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Protein kinase C inhibitors prevent induction and continued expression of cell memory in *Hermissenda* type B photoreceptors

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ABSTRACT **Injections of cAMP-dependent, Ca^{2+}/calmodulin-dependent, or Ca^{2+}/phospholipid-dependent protein kinases into *Hermissenda crassicornis* type B photoreceptors are sufficient to induce many of the changes in B-cell excitability produced by associative conditioning. We report that inhibitors of Ca^{2+}/phospholipid-dependent protein kinases, but not inhibitors of cyclic nucleotide- or Ca^{2+}/calmodulin-dependent protein kinases, prevent the induction as well as continued expression of learning-produced changes in type-B-cell excitability: reductions of voltage-dependent and Ca^{2+}-activated K^+ currents. Our results represent a direct demonstration of long-term (days) experientially induced modulation of ion-channel activity that is dependent upon persistent kinase activity.**

Activation of diverse protein kinases (PKs) can result in the modulation of ion channel activity, receptor function, and synaptic release mechanisms and thus may contribute to induction of several forms of neural plasticity that have been implicated to underlie short- and long-term memory in several well-studied model systems (1). Although activation of a kinase is typically transient, the resulting phosphorylation of substrate proteins may be persistent and is thought to be primarily limited in duration by the rate of dephosphorylation and protein turnover. However, once activated, some kinases exhibit continued catalytic activity in the absence of factors required for initial activation (2). Such a mechanism might underlie the formation of memory in the brain (2, 3). PK involvement in long-term potentiation at hippocampal synapses, a widely studied cellular model of memory in the mammalian brain, is consistent with this suggestion (4–6). Whether persistent kinase activity also contributes to memory-related changes in synaptic transmission and/or cell excitability in other neural systems is unknown.

Hermissenda type B photoreceptors undergo both short- and long-term changes in neural excitability that contribute to associative conditioning-produced changes in phototactic behavior (7–9). For ≈1 hr following repeated pairings of light and statocyst hair-cell stimulation, type B photoreceptors undergo a pairing-specific depolarization accompanied by an increase in resting input resistance (10). Several days following training, retention of memory in B cells is expressed by enhanced photoresponses of dark-adapted cells and increased resting input resistances (7, 8, 11). These changes have been attributed to learning-produced reductions in two distinct K^+ currents: a transient, voltage-dependent, 4-aminopyridinium (4-AP)-inhibitable current (I_A; ref. 12) and a slower, noninactivating Ca^{2+}-dependent K^+ current ($I_{K\text{-}Ca}$; ref. 11).

A training-produced increase in intracellular free Ca^{2+} has been implicated as an initial trigger for K^+-current reductions in B cells (13), possibly through activation of one or more classes of Ca^{2+}-dependent PK. Both the Ca^{2+}/calmodulin-dependent (CaM PK) (14, 15) and Ca^{2+}/phospholipid-dependent (PKC) (16) families have been implicated from studies demonstrating that injections of exogenous kinases mimicked behavioral training effects, as did injections of Ca^{2+} (13) or applications of phorbol esters (16, 17). The availability of potent and partially specific inhibitors of CaM PKs, cyclic nucleotide-dependent PKs, and PKC has prompted us to ask if inhibition of any of these kinase families prevents short-term *in vitro* conditioning-produced changes in type B photoreceptor excitability, or long-term retention of these changes once induced by behavioral training of intact animals.

MATERIALS AND METHODS

Materials. Staurosporine was from Boehringer Mannheim, phosphatidylserine from Avanti Polar Lipids, H-7 from both Seikagaku (Rockville, MD) and Sigma, and H-8 from Seikagaku. 4-AP, diolein, dimethyl sulfoxide (DMSO), histone H1, sphingosine, *N*-stearoyl-D-sphingosine, Et_4N^+, Tris, W-7, and all salts were from Sigma.

