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# Bryostatin Enhancement of Memory in *Hermissenda*

A. M. KUZIRIAN[1,*], H. T. EPSTEIN[1], C. J. GAGLIARDI[1,2], T. J. NELSON[3], M. SAKAKIBARA[4], C. TAYLOR[5], A. B. SCIOLETTI[1,6], AND D. L. ALKON[3]

[1] *Marine Biological Laboratory, Woods Hole, Massachusetts 02543;* [2] *Science Department, Roger Williams University, Bristol, Rhode Island 02809;* [3] *Blanchette Rockefeller Neurosciences Institute, Johns Hopkins University, Rockville, Maryland 20850;* [4] *Department of Biological Science and Technology, Tokai University, Shizuoka, Japan;* [5] *Department of Biology, University of Michigan, Ann Arbor, Michigan 48104; and* [6] *Northeastern University, Boston, Massachusetts 02115*

**Abstract.** Bryostatin, a potent agonist of protein kinase C (PKC), when administered to *Hermissenda* was found to affect acquisition of an associative learning paradigm. Low bryostatin concentrations (0.1 to 0.5 ng/ml) enhanced memory acquisition, while concentrations higher than 1.0 ng/ml down-regulated the pathway and no recall of the associative training was exhibited. The extent of enhancement depended upon the conditioning regime used and the memory stage normally fostered by that regime. The effects of two training events (TEs) with paired conditioned and unconditioned stimuli, which standardly evoked only short-term memory (STM) lasting 7 min, were—when bryostatin was added concurrently—enhanced to a long-term memory (LTM) that lasted about 20 h. The effects of both 4- and 6-paired TEs (which by themselves did not generate LTM), were also enhanced by bryostatin to induce a consolidated memory (CM) that lasted at least 5 days. The standard positive 9-TE regime typically produced a CM lasting at least 6 days. Low concentrations of bryostatin (<0.5 ng/ml) elicited no demonstrable enhancement of CM from 9-TEs. However, animals exposed to bryostatin concentrations higher than 1.0 ng/ml exhibited no behavioral learning.

Sharp-electrode intracellular recordings of type-B photoreceptors in the eyes from animals conditioned *in vivo* with bryostatin revealed changes in input resistance and an enhanced long-lasting depolarization (LLD) in response to light. Likewise, quantitative immunocytochemical measurements using an antibody specific for the PKC-activated $Ca^{2+}$/GTP-binding protein calexcitin showed enhanced antibody labeling with bryostatin.

Animals exposed to the PKC inhibitor bisindolylmaleimide-XI (Ro-32-0432) administered by immersion prior to 9-TE conditioning showed no training-induced changes with or without bryostatin exposure. However, if animals received bryostatin before Ro-32, the enhanced acquisition and demonstrated recall still occurred. Therefore, pathways responsible for the enhancement effects induced by bryostatin were putatively mediated by PKC.

Overall, the data indicated that PKC activation occurred and calexcitin levels were raised during the acquisition phases of associative conditioning and memory initiation, and subsequently returned to baseline levels within 24 and 48 h, respectively. Therefore, the protracted recall measured by the testing regime used was probably due to bryostatin-induced changes during the acquisition and facilitated storage of memory, and not necessarily to enhanced recall of the stored memory when tested many days after training.

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* To whom correspondence should be addressed. E-mail: akuzirian@mbl.edu

*Abbreviations:* CM, consolidated memory; CR, conditioned response; CS, conditioned stimulus; LLD, long-lasting depolarization; LTM, long-term memory; NSW, natural seawater; PKC, protein kinase C; Ro-32, Ro-32-0432, or bisindolylmaleimide-XI; RTE, random training event; STM, short-term memory; TE, training event; UCR, unconditioned response; US, unconditioned stimulus.

## Introduction

Many aspects of memory and recall can be studied especially well in the nudibranch *Hermissenda* because the temporal specificity of experimental effects can be determined within 2 or 3 min, and the process of long-term memory consolidation occurs within 220 to 230 min after conditioning (Epstein *et al.,* 2000, 2004). It is therefore possible to correlate behavior with specific electrophysiological, neurochemical, and structural cytological changes

(Alkon and Fuortes, 1972; Alkon, 1984; Crow, 1988, 2004; Alkon and Nelson, 1990; Alkon *et al.,* 1990, 1998; Kawai *et al.,* 2002; Nelson *et al.,* 2003).

Previous studies have found that two training events (TEs) result in short-term memory (STM) lasting less than 10 min (Epstein *et al.,* 2000, 2004). In *Aplysia,* inhibitors of CREB-2 enhance STM to long-term memory (LTM) that lasts about 1 day (Bartsch *et al.,* 1995; Carew, 1996). However, in *Hermissenda,* nine TEs result in a long-term consolidated memory (CM) that becomes established 220 min after training. This CM lasts at least 6 days (Epstein *et al.,* 2004). There is another distinct memory phase that lasts about 1 day (designated as a subtype of LTM; Epstein *et al.,* 2004). This memory stage becomes established at 60 min post-training, and evidence suggests that it is distinct from the traditionally reported intermediate memory that is formed from existing mRNA (Crow *et al.,* 1999). STM does not require protein synthesis, but both LTM and CM do. Thus, we have been able to readily study the spectrum of events that correlate with the establishment of these memory stages and the transitions between them by using selective transcription and translation inhibitors (Epstein *et al.,* 2000, 2004).

The enzyme protein kinase C (PKC) has long been implicated in the establishment of associative learning and memory in *Hermissenda* and other animal models (McPhie *et al.,* 1993; Sossin *et al.,* 1994; Newton, 1995; Alkon *et al.,* 1998; Weeber *et al.,* 2000; Sutton *et al.,* 2004). In *Hermissenda,* the initial activation of voltage-dependent $Ca^{2+}$ channels and the entry of $Ca^{2+}$ into the photoreceptors triggers PKC autophosphorylation and its translocation from the cytosol to the plasma membrane. Those initial steps subsequently result in the activation of the GTP/$Ca^{2+}$-binding protein calexcitin, which then binds to the ryanodine receptors on the endoplasmic reticulum, triggering a release of internal $Ca^{2+}$ (calcium-induced calcium release). Concurrently, the binding of calexcitin to the plasma membrane closes the rectifying $K^+$-channels. The resultant effect is a long-lasting depolarization (LLD) of the photoreceptors that persists for many minutes. This enhanced excitability produces positive feedback that further enhances $Ca^{2+}$ elevation during memory consolidation (Alkon *et al.,* 1998). Using quantitative immunocytochemistry, we have demonstrated a rise in calexcitin within the B-photoreceptors with the establishment of CM, but not STM (Kuzirian *et al.,* 2001).

Like tumor-promoting phorbol esters, the drug bryostatin-1, which does not promote tumors, is a potent activator of PKC (Smith *et al.,* 1985; Kiss *et al.,* 1991; Pettit, 1991), and is now being tested as an anti-cancer drug (Blackhall *et al.,* 2001). Bryostatin in conjunction with nerve growth factor has been shown to promote the growth of neurites (Burry, 1998). In addition, bryostatin in subnanomolar concentrations has been found to cause the selective translocation of the $\alpha$-isoform of PKC, and the enhanced secretion of soluble amyloid precursor protein-$\alpha$ (sAPP$\alpha$) by the enzyme $\alpha$-secretase. The production of sAPP$\alpha$ has been reported to enhance memory and delay or retard the onset of Alzheimer's disease (Etcheberrigarary *et al.,* 2004). Recently, Sun and Alkon (2005) reported that bryostatin enhanced spatial maze learning in rats. Kortmansky and Schwartz (2003) reported that short-term exposure to bryostatin-1 activates PKC, whereas prolonged exposure promotes significant PKC down-regulation. However, Alkon *et al.* (2005) reported that pre-exposure to bryostatin on 2–3 successive days prior to associative training produced sufficient protein translation that post-training administration of the protein inhibitor anisomycin was completely ineffective. Under these conditions, typical STM was expanded into CM lasting many days. It follows then, that conditions in which PKC is activated and up-regulated should lead to increased memory acquisition and recall; conversely, PKC down-regulation should lead to inhibition of memory formation or lack of recall. This down-regulation of PKC is well known from other systems (Sonneman *et al.,* 2004). Thus, armed with the knowledge of PKC activation by bryostatin and the known effects of phosphorylated PKC on memory formation and recall in *Hermissenda,* a study was undertaken to test the possibility that bryostatin could, depending upon concentration, either promote or inhibit memory acquisition and retention in this model system. Preliminary results of this study have been reported (Scioletti *et al.,* 2004).

