposed as an underlying mechanism in the storage of nonassociative (Kandel et al., 1986) and associative memories (Alkon, 1984, 1989), as well as long-term potentiation, or LTP (Malinow et al., 1988), a potential model of vertebrate learning.

In the nudibranch mollusk *Hermissenda,* several lines of evidence have implicated the second messenger-regulated protein kinase C (PKC) and/or calmodulin-dependent protein kinase (CaM kinase) in the reduction of ionic currents that appear to mediate associative learning. During acquisition of a light-rotation association (behaviorally indexed by reduced phototaxis and foot contraction in the presence of light), the B photoreceptors of the *Hermissenda*'s eye undergo a transient, depolarization-induced rise in levels of intracellular Ca2+ (Connor and Alkon, 1984). This increase in free Ca2+ results in phosphorylation of specific proteins (e.g., 20 kDa) in these cells (Neary et al., 1981; Nelson and Alkon, 1989) that have been related to the inactivation of a fast, voltage-dependent K+ current (I_A) and a slower, Ca2+-dependent K+ current (I_C). Reduction of these currents decreases conductance across the photoreceptor's membrane and thereby increases the cell's resistance to current and, hence, its excitability, which is thought to bias the neural network toward new behavioral responses.

In the *Hermissenda*'s eye (containing 3 B cells and 2 A cells), phosphorylation of the 20 kDa phosphoprotein has been found to increase at least 2-fold in associatively trained animals (Neary et al., 1981). Further studies have indicated that this protein is phosphorylated *in vitro* by both PKC and CaM kinase. Moreover, recent work has found that direct iontophoretic injection of the 20 kDa protein (Nelson et al., 1990), of phorbol ester, an

activator of PKC (Alkon et al., 1986; Farley and Auerbach, 1986), or of PKC itself (Farley and Auerbach, 1986; Alkon et al., 1988), all result in a reduction of I_A and I_C that resembles the reduction normally occurring during associative learning.

In the present experiments, we sought to determine the extent to which PKC and/or CaM kinase regulated the initial storage of short-term (2–10 min) conductance changes indicative of associative memory, and we correlated these neuronal changes with associative behavioral responses. In contrast to earlier reports that activators of PKC and CaM kinase induced conductance changes that mimic those occurring during associative learning, in the present experiments we directly observed the effects of inhibition of protein kinase activation on the acquisition of associative memory. Intact, semi-intact, and in vitro conditioning procedures were employed so that both behavior and protein kinase activity could be examined in parallel. As we were specifically concerned with the induction of the associative memory (as opposed to its maintenance), the conditioning procedures were specifically designed to provide the opportunity to monitor behavioral and cellular responses during and/or immediately after conditioning.

Materials and Methods

Animals. Hermissenda crassicornis (0.65–1.10 gm) were obtained from Sea Life Supply Co. (Sand City, CA). Animals were individually housed in 50-ml flow-through tubes suspended in refrigerated artificial sea water (ASW) kept at 12°C. The animals were maintained on a 12 hr/12 hr light/dark cycle. The intensity of the light where it reached the surface of the water was 20 μW × cm^{-2} and was filtered through yellow acetate (Visual Systems, Rockville, MD, stock 13-01053). All experimental manipulations were conducted during the middle 10 hr of the light cycle.

Behavioral training and testing. After at least 5 d in our laboratory, animals were placed in individual tracks (15.0 × 0.9 × 0.6 cm) milled in clear Plexiglas. The tracks were filled with refrigerated ASW and covered by a clear Plexiglas lid. A field of 16 tracks could be mounted on a Lab Line Instruments shaker (model 4600), which was calibrated to produce a 1000 rpm, 4.0-mm-displacement orbital shaking motion that served as the unconditioned stimulus (US). The shaker was housed in a sound- and light-proof incubator that maintained a temperature of 12°C. Sixty centimeters above the tracks was an electronic shutter through which the emitted light from a 21.5 V, 150 W (nominal) tungsten halogen projector lamp could be passed. With the shutter open, the output of the bulb was adjusted so that the intensity of the light that reached the tracks was 600 μW × cm^{-2}. This light served as the conditioned stimulus (CS). Animals that received paired ($n = 18$) or unpaired ($n = 18$) CS–US presentations were placed in individual tracks where they remained in a darkened incubator for 10 min. Paired (i.e., associatively conditioned) animals then received 9 CS–US pairings at 40-sec intervals. A pairing consisted of a 4 sec light CS presentation that coterminated with 3 sec of rotation. Animals that received unpaired training were exposed to these same stimuli on an explicitly unpaired schedule of alternating CSs and USs at 20-sec intervals. A third group, designated as "naive" ($n = 18$), were placed in tracks but simply maintained in darkness for 10 min. After the last stimulus presentation (or the 10 min of dark adaptation for naive animals), the field of tracks was placed on a stand (still in darkness) that allowed the foot of the animals to be videotaped (during a light CS) by a Panasonic WV-CD 50 video camera fitted with an 8 mm 1:1.4 lens mounted 25 cm from the underside of the tracks. Two and 4 min after the end of the training session, the light CS was again presented, each time in the absence of rotation. Within 100 msec of the onset of the light, and again 100 msec prior to its offset, the length of the animal's foot was measured. Normally (prior to conditioning), these animals exhibit a foot extension when a light is presented in periods of darkness. However, it has been reported that after pairings of light and rotation, the light elicits foot *contraction*, a clinging response normally elicited by rotation [Lederhendler et al., 1986; Matzel et al., in press (b)]. In addition, rate of locomotion was measured during the 10 sec light presentations, as general decreases in locomotor behavior in the presence of light have also been reported following light–rotation pairings [Matzel et al., in press (a)]. The videotapes were scored by an observer who had no knowledge of the treatment conditions of the various groups. This experiment was conducted in several balanced replications.