Enzyme. In collaboration with Sidney Auerbach (Department of Biology, Rutgers University), PKC was purified using minor modifications of an FPLC method (19). This enzyme gave a single silver-stained band at 81 kDa upon SDS/PAGE. In the presence of Ca^{2+} (1 μM), phosphatidylserine, and diolein, the enzyme activity transferred 54 pmol of phosphate per min per mg of protein using histone H1 as substrate. PKC purified by FPLC and hydroxylapatite chromatography (20) was obtained from Alexandra Newton (Department of Chemistry, Indiana University). Enzyme activities of fractions I–III (21) ranged between 400 and 600 nmol of phosphate per min per mg, in the presence of Ca^{2+} (0.5 mM), phosphatidylserine, and diolein, using histone H1 as the substrate. All three fractions ran as major 81-kDa bands in SDS/PAGE, with minor bands at ≈50 kDa. For iontophoretic purposes, the voltage-recording electrode in the voltage-clamp circuit was back-filled via capillary action with PKC (fractions I–III were pooled) and a 1.5 M KCl carrier solution. One-minute negative current (1.0–1.5 nA) steps were used to eject the enzyme. The results obtained from the two preparations of enzyme were very similar and have been pooled in the subsequent data presentation.

Intracellular Recording and *in Vitro* Conditioning. Adult *Hermissenda* (Sea Life Supply, Sand City, CA) were maintained in aquaria with artificial seawater (ASW) as described (11). Intracellular recording and *in vitro* conditioning of the isolated *Hermissenda* nervous system were effected using methods fully described elsewhere (10). Prior to *in vitro*

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Abbreviations: ASW, artificial seawater; 4-AP, 4-aminopyridinium; DMSO, dimethyl sulfoxide; NS, not significant; PK, protein kinase; PKC, Ca^{2+}/phospholipid-dependent PK; CaM PK, Ca^{2+}/calmodulin-dependent PK.

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conditioning, a type B photoreceptor and a statocyst caudal hair cell were impaled and the preparation was dark-adapted for 10 min. Resting membrane potentials and input resistances were measured during the final minute of dark adaptation. A preparation was accepted for further study if the resting membrane potential of the type B photoreceptor was more negative than −45 mV and the resting input resistance exceeded 30 MΩ. Each nervous system was then randomly assigned to one of three conditioning treatments. These entailed exposure to five paired, pseudorandom, or explicitly unpaired 30-sec presentations of whole-field illumination (360 $\mu W/cm^2$) and depolarizing current stimulation of a caudal hair cell (1.0–5.0 nA) sufficient to elicit 2- to 10-Hz trains of action potentials. Simultaneous and completely overlapping pairings of light and hair-cell stimulation were administered every 2 min. In the pseudorandom condition 30-sec light presentations were administered at 0, 2.5, 4.0, 6.0 and 8.0 min; while 30-sec hair-cell stimulation periods were initiated at 0.5, 3.0, 4.0, 5.5, and 7.5 min. In the unpaired condition, a 30-sec interval separated light-offset from the onset of hair-cell stimulation on each conditioning trial. The intertrial interval was 2.0 min. Membrane potentials were continuously monitored for up to 5 min following conditioning. Postconditioning input resistances were measured 2–3 min after the last light step. The standard ASW used for *in vitro* simulations of conditioning contained (in mM) 420 Na^+, 10 K^+, 10 Ca^{2+}, 50 Mg^{2+}, 10 Tris·HCl, 570 Cl^-, pH 7.8. When applied in the bath, W-7, H-7, and H-8 were initially made as 10 mM stock solutions in distilled H_2O. Sphingosine and stearoylsphingosine were made as 10 mM stocks in 9.5% EtOH. Staurosporine was made as a 2.2 μM solution in DMSO. These agents were diluted to their working concentrations in standard ASW on the day of use. Solvent effects (DMSO and EtOH) were assessed at concentrations equal to those present in the final diluted drug solutions. In bath-application experiments, preparations were exposed to the inhibitor for ≥30 min prior to *in vitro* conditioning to allow for drug delivery. When iontophoresed into type B photoreceptors, H-7, W-7, or H-8 was present in the recording electrode in a 1.5 M KCl carrier solution at concentrations (50, 50, and 25 μM) approximately equal to their reported K_i values for PKC, CaM PKs, and cyclic nucleotide-dependent PKs, respectively.