## Materials and Methods

### *Behavioral training*

Individual *Hermissenda* were trained using a Pavlovian conditioning regime adapted from Crow and Alkon (1974) and Lederhendler *et al.* (1986); see Epstein *et al.* (2004) for details. In brief, animals were placed in covered transparent acrylic trays (16 lanes, each about 0.9 cm wide and deep, and 15 cm long) and then dark-adapted in a closed 10–12 °C incubator for 10 min before training or testing. Pharmaceuticals used in the experiments were administered to the animals by immersion. Working solutions were dissolved in natural seawater (NSW) and introduced into the lanes containing *Hermissenda* through Luer syringe hubs cemented into the cover of the training tray. Training consisted of exposing the animals to bright white light (650–700 Lux) for 6 s (conditioned stimulus, CS) paired, after a 2-s delay, with 4 s of vigorous orbital shaking of the training tray (unconditioned stimulus, US). Animals responded to the shaking by contracting lengthwise (conditioned response, CR) (Epstein, 1997). This combination of CS/US stimuli was designated a paired TE and was repeated, depending upon the experiment, at 1-min intervals. Random training events (RTEs) consisted of presenting the CS and US to

animals totally independently and randomly within a maximum 2-min inter-stimulus repeat interval.

Testing for CM was typically done at 4 h using the CS alone (×4 replicates). Animal responses were recorded using a time-lapse VCR and a video camera placed below the transparent training tray. Changes in animal length were measured directly on the video monitor. Inter-millimeter lengths were estimated at ±0.3 mm (*i.e.,* 12.7 or 13.3 mm for animals measuring slightly smaller or larger, respectively, than 13 mm). The measure of learning or memory recall scored was the percentage by which the animal's length changed between light-on and light-off (typically 1%–4%). Contraction of the foot (*i.e.,* shortening of the body length) was deemed a positive CR (Lederhendler *et al.,* 1986). Elongation during locomotion (the normal photopositive response to light) was considered evidence of non-learning or non-recall. Results are presented as means of % Length Change, ± standard error of the mean (SEM). All animals were measured for each of four CS-testing replicates per experiment.

### *Experimental pharmacology, immunocytochemistry, and electrophysiology*

Experiments were first directed at ascertaining the effects of bryostatin on *Hermissenda* memory acquisition and recall, to obtain dose and TE response curves. Bryostatin (stock solution: 10.0 μg/ml in DMSO) was added to the seawater in the lanes of the training tray (exposure by immersion) either prior to training at the start of the 10-min dark-adaption period, or within 3 min post-training. Animals were trained with 2-, 4-, 6-, and the standard 9-paired CS/US TEs, with and without exposure to different concentrations of bryostatin (0.1, 0.25, 0.5, and 1.0 ng/ml [0.113, 0.282, 0.57, 1.13 n*M,* respectively] diluted with buffered seawater; 50 m*M* Tris, pH 8). Additionally, animals receiving random presentations of the CS/UC (4R-TEs) and naive animals were tested with bryostatin for possible drug-induced effects. For the majority of conditions, CS testing was done at 240 min (the minimum time period to verify CM formation). However, for 2-TEs that only generate STM, animals were tested at 90 min, 240 min, and 24 h. To test the efficacy of bryostatin on long-term retention following suboptimal training conditions, animals that had received either 4- or 6-TEs, with and without bryostatin exposure, were CS tested at 4 h and then daily for up to 5 days. Positive control animals were those trained with 9-TEs in NSW.

Memory recall in *Hermissenda* can be blocked by pre- or post-training administration of the translation inhibitor anisomycin (Ramirez *et al.,* 1998; Epstein *et al.,* 2003). To test for the possible range of bryostatin effects, including protein synthesis necessary for memory formation and consolidation, animals were conditioned with paired TEs with bryostatin (0.25 ng/ml) added prior to training (during dark adaptation) followed by anisomycin (1.0 μg/ml) administered immediately post-training. Memory retention of this group was then compared to that of animals receiving just bryostatin or NSW alone. Conditioning with 2-TEs was chosen because it was the most responsive and normally generated only STM that lasted less than 10 min (≈7 min).

Bryostatin is a known activator of PKC, and PKC phosphorylation and translocation from the cytosol to the plasma membrane is required for memory formation in *Hermissenda.* Therefore, a very specific PKC inhibitor, Ro-32 (Ro-32-0432 [bisindolylmaleimide-XI]; 40 n*M*/ml) and its ineffective analog Bis-V (bisindolylmaleimide-V; 40 n*M*/ml) (negative control) were used to test whether bryostatin functioned through the modulation or activation of PKC. Animals were conditioned using 6-paired TEs, and then CS tested at 4 h as an indicator of CM. The pharmaceuticals were administered immediately before the usual 10-min dark-adaptation period preceding training. Ro-32 and bryostatin (0.25 ng/ml) were administered separately to animals: one before the dark-adaptation period; the other immediately post-training. The sequence was then reversed to ascertain if the effects of one could be counteracted by the other, and to elucidate what the activation time domains might be. NSW and Bis-V were used as negative controls, while bryostatin alone applied before training served as the positive control. The percent of change in animal length (CR) was used as the measure of associative conditioning. Behavioral measures were tested for significance using nested analysis of variance (ANOVA), and paired Student *t* test comparisons (*n* values consisted of means of length changes/measure/CS-test replicate/animal/treatment).

As further verification that bryostatin was working through activation of PKC, expression levels of the GTP/$Ca^{2+}$-binding protein calexcitin were quantitatively analyzed immunocytochemically. Kuzirian *et al.* (1998, 2001, 2003) found an associative-conditioning-induced rise in calexcitin levels in the B-photoreceptors that persisted for up to 24 h, and reported that this rise could be demonstrated by antibody-labeling intensity measurements. Separate groups of *Hermissenda* received associative conditioning using either 4-random or 6-paired TEs in the presence or absence of bryostatin (0.25 ng/ml, or only NSW, respectively; administered 10 min prior to training during dark-adaptation). After each experimental treatment and CS testing regime, animals were quickly removed from the training tray lanes and decapitated. Their central nervous systems were fixed (4% paraformaldehyde in Tris-buffered [20 m*M*] NSW), followed by later processing for immunocytochemistry (Kuzirian *et al.,* 2001). In brief, the fixed central nervous systems were washed, dehydrated, and embedded in polyester wax, then sectioned at 4–6 μm, labeled with a rabbit polyclonal antibody to calexcitin (25U2), and finally reacted with the chromogen amino-ethylcarbazole using microperoxidase (ABC method; Vector Laboratories, Burlingame,

CA). Intensity (0–256 greyscale intensity) was measured from digital photomicrographs of at three to five serial images of each eye, using NIH Image ver. 1.36 or Image-J software. Means (B-cell photoreceptor intensities minus background; unstained neuropile regions) from each condition were tested for significance using nested ANOVA and paired Student *t* test comparisons (*n* values consisted of measures/serial-sections/block/animal/treatment).