Training of intact nervous systems. In order to observe neural correlates of memory within minutes of conditioning, it was necessary to prepare the nervous system of the Hermissenda for electrophysiological analysis prior to the conditioning session. The nervous system (which includes the visual and vestibular organs) was dissected out of the Hermissenda, secured to a glass slide with several pins, and treated with 1 mg/ml protease (Sigma, P-8038; dissolved in ASW) for 6 min at 22°C. Proteolysis of the connective tissue sheath facilitated subsequent microelectrode penetration. Dissection was conducted under as low a level of light as was possible. Following proteolysis, a single nervous system was placed in an ASW-filled dish which was then secured to a shaker in a darkened incubator in which the temperature was maintained at 18°C. Three groups of nervous systems ($ns = 6$) were subjected to the same conditioning regimens (paired, unpaired, naive) that were previously described for the whole animal. A fourth group of animals ($n = 6$) was also trained with paired light and rotation, but 100 μM of H7 (Sigma, I-0260), a compound that inhibits the catalytic activity of protein kinases (Hidaka et al., 1984), was added to the ASW bath after proteolysis and for the remainder of the experiment. At the end of the training session, the medial B cell was impaled with a glass microelectrode, and input resistance and responses of the B cell to light were measured 1 min after impalement. Impalement was conducted under dim red light (630 nM) that resulted in a depolarization of the B cell of approximately 10% of the magnitude elicited by the white light test stimulus, with no measurable long lasting depolarization (LLD) measured (in all experiments) 20 sec after the offset of the light. Cells that were not successfully impaled within 5 min of the end of training were discarded.

Electrophysiology. Electrophysiological recordings were obtained using previously published methods (e.g., West et al., 1982). Microelectrodes filled with 3 M KCl had a resistance of 15–30 MΩ and were connected by a chloridized silver wire to the input stage of a high impedance amplifier. Voltage responses were acquired via oscilloscope photography and on a Brush pen recorder. To measure input resistance, 300-msec positive and negative current pulses (0.2, 0.4, and 0.6 nA) were applied through the recording electrode using a balanced bridge circuit. The voltage response was measured 250 msec after the onset of the current. An unfiltered white light (focused on the nervous system) of the same intensity as the light CS used for behavioral training/testing was used to assess the B cell light response. All experiments were conducted at 18°C.

In vitro *conditioning.* The nervous system of the animals was removed, and all impulse activity and synaptic interactions of the medial B cell were eliminated by severing the cell's axon approximately 60 μm from the soma. This procedure produces no measurable changes of light-induced voltage responses (Alkon, 1979). Following dissection, the preparation was subjected to proteolysis as described above. Following impalement with a microelectrode, the cell was maintained in the dark for 10 min. Input resistance was then measured, and conditioning was begun. Each cell received either paired or unpaired presentations of a 4 sec light exposure and 3 sec of positive current injection (approximately 1–2 nA) that was sufficient to depolarize the cell 20–25 mV from its normal rest of −40 to −50 mV. This level of depolarization is sufficient to partially activate a voltage-dependent Ca^{2+} conductance even in the absence of light (Alkon et al., 1984; Farley, 1988). For the paired condition, 9 light-current injections occurred at 40-sec intervals. For the unpaired condition, the light and current injections alternated at 20-sec intervals. Pairings of light and depolarizing current were intended to simulate the effects on the B cell of pairings of light and rotation in the intact animal. This procedure has been previously shown to produce cumulative depolarization (Alkon, 1980), an increase in input resistance, and a prolonged LLD in the B cell. When employed in an intact animal, it has also been found to evoke an associative behavioral change in response to light (Farley et al., 1983).