Voltage Clamp. Standard two-microelectrode voltage-clamp methods for synaptically isolated type B photoreceptors were used (11, 16). The microelectrodes were pulled from microfilament capillary glass and had input resistances of 6 or 15 MΩ (current-passing and voltage-recording electrodes, respectively) when filled with 1.5–3.0 M KCl. Settling time for the voltage-clamp circuit was 0.5–1.5 msec. A type B photoreceptor was accepted for voltage-clamp analysis, following impalement with two microelectrodes, if resting membrane potential was more negative than −40 mV, holding currents at −70 mV were ≤−3.0 nA, and light-induced current was ≥5.0 nA. I_A was measured in standard ASW as the peak outward current 5–10 msec following command depolarizations from a holding potential of −70 mV. $I_{K\text{-}Ca}$ was pharmacologically isolated from other K^+ currents (11, 16, 22) by substitution of 5 mM 4-AP and 50 mM Et_4N^+ for equimolar reductions in Na^+ concentration. $I_{K\text{-}Ca}$ amplitudes were measured as the steady-state outward current ≈398 msec following command depolarizations from a holding potential of −70 mV. Data acquisition and analysis were performed using pClamp software (version 4.1, Axon Instruments, Burlingame, CA) and an IBM/AT-clone computer. After *in vitro* conditioning of a synaptically intact preparation (as described above), type B photoreceptors ipsilateral to the stimulated statocyst hair cell were synaptically isolated by a razor lesion of the optic nerve. A type B photoreceptor was then impaled, fresh ASW (with or without K^+-channel and/or kinase inhibitors) was applied, and K^+ currents were recorded within 5–20 min following the conclusion of conditioning at room temperature (19–20°C). One experimenter carried out *in vitro* conditioning, whereas the other conducted the voltage clamp. In this way, data acquisition and analysis were performed without knowledge of conditioning history or presence vs. absence of a kinase inhibitor. In all experiments, recordings were obtained from a single type B photoreceptor per preparation. Statistical analyses were performed using methods appropriate for independent samples.

RESULTS

Isolated nervous systems from untrained *Hermissenda* were conditioned *in vitro* (10) in the bath-applied presence or absence of the following PKC inhibitors: H-7 (22), staurosporine (23), or sphingosine (24). Five pairings of light and statocyst hair-cell stimulation in standard ASW resulted in a depolarization of 6.00 ± 0.49 mV (mean ± SEM; $n = 12$) and a 21.7 ± 6.2% ($n = 12$) increase in resting input resistance of type B cells. Pseudorandom or explicitly unpaired stimulus presentations (pooled results) produced a modest 2.00 ± 0.40 mV ($n = 12$) depolarization and a negligible 2.0 ± 4.9% ($n = 11$) increase in input resistance. The depolarization produced by pairings was significantly greater than that produced by controls [$t(22) = 6.15$, $P < 0.001$], as was the input resistance increase [$t(19) = 10.56$, $P < 0.001$]. The results from the two control groups failed to differ in either measure. When conditioned in the presence of 10, 60, or 120 μM H-7, type B cells ($n = 4$, 7, or 4) showed dose-dependent reductions in depolarization (4.4 ± 1.7, 2.6 ± 0.7, and 0.5 ± 1.2 mV, respectively; Fig. 1 *A* and *B*). In these conditions, type B cells also failed to exhibit input resistance increases and instead showed small but consistent decreases (−10.4 ± 0.9%, −10.1 ± 5.0%, and −15.7 ± 8.3%; Fig. 1*C*). Bath-applied 20 nM staurosporine also blocked conditioning-produced depolarization (1.6 ± 0.67 mV; $n = 6$) and input resistance increases (−5.8 ± 1.2%; $n = 6$), as did 60 μM sphingosine (depolarization, 1.7 ± 0.45 mV; input resistance increases, 2 ± 5%; $n = 7$) (Fig. 1*C*). *N*-Stearoyl-D-sphingosine (60 μM), which is generally ineffective as an inhibitor of PKC (24), failed to block conditioning changes in B cells (depolarization, 6.5 ± 1.4 mV; input resistance increases, 18 ± 5.2%; $n = 4$). In other experiments, acute application of H-7 (120 μM; $n = 5$), staurosporine (20 nM; $n = 4$), or sphingosine (60 μM; $n = 5$) to type B photoreceptors from untrained animals failed to affect membrane potentials or resting input resistances, as did the solvents in which these agents were dissolved (0.02% ethanol for sphingosine, $n = 4$; 1% DMSO for staurosporine, $n = 3$).

To determine whether the disruption of conditioning-produced changes in B cells by H-7, staurosporine, or sphingosine was attributable to inhibition of a CaM PK (25) or cyclic nucleotide-dependent PK (23), rather than PKC, we used the anticalmodulin agent W-7, a more potent inhibitor of CaM PKs (IC_{50} = 31 μM; ref. 26) than of PKC (IC_{50} = ≈100 μM; ref. 27), and H-8, a more potent inhibitor of cyclic nucleotide-dependent kinases (K_i = 0.5–1.2 μM) than of PKC (K_i = 15 μm; ref. 22).