Electrophysiological recordings were used to measure some initial biophysical properties of the medial B-photoreceptor's light response following bryostatin treatment. Animals were conditioned using training regimes of 4-, 6-, and 9-TEs, with bryostatin treatment and NSW controls. Over the subsequent 24–36 h, animals were sacrificed, the CNS dissected, and sharp-electrode intracellular recordings done for each experimental condition, following previously described methods (Alkon, 1980; Alkon *et al.,* 1982; Sakakibara *et al.,* 1986, 1994). Specifically measured for each B-photoreceptor was the duration of the conditioning induced LLD, and the spike frequency generated by a single light flash equal to that of the training conditions. These parameters were compared for the number of training events used, and the presence, or absence (NSW only), of bryostatin. The means of the LLDs (in s) and spike frequencies (number/s) were recorded and statistically compared using ANOVA, including hierarchic and paired Student *t* test comparisons between experimental conditions.

## Results

Under control conditions used to test bryostatin for possible non-associative conditioning effects in *Hermissenda,* there was no alteration of the expected typical post-conditioning behavior. No excess mortality or overt indications of toxic effects were observed. Bryostatin applied to naive (non-trained) animals produced no CS-induced unconditioned response (UCR; foot [body length] contraction due to agitation). Bryostatin administered with random, suboptimal training regimes (fewer than nine CS/US repetitions) also did not generate any conditioned responses (CR). When the CS was presented, all animals treated under non-learning conditions responded with an elongation in body length—that is, a normal (untrained), positive phototaxis (Table 1). Thus, no drug-induced, consolidated memory (CM) imitating associative conditioning was initiated.

Dose-response results obtained from animals that experienced various associative training regimes (4-, 6-, 9-TEs) indicated that subnanogram concentrations of bryostatin (0.1 to 0.5 ng/ml) all produced CM lasting for days. Thus, for animals dosed and trained under these conditions, positive results obtained with 4-h CS testing were a valid indicator that CM had been established. Bryostatin at higher doses ($\geq$1.0 ng/ml), however, down-regulated the system, and positive CR-results were not obtained (Table 2).

Two paired training events typically had initiated only STM lasting less than 10 min. However, when bryostatin (0.25, 0.5 ng/ml) was administered to animals during a 2-paired TE conditioning regime, CRs were still present at 20 h. There were no statistically different, dose-dependent effects demonstrated at the low concentrations used: 0.25 and 0.5 ng/ml gave identical positive results at 90 and 240 min. However, as with 4-, 6-, and 9-TEs, bryostatin administered concurrently with 2-TEs at concentrations higher than 1.0 ng/ml again demonstrated down-regulation of key biochemical pathways; no positive CR results were obtained with CS testing at 90 min or 4 h. Figures 1 and 2 display results for the various paired training regimes with bryostatin administered at 0.25 ng/ml with 4-h CS testing.

The anisomycin-bryostatin study confirmed the results of other studies (Epstein *et al.,* 2003; Alkon *et al.,* 2005) showing that protein synthesis is involved in memory storage and its subsequent recall. Animals that had received 2-paired TEs plus bryostatin (0.25 ng/ml) and then were tested for recall at 4 h showed positive CRs with the CS alone. However, for similarly treated animals also exposed to anisomycin (1 $\mu$g/ml) immediately following training, no long-term memory was demonstrated; results were statistically equal to those of NSW controls (Fig. 3). Similar findings were obtained with animals conditioned with 4- and 6-paired TEs; anisomycin administered immediately after training gave no CRs when tested with the CS, even if they had been pre-exposed to effective memory-enhancing concentrations of bryostatin (results not presented). Thus, protein synthesis was involved in memory acquisition, in the transition from STM to CM, or in both processes.

**Table 1**

*Behavioral results of 4-h retention test for bryostatin administered to randomly trained and naive* Hermissenda

| Training events (TEs) | Experimental conditions[1] | % Foot change | SEM | Measured differences | Animals tested |
|---|---|---|---|---|---|
| Random: 4-TEs | NSW | +3.51 | 1.53 | 11 | 4 |
| | Bryo (0.25ng/ml) | +2.62 | 0.81 | 38 | 11 |
| Naïve: 0-TEs | Bryo (0.25ng/ml) | +2.48 | 0.81 | 22 | 8 |

[1] NSW, natural seawater; Bryo, bryostatin.

**Table 2**

*Results of behavioral test administered to bryostatin-treated* Hermissenda *under paired associative training conditions*

| Training events (TEs) | Experimental conditions[1] | % Foot change | SEM | Measured differences | Animals tested |
|---|---|---|---|---|---|
| 4-h Retention Tests | | | | | |
| Paired: 9 − TEs | NSW | −2.76 | 0.65 | 33 | 9 |
| | Bryo (0.1 ng/ml) | −1.60 | 0.56 | 16 | 4 |
| | Bryo (0.25 ng/ml) | −2.99 | 0.91 | 59 | 16 |
| | Bryo (1.0 ng/ml) | +2.23 | 0.86 | 35 | 10 |
| Paired: 6 − TEs | NSW | +3.06 | 1.358 | 16 | 4 |
| | Bryo (0.25 ng/ml) | −1.98 | 0.87 | 15 | 4 |
| Paired: 4 − TEs | NSW | +1.97 | 0.69 | 38 | 12 |
| | Bryo (0.1 ng/ml) | −1.61 | 0.56 | 16 | 4 |
| | Bryo (0.25 ng/ml) | −2.89 | 0.91 | 62 | 19 |
| 90-min Retention Test | | | | | |
| Paired: 2 − TEs | NSW | +2.27 | 0.69 | 10 | 4 |
| | Bryo (0.25 ng/ml) | −2.60 | 0.65 | 28 | 8 |
| | Bryo (0.5 ng/ml) | −2.40 | 1.20 | 16 | 4 |
| | Bryo (1.0 ng/ml) | −0.65 | 1.01 | 12 | 4 |
| 4-h Retention Test | | | | | |
| Paired: 2 − TEs | NSW | +3.24 | 2.03 | 11 | 4 |
| | Bryo (0.25 ng/ml) | −1.49 | 1.05 | 26 | 7 |
| | Bryo (0.5 ng/ml) | −2.46 | 1.47 | 14 | 4 |
| | Bryo (1.0 ng/ml) | +1.65 | 0.98 | 14 | 4 |

[1] NSW, natural seawater; Bryo, bryostatin.

PKC antagonist studies yielded the following information (Fig. 4). As expected, *Hermissenda* treated with NSW and the ineffective inhibitor analog Bis-V (bisindolylmaleimide-V; 40 n*M*/ml), showed no CRs following associative conditioning using 6-paired TEs and CS testing at 4 h. Ro-32 (Ro-32-0432 [bisindolylmaleimide-XI]; 40 n*M*/ml), a highly specific PKC inhibitor, was effective at preventing retention recall only when administered during the paired CS/US conditioning period and prior to bryostatin (0.25

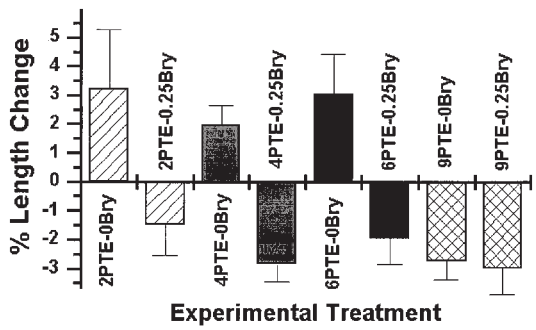


**Figure 1.** Effects of bryostatin on behavioral training and retention. *Hermissenda* receiving optimal (×9) and suboptimal (×2, 4, 6) paired conditioned stimulus/unconditioned stimulus training events (TEs) were tested for the conditioned response (CR) at 4 h. Negative values indicate learning. When treated with bryostatin (0.25 ng/ml; applied during pretraining, dark adaptation; 10 min), animals demonstrated positive learning (conditioned-stimulus-induced conditioned response, CR; foot contraction). With 9-paired TE animals (optimal conditions), there were no significant differences between bryostatin treatment and natural seawater (NSW). However, animals receiving suboptimal TEs without bryostatin (NSW) showed no CR, and foot elongation was as in normal locomotion. ($n$ = 4–20 animals/treatment; ANOVA differences, $P < 0.01$.)