These preparations were maintained in 1 of 4 bathing solutions: ASW (paired, $n = 6$; unpaired, $n = 5$), ASW with 100 μM H7 added (paired and unpaired, $ns = 5$), ASW with 100 μM sphingosine (Sigma, S-3263) added (paired, $n = 6$; unpaired, $n = 5$), or ASW with 100 μM of the cAMP-dependent kinase inhibitor HA1004 (Seikagaku, 120804) added (paired and unpaired, $ns = 6$). While H7 inhibits the catalytic activity of activated PKC and CaM kinase, sphingosine competes with diacylglycerol for PKC activation, although it has also been shown to function
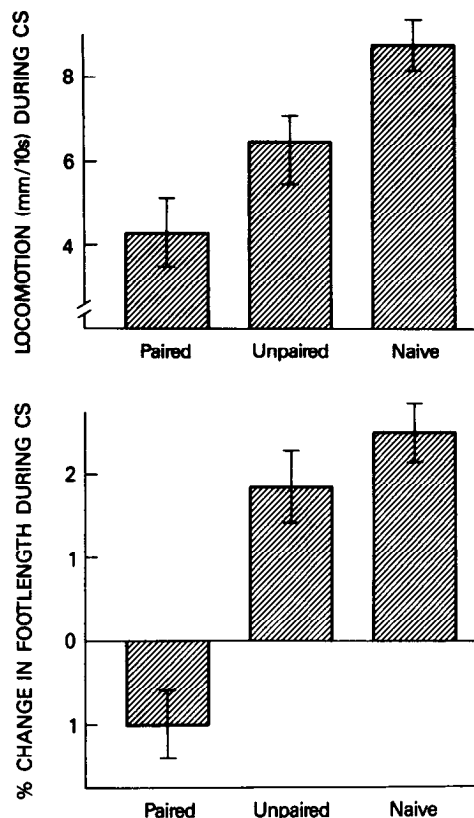
Figure 1. Mean response to 10-sec test light by animals previously exposed to paired light and rotation (9 trials), unpaired light and rotation, or no light and rotation. Responses were measured 180 and 360 sec after the end of the conditioning session and have been collapsed across the 2 times. The *top panel* illustrates the rate of locomotion during the light and the *bottom panel* the percent change in the length of the animals' foot during the middle 9.8 sec of light. Paired light and rotation resulted in generally lower rates of locomotion and a foot contraction relative to the unpaired or naive groups. Note a nonassociative component to the locomotion response absent from the footlength response. *Brackets* indicate standard error.

as a calmodulin antagonist (Jefferson and Schulman, 1988). In contrast, HA1004, although chemically similar to H7, is relatively ineffective against PKC but is a potent inhibitor of the cAMP-dependent protein kinase (i.e., A Kinase; Hidaka et al., 1984). The bathing solution was exchanged at approximate 5 min intervals throughout the experiment.

Statistical analysis. Single- or multi-factor ANOVAs were conducted for each experiment. Follow-up tests based on the error term of the overall ANOVA (i.e., planned comparisons; Keppel, 1982) were used for comparison of individual means.

Results

Behavior

Figure 1 depicts the behavioral responses to light following paired, unpaired, or no exposure to light and rotation. The configuration of the foot during the CS (Fig. 1, *bottom panel*) was influenced by treatment [$F(2,40) = 5.85, p < 0.01$]. Two minutes following the end of the training session, paired exposure to light and rotation resulted in a foot contraction in the presence of light, whereas unpaired or no exposure resulted in a foot extension. The paired group differed from each of the nonassociative control groups [$F(1,40) \leq 6.23, p < 0.05$]. The control groups, however, did not differ from one another ($F = 0.44$).

From the locomotion data, a significant effect of treatment was observed [$F(2,51) = 7.52, p < 0.001$]. Paired exposure to light and rotation resulted in significantly less locomotion in the
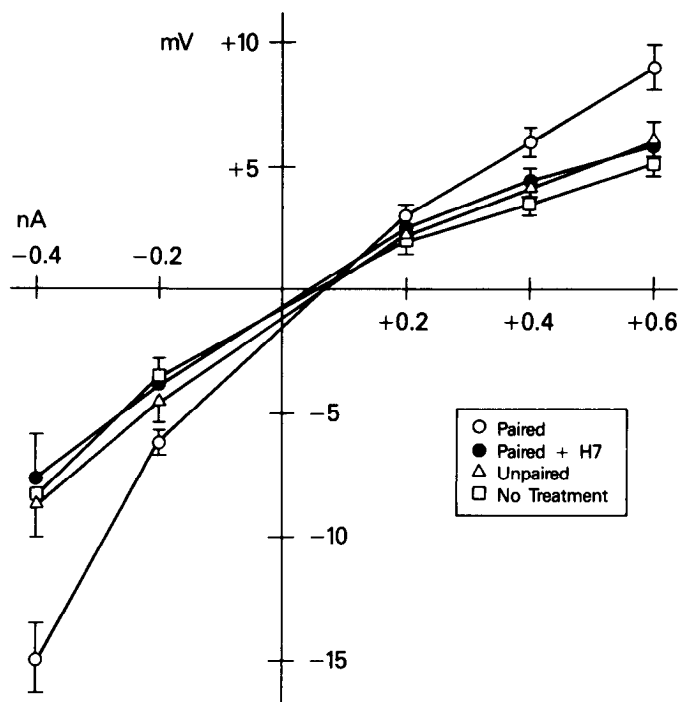