When bath-applied at 15, 60, or 120 μM ($n = 11$, 6, 6) W-7 had negligible effects upon conditioning-produced depolarizations (6.7 ± 0.8, 5.5 ± 0.6, 6.7 ± 1.5 mV) and input resistance increases (8.4 ± 3.1%, 17 ± 4.9%, 15.5 ± 7.5%). Preparations treated with W-7 failed to differ significantly from standard ASW controls in either measure of excitability (Fig. 1). H-8 (25 μM) produced a slight though nonsignificant enhancement of cumulative depolarization (7.37 ± 0.65 mV) and comparable input resistance increases (19.4 ± 6.6%) relative to ASW controls. Acute application of W-7 (120 μM;

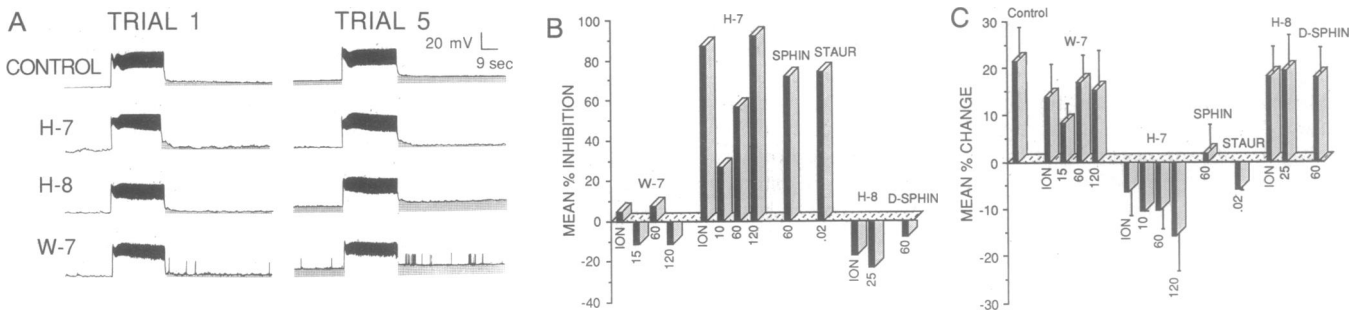

FIG. 1. Reduction of cumulative depolarization and input resistance increases of *in vitro* conditioned type B photoreceptors by PKC inhibitors. (*A*) First- and fifth-trial membrane potentials and light responses of B cells from preparations exposed to pairings of light and statocyst hair-cell stimulation, in the bath-applied presence of the indicated kinase inhibitors. The general kinase inhibitor H-7 (120 μM) completely blocked B-cell depolarization and input resistance increases (data not shown), while a CaM PK inhibitor (W-7; 120 μM) or cyclic nucleotide-dependent PK inhibitor (H-8; 25 μM) did not. Virtually identical results were obtained when H-7, W-7, or H-8 was iontophoresed into B cells prior to conditioning. Prevention of cumulative depolarization and input resistance increases were also observed when preparations were conditioned in the bath-applied presence of other PKC inhibitors [staurosporine (20 nM) or sphingosine (60 μM; data not shown]. (*B*) Summary data for inhibition of cumulative depolarization by kinase inhibitors. Each bar represents the average depolarization observed for ASW controls minus the average observed for the indicated drug treatment, expressed as a percentage of the average control value. All depolarization values were measured 90 sec after the last light step (see text for absolute values). Depolarization was greatly reduced in preparations treated with H-7, sphingosine (SPHIN), or staurosporine (STAUR). W-7 gave small, variable, dose-independent effects. H-8 gave a small but consistent enhancement of cumulative depolarization. *N*-Stearoyl-D-sphingosine (D-SPHIN) had no significant effect. In bath-application experiments, control data were those obtained from preparations conditioned in standard ASW in which no kinase inhibitor was present. In iontophoretic experiments, control data were those obtained from preparations conditioned after exposure to the iontophoresis protocol (carrier-injected) with no inhibitor present in the electrode. Notations under each bar indicate the bath-applied concentration (μM), or iontophoresis condition (ION), for each drug. (*C*) Summary data for inhibition of conditioning-produced increases in resting input resistances of type B cells by kinase inhibitors. Pre- and postconditioning input resistances were estimated for each cell by the straight-line slope of the steady-state voltage–current plot, resulting from injection of negative current steps (−0.10 to −0.50 nA in 0.1-nA increments) into B cells through a balanced and calibrated bridge circuit. Each bar depicts the average post- minus preconditioning input resistance change (MΩ), expressed as a percentage of the average preconditioning value, for the indicated drug and concentration. Error bars = 1 SEM.