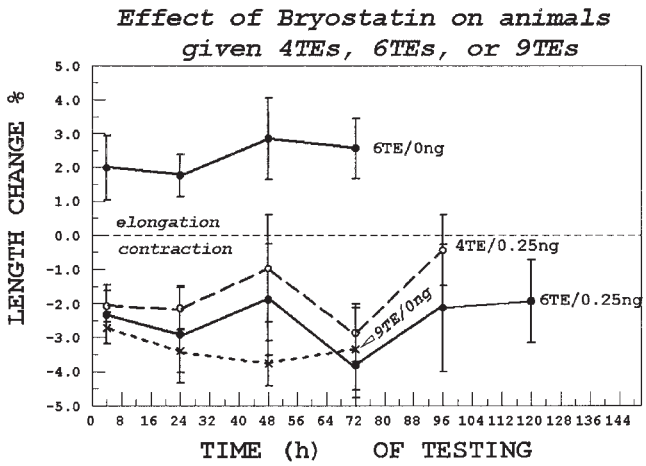


**Figure 2.** Effects of bryostatin on long-term retention of consolidated memory (CM). Animals were trained with suboptimal training regimes of 4- and 6-paired training events (TEs); with bryostatin (0.25 ng/ml) and without (NSW [natural seawater] controls); 9-paired TEs and NSW (9TE/0 ng bryostatin) served as positive controls. All animals were tested with the CS alone at 4, 24, 48, 72, and 96 h (for the 6TE/0.25 ng bryostatin animals, the time was extended to 120 h). Animals trained suboptimally (non-inducing conditions for CM) plus bryostatin all demonstrated CM retention equal to the 9TE/0 ng control animals, thus indicating enhancement by bryostatin to full development of CM. ($n > 12$ animals/treatment; Student's $t$ values/data point, $t > 2.0$; $P < 0.05$.)

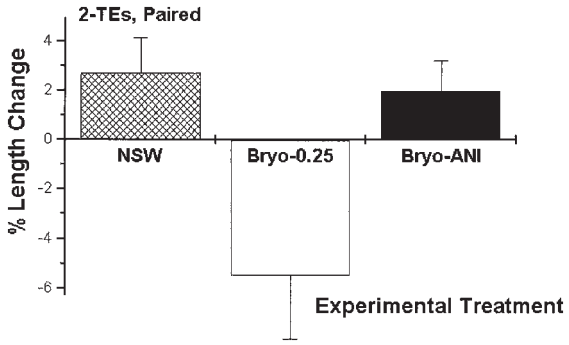


**Figure 3.** Behavioral data indicated enhanced bryostatin (0.25 ng/ml) effects on memory recall involved protein synthesis. Animals receiving post-training administration of the mRNA translation inhibitor anisomycin (1 $\mu$g/ml) following 2-paired training events (TEs) and conditioned stimulus testing at 4 h did not exhibit retention of enhanced consolidated memory. ($n > 4$ animals/treatment; ANOVA, $P < 0.01$.)

ng/ml) exposure. Animals trained similarly but with the drugs applied in the opposite sequence (bryostatin exposure during training preceding Ro-32 administration immediately post-training) and CS-tested at 4 h, showed positive CRs, as did animals exposed to bryostatin alone (Fig. 4). The 16-min exposure period to either drug (10-min dark-adaption plus 6 min of training) was sufficient to allow the full induction of each drug's effect. Importantly, the effect of the initial drug was unable to be canceled or overridden by the other.

The immunocytochemical intensity studies designed to elucidate whether bryostatin worked through PKC activation of calexcitin gave results consistent with the known interactions of the photoreceptor second messenger system (Alkon *et al.,* 1998) and previously published results (Kuzirian *et al.,* 2001, 2003) (Fig. 5A). Conditioning with 4-random or 6-paired (CS/US) TEs produced statistically similar immunolabeling intensities for calexcitin (greyscale levels: 14.2 ± 2.96; 17.5 ± 2.34, respectively) in animals

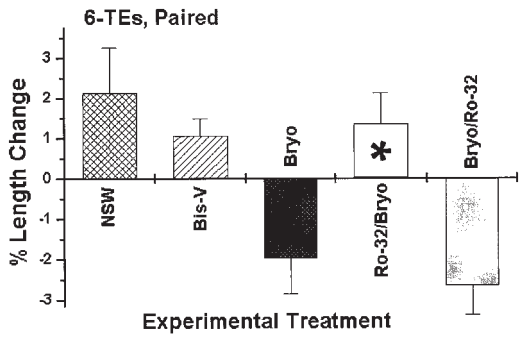


**Figure 4.** Effects of bryostatin on consolidated memory (CM) under control and antagonistic training conditions. *Hermissenda* received 6-paired conditioned stimulus/unconditioned stimulus training events (TEs). Natural seawater (NSW) and Bis-V (bisindolylmaleimide-V; 40 n*M*/ml)-treated animals served as negative controls; CM was not evident nor a positive conditioned response expressed when tested at 4 h. Animals receiving bryostatin (Bryo; 0.25 ng/ml) alone or bryostatin applied before training (during dark adaptation), followed by the PKC inhibitor Ro-32 (Ro-32-0432; bisindolylmaleimide-XI; 40 n*M*/ml) administered within 3 min post-training (Bryo-Ro32), exhibited retention of enhanced CM. However, Ro-32 applied pre-training followed by bryostatin at <3 min post-training (asterisk; Ro32-Bryo) produced no conditioned response; animals elongated in normal locomotion, indicating that CM formation was inhibited. (ANOVA, $P < 0.01$.)