Figure 2. A current–voltage plot of the medial B photoreceptor approximately 5 min after paired, unpaired, or no exposure of the isolated nervous system to light and rotation. Equivalent current injections result in an elevated voltage response (i.e., increased resistance) if the animals were exposed to light–rotation pairings (i.e., associatively conditioned), as indicated by the *open circles*. However, the increase in resistance was blocked if 100 μM of H7, a PKC and CaM kinase antagonist, was present in the bathing solution during the training session (*filled circles*). *Brackets* indicate standard error.

presence of the light relative to naive animals [$F(1,51) = 15.05, p < 0.001$]. However, paired training resulted in only marginally slower locomotion than did unpaired training [$F(1,51) = 3.47; p < 0.10$]. Moreover, unpaired training resulted in marginally slower rates of locomotion relative to naive animals [$F(1,51) = 4.06, p < 0.10$]. These results suggest that 2 min following conditioning, there is both an associative and a nonassociative influence of conditioning on the rate of locomotion.

The present results, like prior work [Matzel et al., in press (b)], suggest that foot contraction and decreased locomotor behavior are independent responses to the CS following CS–US pairings. As in a previous report by Crow (1983), we have found evidence for a nonassociative reduction of locomotion shortly (2 min) after conditioning (cf., Grover et al., 1987). No nonassociative influence on locomotion is typically observed at retention intervals of 1 hr (e.g., Crow, 1983) or longer [Crow and Alkon, 1978; Matzel et al., in press (a)]. However, the present results indicate both an associative and a nonassociative component to the short-term locomotor response. In addition, foot contraction (an active response) developed as a short-term conditioned response that did not appear to be influenced by nonassociative factors.

