Analysis of long-term cognitive-enhancing effects of bryostatin-1 on the rabbit (*Oryctolagus cuniculus*) nictitating membrane response

Desheng Wang[a,d], Deya S. Darwish[b], Bernard G. Schreurs[a,d] and Daniel L. Alkon[c,d]

Previous work demonstrated that protein kinase C (PKC) is implicated in learning and memory. This study investigated whether: (i) PKC activated by bryostatin-1 (Bryo) just before or just after sessions of classical conditioning was capable of enhancing classical conditioning of the rabbit nictitating membrane response; (ii) improved behavioral performance matched the time course of PKC activation induced by Bryo; and (iii) vitamin E (Vit E) enhanced the efficacy of Bryo. Paired rabbits received daily trace conditioning with a tone conditioned stimulus and a corneal air puff unconditioned stimulus. Unpaired rabbits received the same stimuli but in an explicitly unpaired manner. After trace conditioning, all rabbits received daily delay conditioning, and then tone intensity testing. Rabbits pretreated with 10 μg/kg Bryo every other day before a relatively simple trace conditioning task showed more conditioned responses (CRs) during the first 10 trials of each trace conditioning session and a higher likelihood of a CR on the first trial of each trace conditioning session than rabbits pretreated with the vehicle control. Rabbits either posttreated daily with 10 μg/kg Bryo or pretreated with Vit E and subjected to a difficult trace conditioning task showed increased CRs relative to the vehicle control. Neither Bryo nor Vit E or their combination altered nonassociative responding or altered sensitivity to the conditioned stimulus or unconditioned stimulus. These findings demonstrate Bryo has long-term enhancing effects on classical conditioning of the rabbit nictitating membrane response. *Behavioural Pharmacology* 19:245–256 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

A significant number of studies have documented learning-specific changes of endogenous protein kinase C (PKC) activity within memory-related brain structures in animal models as diverse as classical conditioning in the marine snail *Hermissenda* (Alkon *et al.*, 1988), the rabbit nictitating membrane response (NMR) (Olds *et al.*, 1989; Freeman *et al.*, 1998), and spatial maze learning in the rat (Sun and Alkon, 2006). Additional studies have shown that PKC mediates the increased neuronal excitability and enhanced synaptic potentials in hippocampal CA1 neurons that result from classical conditioning (Alkon and Rasmussen, 1988; Freeman *et al.*, 1998) and that these memory-specific changes can be mimicked by application of an exogenous PKC activator (Alkon *et al.*, 1982; Alkon and Rasmussen, 1988). Consequently, PKC may be causally involved in memory acquisition, storage, and loss (Alkon *et al.*, 2005, 2007).

Bryostatin-1 (Bryo), a macrocyclic lactone extracted from the marine bryozoan *Bugula neritina*, has demonstrated antineoplastic effects through activation and downregulation of PKC in various types of cancer cells (Madhusudan *et al.*, 2003; Winegarden *et al.*, 2003). Unlike other PKC activators such as phorbol esters (Kiss *et al.*, 1987), Bryo is devoid of carcinogenic properties and is reasonably well tolerated in clinical trials (Clamp *et al.*, 2003). When compared with other PKC activators, Bryo demonstrates a higher binding affinity and markedly lower release rate to the regulatory domain of PKC (Lorenzo *et al.*, 1997). These unique characteristics may make Bryo a potential memory enhancer and neuroprotectant by acting as a PKC signaling molecule. Recent experiments have demonstrated that Bryo enhances learning and memory in *Hermissenda* (Alkon *et al.*, 2005; Kuzirian *et al.*, 2006) and rat (Sun and Alkon, 2005), and acts as a neuroprotectant in Alzheimer's disease transgenic mice (Etcheberrigaray *et al.*, 2004). To date, there are no systematic in-vivo data showing the long-term cognitive effects of Bryo or whether the compound alters nonassociative responding or modifies sensory or motor processing.