$n = 3$) or H-8 (25 μM; $n = 4$) to untrained cells failed to affect membrane potential or input resistance.

Bath application of PKC inhibitors to the entire *Hermissenda* nervous system might preclude learning-produced changes in B cells for a variety of reasons, including inhibition of PKC activity in type B photoreceptor somata or inhibition of PKC-dependent processes presynaptic to type B cells. To address this issue, we iontophoresed kinase inhibitors into single type B photoreceptors prior to conditioning, to restrict the inhibitor's actions to the injected cell. In these experiments H-7 ($n = 7$), W-7 ($n = 8$), or H-8 ($n = 6$) was iontophoresed into a B cell 2 min before conditioning. In all other respects, the general experimental procedures were identical to those for the bath-application experiments. Iontophoresis of H-7 prevented cumulative depolarization [0.80 ± 0.80 mV; $t(17) = 5.50$, $P < 0.01$, relative to iontophoretic carrier-injected control cells ($n = 12$)] and input resistance increases [6.2 ± 6.1%, $t(12) = 2.60$, $P < 0.05$] (Fig. 1 *B* and *C*). Depolarization and input resistance increases for W-7 (5.7 ± 0.9 mV; 13.9 ± 6.2%), H-8 (7.0 ± 1.3 mV; 18.2 ± 5.4%), and carrier-injected cells (5.9 ± 0.6 mV; 17.3 ± 9.6%) were statistically indistinguishable from each other and ASW controls (Fig. 1 *B* and *C*). As was true for the bath-application experiments, there were no significant differences in membrane potential, resting input resistances, or light responses of B cells among any of the treatment conditions prior to conditioning. In summary, bath application of two potent but only partially selective inhibitors of PKC (sphingosine and staurosporine), as well as a general kinase inhibitor (H-7), prevented the development of conditioning-produced changes in B-cell excitability. Inhibitors of cyclic nucleotide-dependent PKs (H-8) or CaM PKs (W-7) failed to affect these processes.

To determine the role of PKC activation in learning-produced K^+-current reductions, we first confirmed that H-7 prevented PKC-mediated reduction of I_A and $I_{K\text{-}Ca}$ in type B photoreceptors from untrained preparations. In these experiments, PKC purified from rat brain was iontophoresed into B cells under voltage-clamp control. In standard ASW (absence of inhibitor), PKC injections reduced I_A ($n = 5$) by an average of 43% when measured over the potential range −30 to +20 mV and reduced $I_{K\text{-}Ca}$ ($n = 4$) by an average of 62% when measured over the potential range −30 to +15 mV. In the presence of 50–120 μM H-7, however, PKC injections had only small and inconsistent effects on both I_A ($n = 5$; average increase of 5.3%) and $I_{K\text{-}Ca}$ ($n = 3$; average decrease of 0.6%). All measurements were made 1–3 min after kinase injection. We next measured B-cell K^+ currents 2–30 min after *in vitro* conditioning of isolated nervous systems in the presence or absence of 120 μM H-7. Pairings of light and hair-cell stimulation in normal ASW suppressed I_A [$F(2,42) = 6.42$, $P < 0.01$] and $I_{K\text{-}Ca}$ [$F(2,20) = 6.68$, $P < 0.01$] (Fig. 2), when compared with the results from untrained or pseudorandom control animals. However, when B cells were conditioned in H-7, K^+-current amplitudes were not different from those recorded from B cells of untrained or pseudorandom control animals in normal ASW [I_A, $F(2,34) = 0.46$, not significant (NS); $I_{K\text{-}Ca}$, $F(2,16) = 2.22$, NS]. The currents recorded from B cells conditioned in the presence of H-7 were significantly greater than those recorded from cells conditioned in its absence [I_A, $F(1,24) = 3.75$, $P < 0.05$; $I_{K\text{-}Ca}$, $F(1,9) = 5.19$, $P < 0.05$] (Fig. 2).