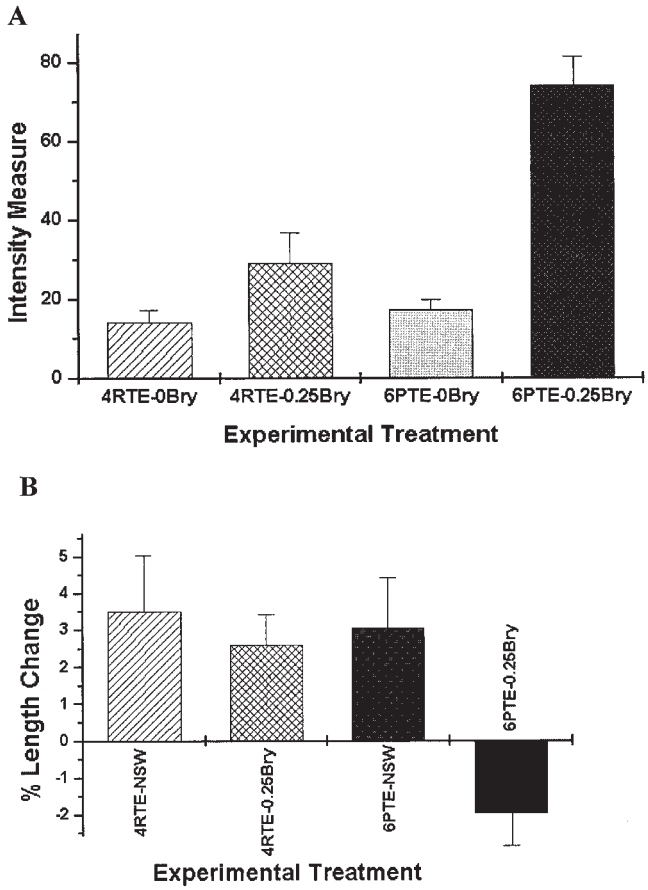


**Figure 5.** (**A**) Immuno-intensity measurements (as 0–256 greyscale intensities) of calexcitin antibody labeling in B-photoreceptors as a function of bryostatin and training regime. Animals experiencing random conditioned stimulus/unconditioned stimulus training (4RTE-NSW) and those receiving paired associative training (6PTE-NSW) in natural seawater (NSW) without bryostatin demonstrated base levels of calexcitin comparable to naive animals (Kuzirian *et al.,* 2001, 2003). Bryostatin (0.25 ng/ml) administration increased calexcitin levels for both training paradigms. The elevated calexcitin levels seen with 4-random TEs (4RTE-0.25Bryo) (2× increase) were not accompanied by concurrent positive testing responses and consolidated memory (CM) retention. However, calexcitin levels increased >4.3× in animals receiving 6-paired TEs (6PTE-0.25Bryo) plus bryostatin. These animals thus treated exhibited conditioned responses and CM recall. (**B**) Behavioral responses of animals trained and used for the calexcitin immunocytochemical intensity measures (**A**). Only paired, 6-TEs training plus bryostatin produced positive learning and demonstrated recall of CM. Labels on graph as described above. For **A,** all Student's $t$ comparisons were significantly different ($P < 0.05$ to $P < 0.001$) except the controls of 4- and 6-TEs without bryostatin.

trained in NSW. As illustrated in Figure 5B, neither training regime induced acquisition or retention of the conditioned behavior (no recall demonstrated). Bryostatin (0.25 ng/ml)-exposed animals given 4-RTEs showed a significant increase in calexcitin levels (2× increase; greyscale level: 29.2 ± 7.7) over animals kept in NSW. However, the random training regime did not induce a positive CR in those animals when tested for CM retention at 4 h. *Hermissenda* trained with 6-paired TEs plus bryostatin demonstrated the positive CR as well as a greater than 4.3× increase in calexcitin levels (greyscale level: 74.4 ± 7.3; an intensity level within the statistical limits for calexcitin levels from animals experiencing 9-paired TEs; data not graphed). Analysis of the microscopic images used to obtain the intensity measurements indicated that calexcitin immunolabeling was confined to the B-photoreceptors. Neither of the two A-type photoreceptors stained positively (Fig. 6A, B). Additionally, a preliminary comparison of the staining

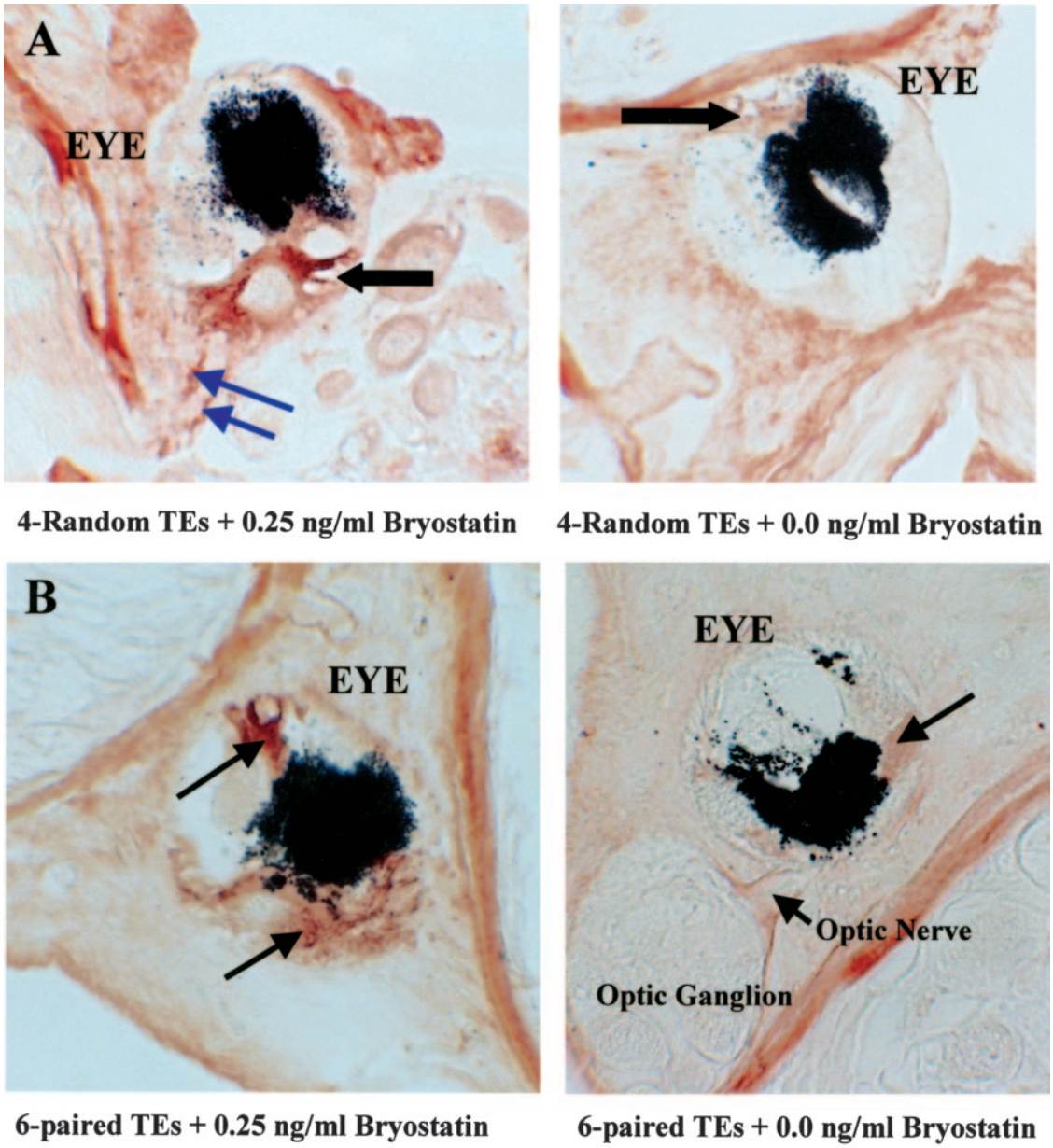


**Figure 6.** Photomicrographs of eyes illustrating the calexcitin immunocytochemistry of bryostatin-treated and non-treated control (natural seawater, NSW) *Hermissenda.* Black arrows indicate B-photoreceptors, which in the left-hand panels were highly immunolabeled in animals trained and exposed to bryostatin (0.25 ng/ml); the photoreceptors in the right-hand panels (from NSW control animals) were significantly less stained. Blue arrows in upper left panel indicate two stained photoreceptor axons.

intensities of the three B-cells indicated that the medial B-cell (photoreceptor located nearest the cerebropleural ganglion) consistently labeled most intensely (data not presented).