Conditioning of intact nervous systems

Figure 2 illustrates the voltage responses of the B cell soma membrane to current injection 4–8 min following conditioning. At that time, no differences in the resting membrane potential were detected ($F = 0.86$). However, membrane resistance was
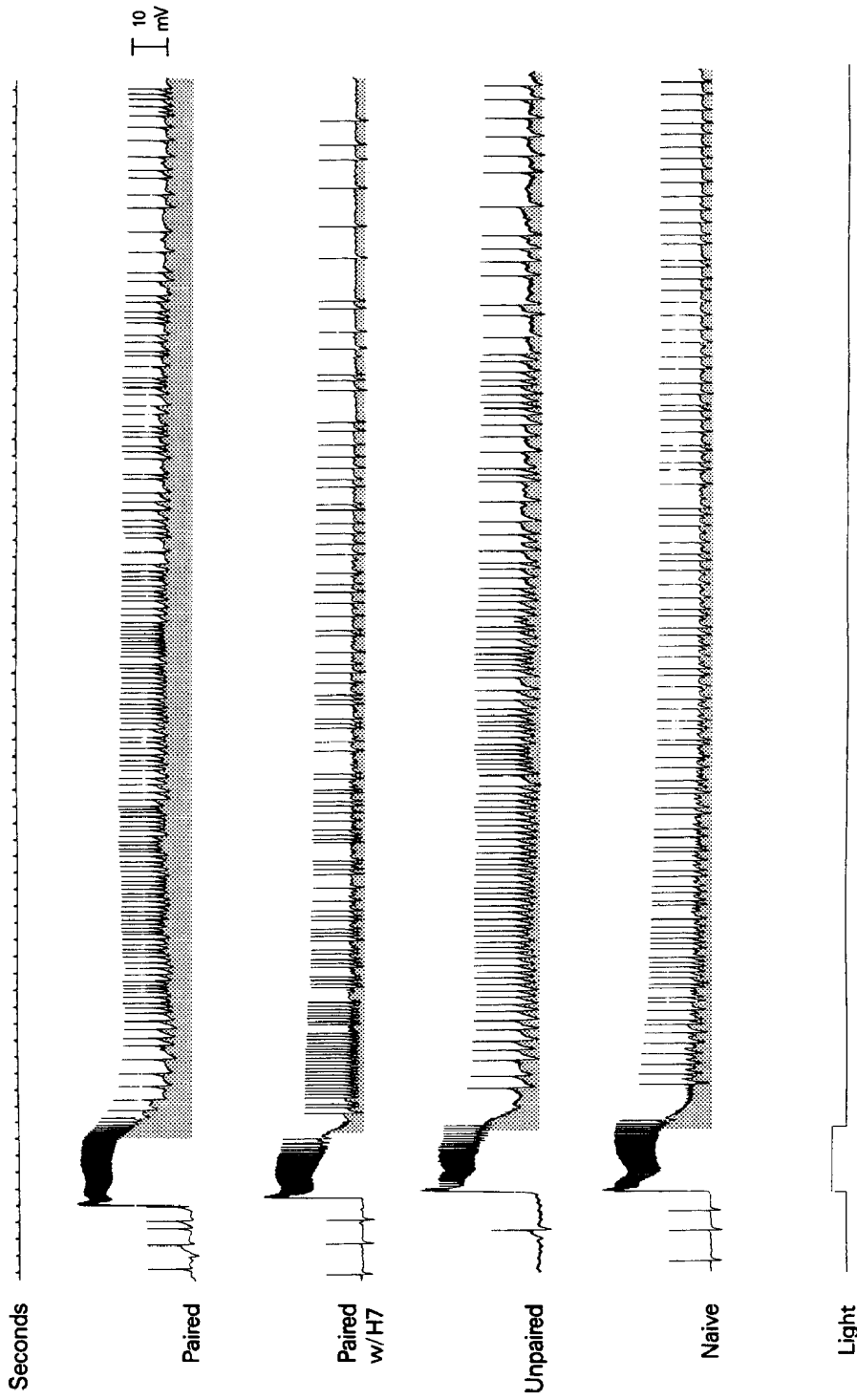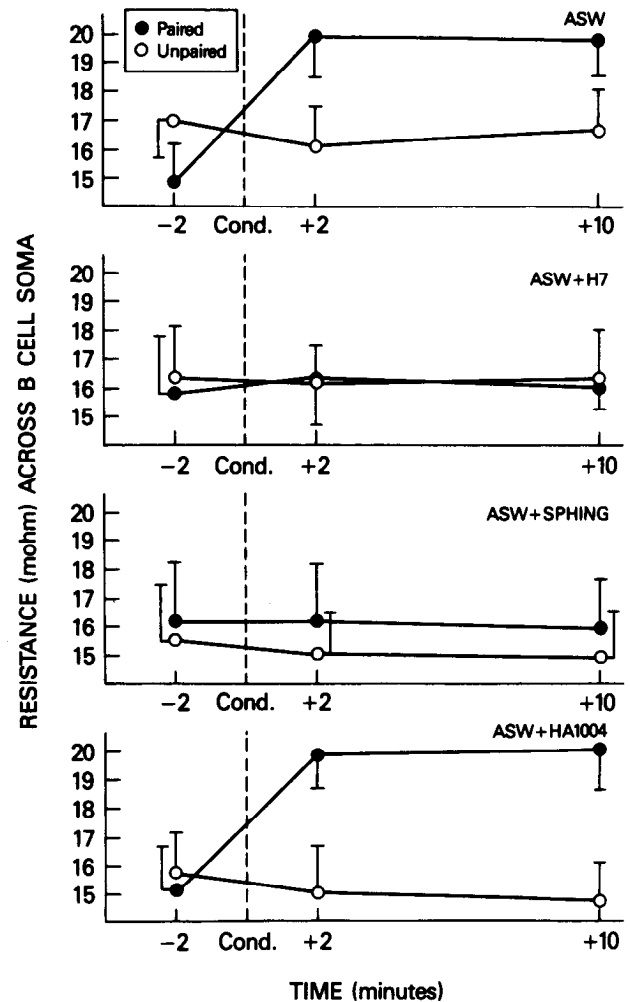
Figure 3. Representative light responses taken from B photoreceptors following training of the isolated nervous system with either paired, unpaired, or no exposure to light and rotation. *Shaded areas* indicate depolarization above the resting potential after the offset of the light. Pairings of light and rotation resulted in a greater steady-state light response and an enhanced depolarization after the offset of the light. These responses are influenced by the resistance of the soma membrane, which reflects ion channel conductance. Note that there is no elevation of the steady-state response or the long-lasting depolarization if light–rotation pairings occurred in the presence of H7.
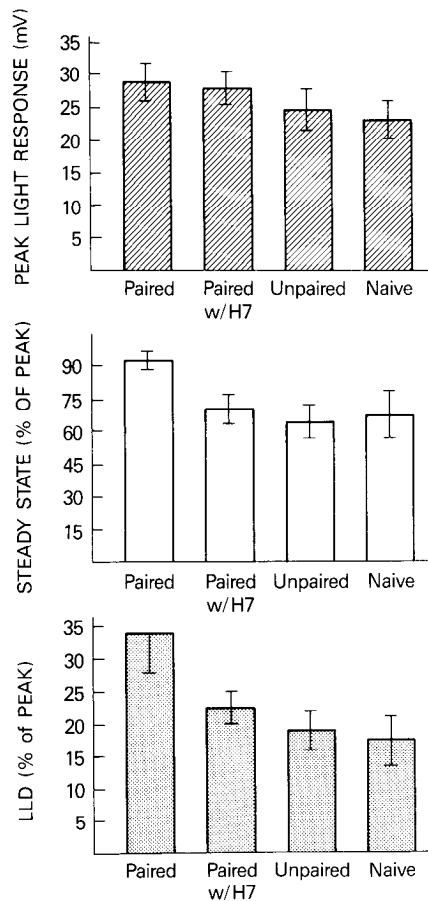
Figure 4. Group means of 3 responses of medial B cells to a 4-sec light taken approximately 5 min after paired, unpaired, no exposure of the isolated nervous system to light and rotation, or paired exposure with H7 present in the ASW bath. The peak light response is illustrated in the *top panel*, the steady-state light response (as a percentage of the peak) in the *middle panel*, and the level of depolarization (as percentage of the peak) in the *bottom panel* 20 sec after the offset of the light. While the peak response did not differ, the steady-state and the long-lasting depolarization was enhanced in the B cells exposed to pairings, provided that H7 was not in the bath during conditioning.