To determine whether PKC activity is also necessary for expression of long-term memory in type B cells, K^+ currents were measured 1–2 days following standard behavioral conditioning of the intact animal (7, 8, 11). All animals were trained in standard ASW, in the absence of exogenous kinase inhibitors. Pairings of light and rotation resulted in suppression of phototactic behavior similar to previous studies. The average suppression score (7, 8) for associatively trained animals was 0.29 ± 0.005 (mean ± SEM, $n = 93$) 1 day postconditioning, which was significantly less than that of random control animals at the same time [0.49 ± 0.01; ($n = 32$); $t(123) = 4.14$, $P < 0.005$]. K^+ currents were then measured 1 or 2 days after conditioning, in either standard ASW (no inhibitors) or the bath-applied presence of H-7 (120 μM), sphingosine (60 μM), W-7 (120 μM), or H-8 (25 μM). In standard ASW, I_A [$F(2,54) = 9.10$, $P < 0.01$] (Fig. 3) and $I_{K\text{-}Ca}$

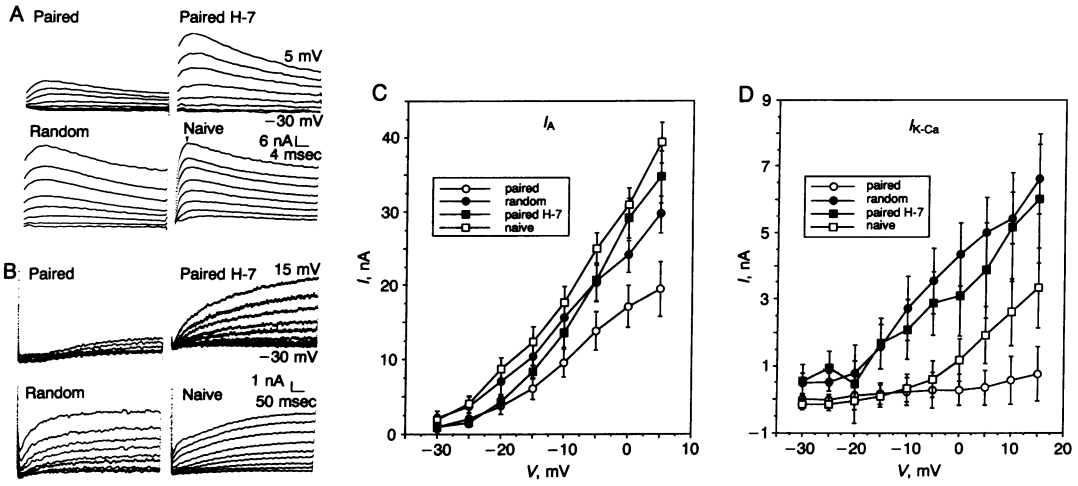

FIG. 2. H-7 prevents *in vitro* conditioning-produced reductions of transient (I_A) and delayed, Ca^{2+}-dependent ($I_{K\text{-}Ca}$) K^+ currents of type B photoreceptors. (*A* and *B*) Samples of I_A (*A*) and $I_{K\text{-}Ca}$ (*B*) current traces recorded from type B photoreceptors of preparations trained in indicated manner. When preparations were conditioned in the presence of 120 μM H-7 (paired H-7), K^+ currents were on average equal in magnitude to those recorded from nonconditioned control preparations [naive or (pseudo)random]. In the absence of H-7, pairing of light and hair-cell stimulation (paired) resulted in pairing-specific reductions of K^+ currents at all potentials equal to or more positive than −30 mV. Note differences in amplitude and time scales for traces in *A* and *B*. All traces were filtered at 500 Hz. Capacitative transients distorted the early phase of activation of I_A and have been suppressed in plots of the current traces. (*C* and *D*) Summary current–voltage (*I–V*) relations for I_A (*C*) and $I_{K\text{-}Ca}$ (*D*) of *in vitro* conditioned type B cells. Currents from paired H-7 preparations (■) (n = 8 and 5 for I_A and $I_{K\text{-}Ca}$, respectively) were not significantly different from naive (□) (n = 12 and 9) or (pseudo)random (●) (n = 17 and 5) controls. In normal ASW, currents were significantly smaller for paired preparations (○) when compared to controls. All current values have been leakage-corrected. Values are means ± SEM.