Immunocytochemical intensity data from past experiments conducted under identical cytological conditions were compared and analyzed for any apparent trends (Table 3; Fig. 7). The analyses revealed that calexcitin immunointensity measurements fell into four distinct groups. The base level expressed for calexcitin had a greyscale intensity level below 20 (mean level, 17.3 ± 0.5). Grouped within this level were naive animals, animals that had received suboptimal training that did not generate acquisition of associative conditioning or its retention, and 48-h post-trained animals. A second category denoted an initial activation level (expressed greyscale levels between 30 and 35, occurring between 5 and 30 min). This level of calexcitin activation represented training conditions that produced only STM, or was an intermediate, post-training level expressed in animals sampled during the ramp-up phase from training regimes that eventually generated consolidated memory. The third category, greyscale levels greater than 55, delineated full learning acquisition. It included animals that had received paired, 9-TE training regimes as well as animals given suboptimal numbers of paired training replicates (4–6 TEs) augmented by bryostatin exposure. This intensity level was attained within 40 min post-training with 9-paired TEs, and it was sustained for more than 200 min. The last category represented a memory/retention-state level (greyscale levels ≈27). Animals receiving 9-paired TEs returned to this slightly-above-baseline calexcitin intensity level within 240 min following training. Calexcitin intensities were sustained at this level for more than 24 h, but returned to baseline by 48 h post-training.

The initial electrophysiologic data obtained were consistent with demonstrated behavioral results and known physiology (Table 4; Fig. 8). Animals trained in NSW with 4- and 6-paired (CS/US) TEs for these experiments did not demonstrate retention of the CR at the 4-h test time (CS alone) (data not graphed). For this group, the length of the after-spike depolarizing wave measured *in vitro* was short

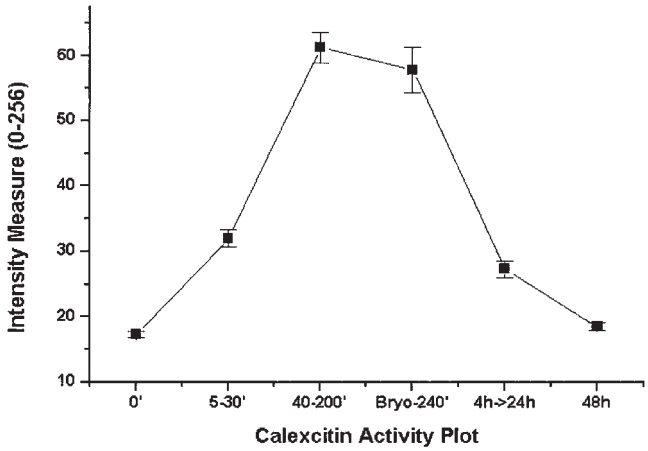


**Figure 7.** Cumulative data from calexcitin immunocytochemical studies depict the time course of calexcitin levels induced by various training regimes and bryostatin administration. Base level (0 min) represents intensity levels in naive, untrained animals, and those who had received suboptimal, non-acquisition training conditions. Between 5 and 30 min, calexcitin rose to this level in animals that demonstrated only short-term memory. Maximal levels were obtained between 40 and 200 min in animals that expressed full, long-term consolidated memory (CM) lasting days, whether by paired, 9-TEs (training events) conditioning or paired suboptimal TEs (4-, 6-TEs) plus bryostatin (0.25 ng/ml). This level was also found in animals that had been randomly trained or in naive animals receiving bryostatin only and tested with the conditioned stimulus at 240 min. Lower but sustained calexcitin levels lasting 4 to >24 h post-training occurred in animals that develop full CM. All categories are significantly different from each other ($n = 6–47$ animals; Student's $t$ comparisons, $P < 0.01$).

(23 ± 2.48, 18.5 ± 1.6 s, respectively; Fig. 8A). Animals similarly trained but in the presence of bryostatin (0.25 ng/ml; administered during dark-adaption prior to training) all tested positive for training acquisition and CM retention. The B-photoreceptors of these animals exhibited an extended period of depolarization (long-lasting depolarization; LLD) after light-induced spiking activity (Fig. 8B). The duration of the bryostatin-induced LLDs for 4- and 6-paired TEs were statistically similar to light-induced responses in photoreceptors from animals experiencing 9-paired CS/US training regimes with or without bryostatin being present (*t*

**Table 3**

*Cumulative calexcitin (CE) immuno-intensity data illustrating the calexcitin activation levels obtained under different conditions of memory acquisition and recall*

| Experimental conditions[1] | Greyscale (0 to 256) Intensity | SEM | Measured differences | Animals tested |
|---|---|---|---|---|
| Base level: Non-acquisition; Non-retention state | 17.33 | 0.50 | 145 | 49 |
| CE-activation: STM acquisition; No long-term retention (<4-h) | 32.05 | 1.24 | 35 | 22 |
| CE-activation: CM (>24-h) acquisition/retention | 61.38 | 2.34 | 109 | 47 |
| Non-associative conditioned; bryostatin activation alone | 57.90 | 3.45 | 14 | 6 |
| CM: Long-term retention state | 27.32 | 1.24 | 51 | 18 |

[1] STM, short-term memory; CM, consolidated memory.

**Table 4**

*Long-lasting depolarization (LLD) data obtained from* Hermissenda *photoreceptors under various control (NSW alone) and bryostatin-administered, associative training conditions*

| Experimental conditions[1] | LLD (s) | SEM | Animals tested |
|---|---|---|---|
| NSW Control: 4-TEs | 23.05 | 2.48 | 21 |
| Bryostatin (0.25 ng/ml): 4-TEs | 54.67 | 0.72 | 12 |
| NSW Control: 6-TEs | 18.50 | 1.64 | 12 |
| Bryostatin (0.25 ng/ml): 6-TEs | 49.17 | 1.11 | 6 |
| NSW Control: 9-TEs | 55.42 | 0.40 | 12 |
| Bryostatin (0.25 ng/ml): 9-TEs | 52.61 | 1.26 | 18 |

[1] Animals received either 4-, 6- or 9-paired conditioned stimulus/unconditioned stimulus training events (TEs) at 1-min inter-trial intervals. NSW, natural seawater.

values $<1.49$, $P > 0.09$; $n > 6$ animals/treatment) (Fig. 8A). In addition, spike frequency analyses between the experimental conditions matched the LLD results. The mean spike frequencies were 2.75 times higher for the bryostatin-treated, 4- and 6-paired TEs animals (Fig. 8B) than for the NSW controls (ANOVA; $n = 17$, $F = 208$, $P = <0.05$).

## Discussion

The results described in this study concur well with the known PKC physiology previously described for *Hermissenda* (Alkon *et al.,* 1998) and the pharmacology of bryostatin (Smith *et al.,* 1985; Burry, 1998; Kortmansky and Schwartz, 2003). It has been well demonstrated in *Hermissenda* that associative-conditioning-induced $Ca^{2+}$ entry into the B-photoreceptors initiates PKC activation and its autophosphorylation (Farley and Auerbach, 1986; Alkon and Nelson, 1990; Farley and Schuman, 1991; Alkon *et al.,* 1998; Crow *et al.,* 1998; Crow, 2004). Similar PKC activation has been reported in other learning models, including *Aplysia* and vertebrates (Bank *et al.,* 1988; Scharenberg *et al.,* 1991; Sossin *et al.,* 1994; Vianna *et al.,* 2000; Weeber *et al.,* 2000; Abel and Lattal, 2001; Sutton *et al.,* 2004). In *Hermissenda,* this activation causes PKC to translocate to the cell membrane (McPhie *et al.,* 1993). Subsequently, this translocation induces secondary pathway cascades that lead to the activation and increased production of the GTP/$Ca^{2+}$-binding protein calexcitin (Nelson *et al.,* 1990, 1996, 1999; Ascoli *et al.,* 1997; Kuzirian *et al.,* 2001), the release of internal calcium from the endoplasmic reticulum, and the reduction of outward $K^+$ currents that triggers a rise in the resting potential and the establishment of a long-lasting depolarization. Early gene activation is also associated with PKC up-regulation caused by these same associative conditioning regimes (Alkon *et al.,* 1998).