Figure 5. Group means of resistance 2 min before, 2 min after, or 10 min after either paired (*filled circles*) or unpaired (*open circles*) exposure of the isolated B photoreceptor to light and depolarizing current. This procedure mimics the effects on the B cell of light-rotation presentations in the intact nervous system. These treatments were conducted with either ASW, H7, sphingosine, or HA1004 (all 100 μM) present in the bathing solution. Although none of the latter 3 compounds affected the resting membrane potential or basal levels of resistance, H7 and sphingosine, which inhibit the activity and activation (respectively) of PKC, blocked the increase in resistance that occurred with conditioning. This was not true of HA1004, a cAMP-dependent protein kinase inhibitor.

significantly affected by treatment [$F(3,20) = 3.62$, $p < 0.05$]. Animals that received paired light and rotation were found to have higher membrane resistance than did animals that received unpaired, no exposure, or paired exposure to those stimuli in the presence of the protein kinase inhibitor H7 [$F(3,20) \geq 6.14$, $p < 0.05$]. These latter 3 groups did not differ among themselves ($F \leq 0.30$).

Representative responses of the B photoreceptor to a 4-sec light flash are depicted in Figure 3. The peak light response did not differ between groups ($F = 0.33$). In contrast, the LLD, measured as the percent of the peak light response 20 sec after the offset of the test light, differed between groups [$F(3,20) = 3.22$, $p < 0.05$]. Comparisons of individual means indicated that paired training resulted in a greater LLD than did unpaired or no training, or paired training in the presence of H7 [$F(1,20) \geq 5.62$, $p < 0.05$]. These latter 3 groups did not differ among themselves ($F \leq 0.83$). The steady-state light response of paired animals, measured at asymptote approximately 2 sec after the onset of the light and converted to a percentage of the peak light response, was also greater than that exhibited by unpaired and

untreated animals [$F(1,20) \geq 4.36$, $p < 0.05$] and was marginally greater than in paired animals in which H7 was present in the bath [$F(1,20) = 3.35$, $p < 0.10$]. Group means for these light responses are illustrated in Figure 4.