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When Bryo was tested as a new anticancer therapy in phases I and II clinical trials (Winegarden *et al.*, 2003), dose-dependent side effects including myalgia, nausea, and vomiting were a major concern. Vitamin E (Vit E) has been clinically tested as a way of reducing PKC-mediated microvascular complications of diabetes through peripheral PKC inhibition (Wigg *et al.*, 2004). Moreover, Vit E cannot readily cross the blood–brain barrier (Pappert *et al.*, 1996), suggesting that administration of Vit E together with Bryo may decrease Bryo-induced PKC activation in the peripheral nervous system and reduce Bryo-related toxicity without influencing Bryo-induced PKC activation in the central nervous system (CNS). Earlier studies have shown that Vit E can significantly prevent the memory deficit induced by different manipulations and diseases (Yamada *et al.*, 1999; Tuzcu and Baydas, 2006). No examination of the effects of Vit E on classical conditioning in normal adult rabbits has been, however, carried out, nor have the effects of combining Bryo and Vit E been assessed.

This study used classical conditioning of the rabbit NMR, which provides a systematic set of paradigms capable of assessing a drug's effect on trace conditioning, delay conditioning, nonassociative responding, and on sensory and motor processing (Gormezano and Harvey, 1980; Schindler *et al.*, 1984). Trace classical conditioning was chosen because it engages the hippocampus in which increased PKC is expressed as a result of learning and memory. Experiments were conducted to test whether PKC activated by Bryo before or after classical conditioning of the rabbit NMR might be helpful in memory acquisition, consolidation, and storage. A relatively easy trace conditioning paradigm was used to determine whether changes in behavioral performance matched the time course of PKC activation produced by Bryo. A difficult trace paradigm was used to determine whether Bryo could be more than a simple facilitator of the rate of acquisition in trace conditioning. We also examined the effects of Vit E to potentiate Bryo effects on learning and ameliorate its side effects.

Methods

Subjects

A total of 127 male New Zealand white rabbits (*Oryctolagus cuniculus*) weighing approximately 2.0 kg were housed individually, given free access to food and water, and maintained on a 12 : 12-h light–dark cycle. All behavioral testing was conducted during the light cycle. All procedures followed the guidelines of the American Psychological Association and the National Institutes of Health, and the research was approved by the West Virginia University Animal Care and Use Committee.

Apparatus

The apparatus and recording procedures for the rabbit NMR, developed and first described by Gormezano (Gormezano *et al.*, 1962), have been detailed previously (Schreurs and Alkon, 1990; Schreurs *et al.*, 2000). Each rabbit was restrained in a Plexiglas box and placed in a sound-attenuating, ventilated chamber. A stimulus panel containing a speaker and a 10-W house light was mounted above the rabbit's head. Ambient noise (65 dB) was provided by an exhaust fan. Air puffs (APs) to the cornea of the right eye were delivered by a programmable pressure regulator (ER3000, Tescom, Elk River, Minnesota, USA) connected to a 1-mm diameter tube positioned 5–7 mm from the center of the cornea. Transducing NMRs involved a hook and L-shaped lever attached to a 6-0 nylon loop sutured into but not through the nictitating membrane. The other end of the lever was attached to either a rotary encoder or a potentiometer connected to a 12-bit analog-to-digital (A/D) converter (5-ms sampling rate; 0.05-mm resolution). Individual NMR A/D outputs were stored on a trial-by-trial basis for subsequent analysis. Data collection, analysis, and stimulus delivery were accomplished using a LabVIEW (Austin, Texas, USA) software system (Schreurs *et al.*, 2000).

Procedure

Bryostatin pretreatment

After being housed for 1 week, rabbits were randomly allocated to four groups that comprised the cells of a 2×2 factorial design with the factors of drug (Bryo, saline) and pairings (paired, unpaired). Rabbits in the paired ($n = 18$) or unpaired group ($n = 12$) were equally divided between two drug conditions: Bryo (10.0 μg/kg) and vehicle. Bryo or vehicle was injected into the marginal vein of the rabbit ear 10 min before every other day of trace conditioning. Bryo or vehicle was also injected 10 min before the second day of tone intensity testing. This Bryo administration regime was chosen to match the time course of Bryo-induced PKC activation and subsequent increased protein synthesis required for memory formation (Alkon *et al.*, 2007) and minimize the side effects observed at high doses in clinical trials (Winegarden *et al.*, 2003) that might interfere with the performance.