[$F(2,25) = 4.30$, $P < 0.05$] (Fig. 4) amplitudes of B cells from conditioned animals were reduced relative to those of untrained or random control cells. Suppression of K^+ currents was evidently dependent upon ongoing PKC activity, since currents measured from conditioned animals in the presence of H-7 were not different from those measured from untrained or random control animals in ASW [I_A, $F(2,47) = 1.41$, NS; $I_{K\text{-}Ca}$, $F(2,22) = 1.83$, NS] but were greater than the currents measured from trained animals when H-7 was omitted from the bath at the time of recording [I_A, $F(1,33) = 6.13$, $P < 0.025$; $I_{K\text{-}Ca}$, $F(1,17) = 4.47$; $P < 0.05$] (Figs. 3 and 4). Similar results were obtained for sphingosine. K^+ currents measured from conditioned animals in the presence of sphingosine were not different from random or untrained controls [I_A, $F(2,38) = 1.42$, NS; $I_{K\text{-}Ca}$, $F(2,19) = 1.64$, NS] but were greater than those recorded from conditioned animals in the absence of sphingosine [I_A, $F(1,24) = 4.38$, $P < 0.05$; $I_{K\text{-}Ca}$, $F(1,14) = 5.89$, $P < 0.025$] (Figs. 3 and 4). I_A and $I_{K\text{-}Ca}$ amplitudes from conditioned animals were suppressed, regardless of whether either W-7 or H-8 was present or absent from the bath at the time of recording [I_A, $F(2,31) = 0.70$, NS; $I_{K\text{-}Ca}$, $F(2,24) = 0.29$, NS] (Figs. 3 and 4).

DISCUSSION

We conclude that activation of PKC within type B photoreceptors is necessary for the induction as well as expression of persistent memory-related changes in B-cell excitability. Previous studies (16, 17) have demonstrated that PKC activation is sufficient to produce many of the same B-cell excitability changes produced by behavioral conditioning. Single-channel studies of ripped-off patches of type B photoreceptor membranes indicate that one mechanism involved in PKC-mediated reduction of $I_{K\text{-}Ca}$ is likely to involve phosphorylation of the ion-channel complex or a closely associated molecule (28).

A common view is that although transient activation of a kinase may be necessary for the initial induction of learning-induced neural plasticity, persistent changes are more likely to reflect the duration of covalent modification(s) of proteins

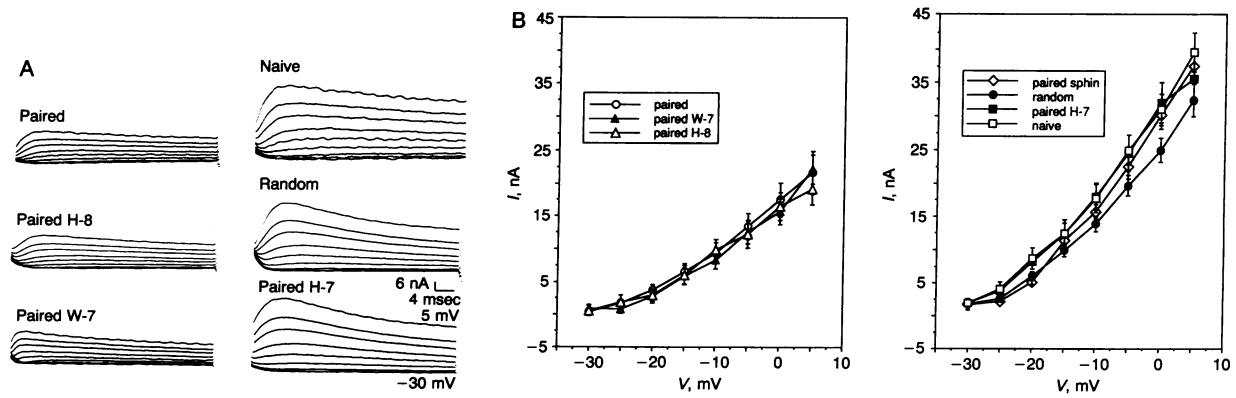

FIG. 3. H-7 and sphingosine reverse associative-training produced reductions in I_A. (*A*) Sample current traces of I_A from type B photoreceptors of behaviorally conditioned animals, 1 day after training. (*B*) Summary *I–V* plots for I_A from animals trained in indicated manner. Currents from paired (○) (n = 21), paired W-7 (▲) (n = 7), and paired H-8 (△) (n = 6) animals were on average indistinguishable from one another but were significantly smaller than those from paired H-7 (■) (n = 14), paired sphingosine (◇) (n = 5), random (●) (n = 24), or naive (□) (n = 12) animals. Values are means ± SEM.