In vitro *conditioning*

Like presentations of light paired with rotation to the isolated nervous system, pairings of light and depolarizing current to the isolated B cell resulted in an increase in resistance if the pairings occurred in normal ASW, as depicted in the top panel of Figure 5. This effect was blocked by H7 (second panel), presumably via inhibition of the catalytic activity of PKC and/or CaM kinase, or if the activation of these kinases was blocked by sphingosine (third panel). However, the cAMP-dependent kinase inhibitor HA1004 was ineffective in blocking the effects of this *in vitro* conditioning procedure.
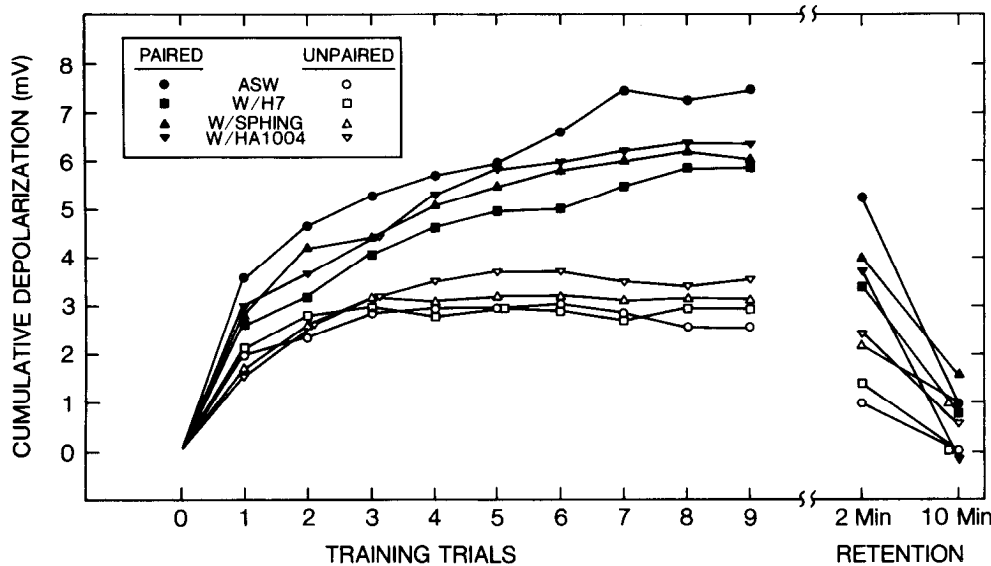
Figure 6. Mean accumulation of depolarization during paired or unpaired presentations of light and depolarizing current to the isolated B photoreceptor for each of 4 bathing mediums (ASW, or ASW with H7, sphingosine, or HA1004 added). Membrane potential was measured 20 sec after light offset. The sustained depolarization is presumably mediated by a rise in intracellular Ca^{2+}. Regardless of the bathing medium, pairings resulted in a progressive increase for at least 5 trials for cells exposed to paired stimuli. In contrast, the weaker accumulation was largely complete after the second trial in cells exposed to unpaired stimuli. Following the last pairing, the depolarization was still evident 2 min later but had dissipated by 10 min in all groups.

These observations were validated by statistical tests. A 3-factor ANOVA of treatment (paired or unpaired), bathing medium (ASW, H7, sphingosine, or HA1004), and time of observation (before conditioning, 2 min after conditioning, or 10 min after conditioning) was conducted. No main effects of treatment or bathing medium were found, indicating that the drugs employed had no effect on the baseline resistance of the cells. A main effect of time of observation was observed [$F(2,72)$ = 6.36, $p < 0.01$]. Moreover, time interacted with the type of bathing medium [$F(4,72)$ = 2.67, $p < 0.05$] and with treatment [$F(2,72)$ = 14.55, $p < 0.001$]. Finally, a treatment × medium × time interaction was observed [$F(6,72)$ = 4.29, $p < 0.001$]. These interactions indicate that, although the groups had initially comparable levels of resistance, resistance increased as a function of CS–US pairings relative to when the CS and US were unpaired. However, this effect was dependent on the solution in the bathing medium, as the increase in resistance was attenuated by H7 and sphingosine, but not HA1004.

In the intact, isolated nervous system, pairings of light and rotation produce cumulative depolarization of the B photoreceptor (Alkon, 1980). A strong, progressive depolarization was observed in the present experiment during light–current pairings, but a weaker depolarization was observed if the light and current injections were unpaired. These results are plotted in Figure 6 as a function of conditioning trials and retention interval for cells having received either paired or unpaired light and current presentations in each of the 4 bathing mediums. It is conceivable that H7 or sphingosine attenuated conditioning by disrupting this cumulative depolarization. This, however, was not the case, as comparable levels of cumulative depolarization were observed in all groups exposed to light–current pairings, as was the weaker depolarization in the groups exposed to light and current unpaired. An ANOVA was conducted on the resting membrane potentials of cells exposed to the paired and unpaired regimen immediately prior to the first stimulus exposure, 20 sec after the final stimulus exposure, 2 min after the final final exposure, and 10 min after the final exposure. A significant effect of time of observation was observed [$F(3,108)$ = 114.7, $p < 0.001$], as was an interaction of pairings and time of observation [$F(3,108)$ = 7.33, $p < 0.001$], but no main effect

of pairings ($F = 0.53$). These results are indicative of an initially comparable, but a larger, progressively increasing depolarization undergone as a result of pairings of light and current injection, relative to when light and current were unpaired. No main effect of the drug in the bathing medium was observed ($F = 0.18$), nor did the drug type interact with any other factor ($F \leq 0.86$), indicating that the pairing-induced cumulative depolarization was not affected by the drug in the bath. This latter result suggests that the absence of a resistance increase when conditioning occurred in the presence of H7 or sphingosine was not a result of an antagonism of Ca^{2+} influx, but rather an antagonism of Ca^{2+} interaction with the target kinase.