All rabbits in the paired group received 1 day of adaptation and eight daily sessions of paired trace conditioning to assess their ability to learn a difficult conditioning task. All rabbits in the unpaired group received 1 day of adaptation and 8 days of explicitly unpaired stimulus presentations to assess the level of nonassociative responding. All rabbits then received six daily sessions of delay classical conditioning to test their ability to learn a simpler, hippocampally independent conditioning task and two daily sessions of tone intensity testing to assess their hearing.

On the adaptation day, the rabbits were prepared for AP and recording of the NMR and then adapted to the training chambers for the length of time of subsequent

Table 1 Conditioning paradigms

	Trace		Delay	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Tone-CS				
Duration (ms)	250	100	400	400
Frequency (kHz)	1	1	1	1
Intensity (dB)	90	82	90	82
Air puff-US				
Duration (ms)	100	100	100	100
Pressure (psi)	4	4	4	4
Trace (ms)	250	500	0	0
Interstimulus interval (ms)	500	600	300	300
Length of session (min)	30	60	30	60

Pretreatment: Bryo (bryostatin-1) pretreatment; posttreatment: Bryo posttreatment. CS, conditioned stimulus; US, unconditioned stimulus.

training sessions (30 min). Each of the eight paired trace conditioning sessions (Table 1) consisted of 30 presentations of a 250-ms, 1-kHz, 90-dB tone that was followed by a 250-ms trace interval and then a 100-ms, 4-psi AP (i.e. 500-ms interstimulus interval). Paired stimulus presentations were delivered, on average, every 60 s (50–70 s range). Sessions for unpaired rabbits consisted of 30 conditioned stimulus (CS)-alone and 30 unconditioned stimulus (US)-alone presentations that occurred in an explicitly unpaired manner delivered, on average, every 30 s (20–40 s range). Each of the six delay conditioning sessions (Table 1) for all rabbits consisted of 30 presentations of a 400-ms, 1-kHz, 90-dB tone that coterminated with a 100-ms, 4-psi AP (i.e. 300-ms interstimulus interval). The two tone intensity testing sessions consisted of the presentation of one of eight 400-ms tone intensities (55, 60, 65, 70, 75, 80, 85, 90 dB) or a zero intensity (0 dB) that coterminated with a 100-ms AP. Each of the tone intensities was presented in a randomized sequence that occurred eight times with each trial delivered, on average, every 60 s (50–70 s range).

The length of each session (30 min) was designed so that stimulus presentations coincided with the maximal activation of PKC induced by Bryo. Given the strong dependence of classical conditioning on stimulus parameters, we selected an intensity of 90 dB and a duration of 250 ms for the tone CS and an interstimulus interval of 500 ms to reduce the amount of time required for acquisition of the rabbit NMR (Kehoe and Macrae, 2002).

Bryostatin posttreatment with or without vitamin E

Sixty-three rabbits in the paired group were divided among seven different drug conditions: vehicle control ($n = 12$), 1.0 μg/kg Bryo ($n = 12$), 5.0 μg/kg Bryo ($n = 6$), 10.0 μg/kg Bryo ($n = 12$), vehicle control + Vit E ($n = 6$), 5.0 μg/kg Bryo + Vit E ($n = 7$), 10.0 μg/kg Bryo + Vit E ($n = 8$). Thirty-four rabbits in the unpaired group were divided among five different drug conditions: vehicle control ($n = 8$), 1.0 μg/kg Bryo ($n = 8$), 10.0 μg/kg Bryo ($n = 8$), vehicle control + Vit E ($n = 6$), 10.0 μg/kg Bryo + Vit E ($n = 4$). Bryo or vehicle was injected into the marginal vein of the rabbit ear within 20 min of the end of each session of trace conditioning and at the end of the first session of tone intensity testing (see below). Vit E 300 (15 mg/kg) purchased from Durvet (Blue Springs, Missouri, USA) was given via intramuscular injection before adaptation, prior to each session of trace conditioning, and before both tone intensity testing sessions.