Bryostatin, a macrocyclic lactone, is a potent, specific activator of PKC. It acts similarly to phorbol esters by binding to the regulatory domain of PKC (Smith *et al.,* 1985; Singh *et al.,* 1994; Burry, 1998) and causing increased activity in membranes. In addition, both agents activate signaling pathways that lead to increased transcription and rapid early protein synthesis (Burry, 1998). Bryostatin has the advantages that it is not a tumor promoter and it induces PKC activation in subnanomolar concentrations. In addition to stimulating PKC-induced outgrowth of nerve growth factor-dependent neurites (Burry, 1998), it favors the generation of the nontoxic form of soluble $\alpha$-amyloid precursor protein by differentially activating the isoform, PKC-$\alpha$ (Etcheberrigaray *et al.,* 2004). Bryostatin has recently been reported to enhance spatial maze learning in rats (Sun and Alkon, 2005), and learning in rabbits experiencing tone and nictitating membrane associative training paradigms (as reported in Alkon *et al.,* 2005). Its effects can be readily inhibited by many bisindolymaleimide compounds (Burry, 1998).

The dose response results with *Hermissenda* were congruous with the published data for several model systems (Favit *et al.,* 1998; Wender *et al.,* 1998; Etcheberrigaray *et al.,* 2004; Scioletti *et al.,* 2004; Alkon *et al.,* 2005). Subnanogram concentrations of bryostatin administered before paired associative conditioning up-regulated PKC activity, and specifically for *Hermissenda,* enhanced both acquisition and retention initiated by suboptimal paired CS/US training regimes. However, dosages of bryostatin exceeding 1.0 ng/ml were sufficiently high to cause PKC down-regulation, and there was no recall of any acquired learned behavior (Table 2). This down-regulation phenomenon has been reported in other neuronal systems and has been associated with the ubiquitin-proteasome system and apoptosis (Burry, 1998; Vrana and Grant, 2001). Importantly, bryostatin-exposed control animals exhibited no deviations from the normal expected behavior of naive animals; all behavioral modifications were specific to animals exposed to the associative training regimes.

Of significance was the transition, induced by the addition of bryostatin, for 2-TE conditioning from short-term memory (STM) to a long-term memory (LTM) lasting nearly 24 h. Typically this suboptimal training regime produced only STM lasting about 7 min (Table 2; Fig. 1). This long-term enhancement was even more dramatic for 4- and 6-TE regimes in which STM was extended by bryostatin to full, consolidated memory (CM) lasting for many days; comparable in numbers to the positive standard of 9-TEs (Figs. 1–2) (Epstein *et al.,* 2000, 2003, 2004). The blockade of bryostatin-enhanced training effects initiated when anisomycin, an inhibitor of protein translation, was applied immediately post-training, indicated that bryostatin's enhancement to CM formation required the synthesis of new protein. These anisomycin results concur with those published earlier for *Hermissenda* (Epstein *et al.,* 2003). Although the exact time-window of the synthesis was not investigated here, it is contemplated for follow-up studies.

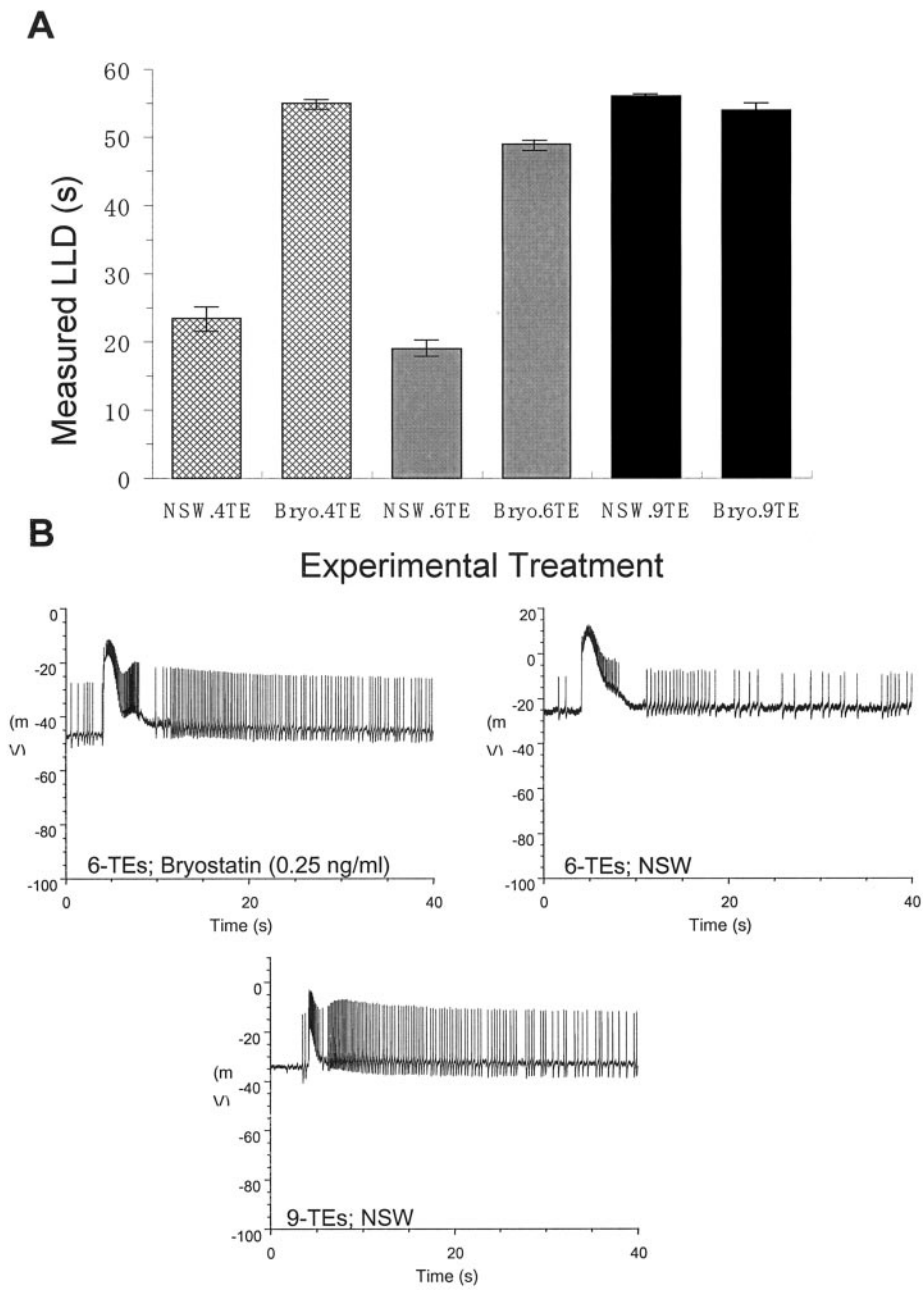


**Figure 8.** Enhanced effects of bryostatin in producing a light-induced, long-lasting depolarization (LLD) response (measured from sharp-electrode intracellular electrophysiologic recordings) in the B-photoreceptors of associative conditioned *Hermissenda.* (**A**) In NSW (natural seawater) control animals, LLDs were produced only with 9 training events (TEs) (4-, 6- *vs.* 9-TEs: $P < 0.001$). However, bryostatin produced significant LLDs with 4- and 6-TEs, equal to 9-TEs. (**B**) Representative electrophysiologic records from animals that had received 6-TEs with or without bryostatin (0.25 ng/ml), plus 9-TE positive controls ($n = 12$; Student's $t$ comparisons, $P < 0.001$).