Discussion

Two conditioned behavioral responses indicative of associative memory were observed within minutes of the completion of 9 training trials. One response, decreased locomotion, appeared to contain both an associative and a nonassociative component, while the other, foot contraction, was exclusively associative. During the time that the short-term conditioned responses were observed, an increase in resistance of the medial B cell soma membrane was detected in cells that were trained in the isolated nervous systems. The medial B photoreceptor synapses on interneurons that in turn synapse on the motor neurons that control turning toward light (Goh et al., 1985). Other evidence suggests that the medial B cell also synapses on interneurons that mediate foot contraction (Akaike and Alkon, 1980).

Resistance is inversely related to K^+ channel conductance and directly related to excitability. This conductance decrease influences the magnitude of the LLD following a light step, as well as the steady-state light response (Alkon, 1984). Each of these responses to light were elevated as a result of light–rotation pairings in the present experiments.

Although a nonassociative influence on locomotion was observed 2 min after conditioning, this response did not appear to arise from the medial B cell, as unpaired exposure to light and rotation did not affect the properties of this cell relative to cells that received no exposure to these stimuli. Since no nonassociative effects of conditioning on the B cell were observed even in the presence of a nonassociative behavioral response,

it would appear that in this system, associative learning, as it occurs in the B cell, is not an enhancement of a nonassociative process.

Conditioning of the isolated nervous system, as well as *in vitro* conditioning of the isolated B cell, indicated that protein phosphorylation, initiated by a cumulative depolarization of the B cell (and probably the associated Ca^{2+} influx), regulated the acquisition of the memory. Memory was indexed by enhanced steady-state response to light, as well as an enhanced LLD after the offset of the light and/or increased input resistance. These correlates of memory were blocked by sphingosine, which competes with diacylglycerol for the activation of PKC, and H7, which inhibits the activity of the kinase. This result suggests the possibility than an initial biochemical step in the memory acquisition is the translocation of PKC from the cytosol to the membrane, where phospholipid, a critical cofactor for its activation, is present (Hannun et al., 1986). In the membrane, phosphorylation of ion channels or associated proteins may result in an increase in neuronal excitability (cf., Alkon et al., 1988). That a Ca^{2+}-dependent protein kinase (PKC or CaM kinase), as opposed to cAMP-dependent kinase, regulated this process was demonstrated by the inability of HA1004 to attenuate the effects of *in vitro* conditioning. Our implication of PKC in this process is consistent with a report by Crow et al. (1989) that chronic down regulation of PKC retards the acquisition of neural correlates of memory in an *in vitro* conditioning preparation.

It is possible that cAMP-dependent protein kinase plays a role in memory acquisition *in vivo* if synaptic interactions are required for its activation (e.g., Schachter et al., 1988; for related data, see Grover et al., 1989). Nevertheless, a Ca^{2+}-dependent kinase appears to be an important regulator of the process underlying the cumulative depolarization-induced formation of memory. Since the magnitudes of the resistance increases were similar whether conditioning was performed on the intact nervous system with the CS and US or simulated in the isolated eye, the *in vitro* simulation employed here may initiate many of the same processes as does conditioning of the intact nervous system with natural CS and US.

As previously described, a wealth of indirect evidence has implicated Ca^{2+}-dependent protein kinase activity in the storage (i.e., at least 24 hr) of associative memory in *Hermissenda*. This evidence includes the analogous biophysical effects on the B cell of conditioning with those induced by activation of C-kinase by phorbol ester and/or direct injection of PKC in conjunction with Ca^{2+} elevation. Here, we report direct evidence that inhibition of PKC and/or CaM kinase blocks the biophysical correlates of memory induction, suggesting that protein kinase activity regulates both the short-term induction and the longer-term maintenance of the associative memory. Related to this latter point, we have recently found that 1 d after light–rotation pairings, membrane-bound PKC activity, as indexed by tritiated phorbol ester binding, is elevated in the medial and intermediate B cells (D. L. McPhie, L. D. Matzel, J. L. Olds, A. W. Kuzirian, and D. L. Alkon, unpublished observations).

An NMDA-mediated rise in intracellular Ca^{2+}, accompanied by an activation of PKC, has been implicated in the induction of LTP in the rabbit hippocampus (e.g., Malenka et al., 1986; Malinow et al., 1988; but see Muller et al., 1988). Like the results reported here, Malinow et al. (1988) found that H7 and sphingosine blocked the induction of LTP, while HA1004 was largely ineffective. Consistent with the hypothesis that H7 inhibits the activity of PKC, Malinow et al. (1988) also reported that es-

tablished LTP could be reversed by the application of H7 but not by sphingosine, which would affect only the activation of the kinase. We have also found that application of H7 within 2 min of the completion of conditioning results in at least a partial reversal of the elevated resistance arising from *in vitro* conditioning in *Hermissenda* (L. D. Matzel and D. L. Alkon, unpublished observations). These observations therefore suggest a possible conservation of aspects of the neural mechanisms supporting LTP in rabbits and classical conditioning in *Hermissenda*.

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