All rabbits received the same general training protocol as that used for bryostatin pretreatment except that the number of trials for paired rabbits was increased to 60 per session, the number of trials for the unpaired rabbits was increased to 120, and a number of parameters were changed to make the trace conditioning paradigm more difficult (Table 1). The tone duration was reduced to 100 ms and its intensity reduced to 82 dB. The trace interval was increased to 500 ms. Consequently, eight trace conditioning sessions for paired rabbits consisted of 60 presentations of a 100-ms, 1-kHz, 82-dB tone that was followed by a 500-ms trace interval and then a 100-ms, 4-psi AP (i.e. 600-ms interstimulus interval). Sessions for unpaired rabbits consisted of 60 CS-alone and 60 US-alone presentations that occurred in an explicitly unpaired manner delivered, on average, every 30 s (20–40 s range). After trace conditioning, all rabbits received 6 days of paired delay conditioning. Each of the six delay conditioning sessions consisted of 60 presentations of a 400-ms, 1-kHz, 82-dB tone that coterminated with a 100-ms, 4-psi AP (i.e. 300-ms interstimulus interval). Each of the tone intensities presented in the two tone intensity testing sessions occurred only four times with each trial delivered, on average, every 60 s (50–70 s range).

A conditioned response (CR) was defined as any NMR exceeding 0.5 mm that was initiated after CS onset but before US onset. An unconditioned response (UR) was defined as any NMR exceeding 0.5 mm that was initiated within 1200 ms of US onset. Amplitude of a response was scored in millimeters as the maximum extension of the nictitating membrane. Onset latency of a response was identified as the latency in milliseconds from stimulus onset at which a response rose 0.1 mm above the baseline. Peak latency of a response was determined as the latency in milliseconds from stimulus onset to maximum extension of the nictitating membrane. Area of a response was calculated as the total area under the response curve from US onset to the end of the trial.

Drug

Bryo (NSC 339555) supplied by National Cancer Institute (NCI, Bethesda, Maryland, USA) was first dissolved in PET diluent (60/30/10, NSC 641159), which was a 60, 30, and 10% (v/v) mixture of polyethylene glycol 400 (PEG400), dehydrated alcohol, and polysorbate 80

(Tween 80). The Bryo and PET were then diluted with 0.9% sodium chloride solution before administration. Bryo purchased from L.C. Laboratories (Woburn, Massachusetts, USA) was dissolved in dimethyl sulfoxide (Fisher Chemicals, Fair Lawn, New Jersey, USA) at a concentration of 1 μg/μl, and then diluted in 1% Tween 20/25% dimethyl sulfoxide/74% phosphate-buffered saline before administration.

Data analysis

Three rabbits receiving Bryo posttreatment did not complete the study and were excluded from the data analyses. Specifically, two rabbits from Bryo 10.0 μg/kg + Vit E group died and one rabbit from the paired vehicle control group was removed because of an unusual orientation of the eyeball that prevented the proper delivery of AP at the cornea. Repeated measures analyses of variance (SPSS 14.0, Chicago, Illinois) were performed on the data with follow-up analyses used to localize significant sources of variation. The significance level was set at $P < 0.05$.

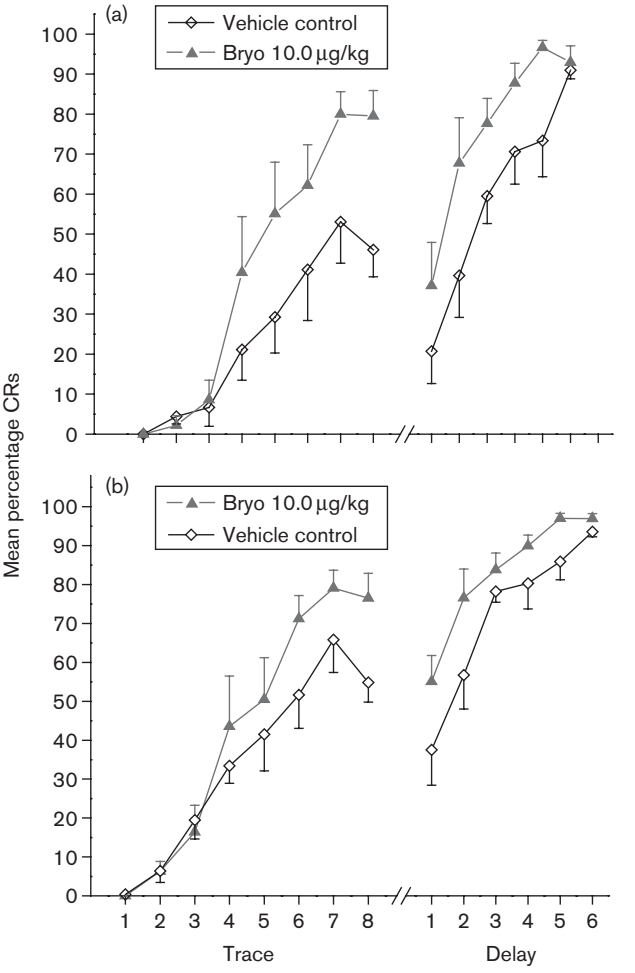
Results

Bryostatin-1 pretreatment facilitates acquisition of the classically conditioned rabbit nictitating membrane response