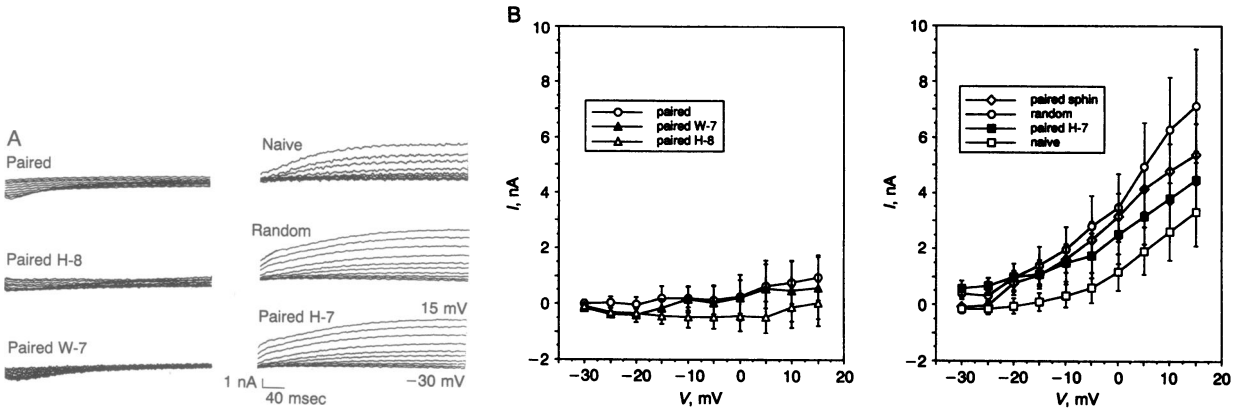

FIG. 4. H-7 and sphingosine reverse associative-training produced reductions in $I_{K\text{-}Ca}$. (*A*) Sample current traces of $I_{K\text{-}Ca}$ from type B photoreceptors of behaviorally conditioned animals, 1 day after training. (*B*) Summary *I–V* plots for $I_{K\text{-}Ca}$ from animals trained in indicated manner. Currents from paired (n = 11), paired W-7 (n = 11), and paired H-8 (n = 5) animals were not different from each other but were significantly smaller than those from paired H-7 (n = 8), paired sphingosine (n = 5), random (n = 8), or naive (n = 9) controls. Values are means ± SEM.

that are substrates for the kinase, rather than persistent kinase activation *per se*. This view may require modification since chemical modification of some enzymes (e.g., autophosphorylation of multifunctional CaM PK II) can lead to their persistent catalytic activity, even in the absence of activators (2). Several candidate mechanisms have been identified from *in vitro* biochemical studies of PKC which could in principle play a role in the persistent activation of PKC of the kind that appears to underlay long-term changes in type B cells. These include irreversible association of PKC with acidic phospholipid membranes (29), oxidative modification of the regulatory domain (30), and changes in basal levels of endogenous activators or inhibitors (31). Training may also result in differential expression of those PKC isozymes (21, 32) with less stringent activation requirements for Ca^{2+} or diacylglycerol. Our results do not at present allow us to decide among these or other alternatives. Our finding that either sphingosine or H-7 reversed the training-produced reductions in K^+ currents seems at face value to exclude the possibility that training results in a limited proteolysis of PKC, thereby producing a catalytically active fragment (33). Had this occurred, the K^+-current reduction reversal by sphingosine would be unexplained, since the sphingosine-binding site on PKC is thought to be located within the conserved C_2 region of the enzyme, which would be removed from the kinase domain during proteolysis. However, it is possible that sphingosine's restoration of K^+-current amplitudes is unrelated to PKC inhibition (34). Regardless of the precise mechanisms responsible for continued PKC activity, our results indicate that it plays a role in the expression of cellular memory in *Hermissenda* type B cells lasting for several days. Thus, our studies add to the rapidly growing literature which indicates that PKC activity may play pivotal roles in a variety of developmental and experimentally induced changes in neuronal structure and function (18).

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