A regimented, post-training schedule of anisomycin administration, alone or in conjunction with the PKC inhibitor Ro-32 (see below), would yield the desired data. It should also be noted that *Hermissenda* can be preconditioned, or primed, for enhanced memory acquisition and recall by administering bryostatin on 3 successive days prior to training. Under those conditions, anisomycin had no effect when applied immediately after suboptimal training (Alkon *et al.,* 2005). The protein synthesis required for long-term retention and recall was initiated by bryostatin and completed prior to training. As a result, the associative conditioning putatively needed only to transform the training stimuli into CM.

Use of the specific PKC inhibitor Ro-32 (Ro-0432; bisin-

dolylmaleimide-XI, Bis-XI) confirmed the hypothesis that bryostatin-enhanced CM occurred through PKC activation (Burry, 1998; Alkon *et al.,* 2005). Due to the specificity of Ro-32, it can be stated conclusively that PKC-$\alpha$ was the predominant isoform affected (a 10-fold selectivity; Wilkinson *et al.,* 1993). Further, the experiments indicated that PKC activation could be initiated either before training by bryostatin itself, or synergistically if applied immediately after paired training. Ro-32 inhibited CM only if it was applied before training (*i.e.,* purely associative-conditioning-induced PKC activation) or bryostatin exposure. Post-training or post-bryostatin administration of Ro-32 had no inhibitory effects. The non-effective PKC inhibitor Bis-V served as an effective negative control.

The increased levels of bryostatin-induced calexcitin were consistent with those of initial bryostatin studies (Scioletti *et al.,* 2004) and with levels induced solely by associative conditioning (Kuzirian *et al.,* 2001, 2003; Borley *et al.,* 2002). This study's data, however, indicated that bryostatin acting through PKC activation could induce calexcitin levels to rise irrespective of the training paradigm (Figs. 5, 6A, B; 7). Bryostatin-enhanced calexcitin levels in naive animals were comparable to those in training conditions that generated CM (*i.e.,* 9-paired TEs). Four random CS/US stimuli plus bryostatin apparently down-regulated the system, because the levels of calexcitin only doubled. The random levels were comparable to those seen for STM activation or to the declining levels exhibited by (4–24 h) post-trained animals. This latter situation of declining levels would reflect down-regulated conditions brought about by a triggered ubiquitin-proteasome pathway, as recently reported by Alkon *et al.* (2005). It can be concluded on the basis of the behavioral data and the calexcitin intensity levels obtained, that full acquisition of learned behavior and its demonstrated recall in the presence of bryostatin depended upon the nature of the stimulus presentation regimes used (paired *vs.* random). Learned acquisition and its recall required *paired* CS/US stimuli. Random presentations, even though accompanied by enhanced calexcitin levels, were insufficient to modify the animal's behavior. Additionally, the actual length of memory retention induced by bryostatin was again governed by the quality and number of training events presented: *e.g.,* 2-paired TEs plus bryostatin-enhanced STM to the LTM state of about 20 h; 4- and 6-paired TEs augmented conditions that generated true CM lasting many days. It can also be stated that the temporal rise in calexcitin was transient. Calexcitin reached its highest level within 40–60 min post-training, and remained elevated until 200 min. It then decreased rapidly to about half that level by 240 min (Kuzirian *et al.,* 2003), and remained at that level until returning to baseline by 48 h. Thus, it appeared that the induction and stabilization of CM that was dependent upon sustained calexcitin levels was completed within 48 h.

In *Hermissenda,* the characteristic biophysical change generated by 9-paired TEs and the subsequent formation of CM has long been associated with an LLD in the B-photoreceptors, as revealed by a single light flash (Crow and Alkon, 1974; Alkon, 1980; Lederhendler *et al.,* 1986). Under all conditions in this study when bryostatin was present and positive behavioral acquisition was obtained, a distinct LLD was demonstrable in the B-photoreceptors. The duration of the experimental LLDs recorded was statistically the same for all conditions that produced CM, whether 9-TEs, or bryostatin-enhanced, suboptimal training conditions (4-, 6-TEs). Physiologically, according to published reports, this LLD was produced by increased PKC activation coupled with calexcitin activation and its suppression of, and reduction in, the outward $K^+$ currents ($I_A$; $I_{K\text{-}Ca}$), and the release of $Ca^{2+}$ from internal stores (Farley *et al.,* 1983; Farley and Auberbach, 1986; Alkon, 1989; Matzel *et al.,* 1992; Sakakibara *et al.,* 1993; Alkon *et al.,* 1998). Thus, the acquisition of CM generated by bryostatin administered in conjunction with suboptimal training conditions sufficiently mimics the initial biophysical conditions produced by 9-paired training regimes with respect to the biophysics of LLD induction.

Toward the question of what stages of memory formation and recall are affected by bryostatin, the following points are discussed. The most probable events affected by bryostatin-enhanced memory in *Hermissenda* are those associated with acquisition and consolidation. Studies on conditioned taste aversion memory conducted with highly specific PKC inhibitors have revealed that in rats, PKC activation dominates the processes involved with memory acquisition (Bielavska and Krivanek, 1994; Krivanek, 1997; Sacchetti and Bielavska, 1998). Similar results from studies using chicks were reported by Serrano *et al.* (1995). The authors, using the highly specific PKC inhibitor chelerythrine, reported that PKC activation must occur temporally very near the training events, but that the height of PKC activity and LTM consolidation appeared only after 60 min. PKC involvement in memory consolidation and retention has also been reported for mice (Mathis *et al.,* 1992; Sato *et al.,* 2004). Like *Hermissenda,* conditioned rats showed an immediate translocation of cytosolic PKC to the membrane that was later followed by a rise in cytosolic levels (but not membrane-activated PKC) after acquisition (Krivanek, 1997). Krivanek's results taken together with the results of Serrano *et al.* (1995) appear to be mimicked by the time course of calexcitin increases reported here. To induce the enhanced memory, bryostatin must be applied either immediately before or after paired-training conditions. To inhibit the enhancing effects of bryostatin, Ro-32 must precede training or bryostatin administration. To inhibit protein synthesis, anisomycin must also be applied immediately after PKC activation. Calexcitin rose to its highest level within 40–60 min post-training and remained high until 200 min. Epstein *et al.* (2000, 2003) reported that an initial LTM was

established 60 min post-training, and that CM was present at 240 min. This CM formation followed a period of DNA transcription that ended at 180 min and protein translation that was completed by 220 min. Bryostatin's induction of biophysical changes reflected in the generation of LLD also had to occur before or immediately after training. Together, all these results lead to the conclusion that bryostatin initially enhances memory acquisition and likely contributes to its transition from STM to CM. Indeed, the speculation by Alkon *et al.* (2005) that the *de novo* protein synthesis (including that of calexcitin) induced by bryostatin exposure on consecutive days prior to training supports the assumptions that PKC activation is involved with memory acquisition and transition. Critical memory proteins were produced by activated PKC and were immediately available following training to acquire and store that memory long-term.

Memory retrieval has not been reported to involve PKC in murine systems (Mathis *et al.,* 1992; Sacchetti and Bielavska, 1998). Sato *et al.* (2004) hypothesized that in mice, memory retrieval most likely occurs *via* the phospholipase C/inositol triphosphate pathway, leading to greater $Ca^{2+}$ release from internal stores and PKA activation by cAMP. Co-activation of PKA and PKC has been reported in vertebrate and invertebrate memory systems, principally involving modulation of synapses, receptors, and channel activation (Byrne and Kandel, 1996; Li *et al.,* 2002; Millan *et al.,* 2003). It has been reported for *Hermissenda* that PKA modulates synaptic facilitation between the vestibular hair cells and photoreceptors (Tamse *et al.,* 2003). Thus, the roles PKC and PKA may play in the processes of memory retrieval are accessible for study in this model system.

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