Figure 1 depicts mean percent CRs across the 8 days of trace conditioning and 6 days of delay conditioning for rabbits pretreated with Bryo or vehicle. Compared with rabbits in the vehicle control group, rabbits pretreated with Bryo showed more CRs during the first 10 trials (Fig. 1a) but not during the entire 30 trials (Fig. 1b) of each trace conditioning session. Analysis of percent CRs for the first 10 trials of each trace conditioning session yielded significant effects of days [$F(7,112) = 34.36$, $P < 0.001$] and group [$F(1,16) = 5.85$, $P < 0.05$] and a significant day × group interaction [$F(7,112) = 2.30$, $P < 0.05$]. An analysis of percent CRs for the entire 30 trials of each trace conditioning session yielded a significant main effect of days [$F(7,112) = 49.13$, $P < 0.001$], but failed to show a main effect of group [$F(1,16) = 2.73$, NS] or day × group interaction [$F(7,112) = 1.38$, NS]. Clearly, Bryo-treated rabbits learned the simple trace conditioning task sooner during the trace conditioning session than the vehicle controls. Moreover, the facilitating Bryo effect also seems to have been sustained because an analysis of the 30 trials on the last day of trace conditioning revealed a significant difference between the groups [$F(1,16) = 7.98$, $P < 0.05$].

When shifted to delay conditioning, rabbits pretreated with Bryo continued to show higher levels of responding and more rapid acquisition of CRs than those pretreated with vehicle. Analysis of percent CRs for the first 10 trials of each delay conditioning session indicated significant main effects of days [$F(5,80) = 28.60$, $P < 0.001$] and group [$F(1,16) = 7.57$, $P < 0.05$]. Interestingly, an analysis of percent CRs for the entire 30 trials of each delay conditioning session also showed main effects of days [$F(5,80) = 30.562$, $P < 0.001$] and group [$F(1,16) = 7.29$, $P < 0.05$]. These data suggest pretreatment with Bryo had a facilitative effect on the acquisition of a classically conditioned rabbit NMR – an effect that coincides with the maximal PKC activation induced by Bryo and the subsequent increased protein synthesis required for memory formation and transformation.

Fig. 1

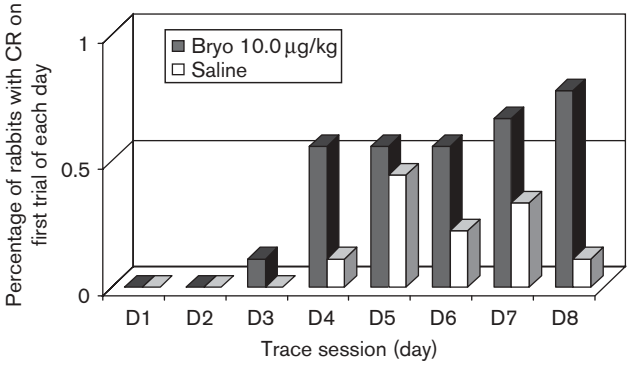


Mean percent (± SEM) conditioned responses (CRs) during the first 10 trials (a) and the entire 30 trials (b) of each of 8 days of trace conditioning and 6 days of delay conditioning for rabbits pretreated with bryostatin-1 (Bryo) or vehicle. Trace conditioning consisted of eight 30-trial sessions in which a 250-ms, 90-dB, 1-kHz tone-conditioned stimulus (CS) was presented 500 ms before a 100-ms, 4-psi corneal air puff (AP) unconditioned stimulus. Delay conditioning consisted of six 30-trial sessions in which a 400-ms, 90-dB, 1-kHz tone CS coterminated with a 100-ms, 4-psi AP.

Bryostatin-1 pretreatment enhances memory retention of the classically conditioned rabbit NMR

Figure 2 depicts the mean percent of rabbits with a CR on the first trial of each day during trace conditioning.

Fig. 2



Mean percent of rabbits with a conditioned response (CR) on the first trial of each trace conditioning session for rabbits pretreated with bryostatin-1 (Bryo) or vehicle. Trace conditioning consisted of eight 30-trial sessions in which a 250-ms, 90-dB, 1-kHz tone-conditioned stimulus was presented 500 ms before a 100-ms, 4-psi corneal air puff unconditioned stimulus.

When compared with the vehicle control group, rabbits pretreated with Bryo showed a significantly higher likelihood of eliciting a CR on the first trial of each trace conditioning session. The occurrence of a CR on the first trial is a measure of the rabbit's ability to recall the association between the CS and US from the previous day's CS–US pairings because the US does not occur on this first trial until 500 ms after tone onset – the interval used to determine the presence of a CR. In other words, the first US had not yet occurred to 'remind' the rabbit of the CS–US association. Analysis of percent CRs on the first trial during trace conditioning indicated significant effects of days [$F(7,112) = 8.83$, $P < 0.001$] and group [$F(1,16) = 4.87$, $P < 0.05$] and day × group interaction [$F(7,112) = 2.48$, $P < 0.05$]. This indicates that pretreatment with Bryo enhanced retention of the previously acquired CR. As Bryo was given every other day during trace conditioning, the data also indicate enhanced retention was due to the long-term cumulative effects of Bryo. Finally, the mean percent of rabbits with a CR on the first trial of each trace conditioning session did not increase until day 4, indicating that several days were required for Bryo to become effective in consolidating the memory trace.

Bryostatin-1 pretreatment does not sensitize the rabbit to air puff

Comparisons of percent URs, peak latency, amplitude, and area for the vehicle control and the Bryo groups across the eight sessions of unpaired stimulus presentations revealed no systematic differences in responding between groups. A repeated-measures analysis of percent URs during unpaired stimulus presentations indicated neither main effects of days and group, nor was there a significant day × group interaction ($P > 0.05$). Repeated measures analyses of UR peak latency, amplitude, and area during the 8 days of unpaired stimulus presentations did not show any significant effects of days or group, or day × group interaction. These results indicate that Bryo-induced facilitation of classical conditioning of the rabbit NMR was not due to a systematic alteration in motor activity. Pretreatment with Bryo did not sensitize the rabbits to AP.

Bryostatin-1 pretreatment does not sensitize the rabbit to tone

Comparison of mean percent responding to the tone for vehicle control and Bryo groups across the eight sessions of unpaired stimulus presentations and 6 days of paired delay conditioning revealed no systematic changes in the very low levels of responding to the tone during the unpaired stimulus presentations. A repeated-measures analysis of percent responses indicated that there were no significant main effects of days or group, or day × group interaction, during the unpaired stimulus presentations. As a result, it seems that Bryo did not sensitize responding to the tone CS.

Analysis of amplitude, area, onset latency, and peak latency of responses to the tone during unpaired stimulus presentations yielded no significant main effects of days, group, or a day × group interaction. These results further support the conclusion that Bryo did not influence the baseline levels of responding to the tone.

When the unpaired rabbits were shifted to delay conditioning, all rabbits showed rapid acquisition of CRs. Analysis of percent CRs during delay conditioning indicated significant main effect of day [$F(5,50) = 35.88$, $P < 0.001$], but no significant group effect [$F(1,10) = 1.69$, NS] or day × group interaction [$F(5,50) = 0.45$, NS]. This indicates that rabbits previously given unpaired trace conditioning were still able to learn the easier delay conditioning task regardless of the drug administration.

Tone intensity testing for all paired groups and previously unpaired groups indicated that the level of CRs increased as a function of tone intensity but that there were no differences among these groups on either day 1 or day 2 (data not shown). These observations were corroborated by an analysis of variance that yielded a significant main effect of tone intensity [for paired groups: $F(8,128) = 123.13$, $P < 0.001$ on day 1, $F(8,128) = 88.76$, $P < 0.001$ on day 2; for unpaired groups: $F(8,56) = 63.81$, $P < 0.001$ on day 1, $F(8,56) = 52.87$, $P < 0.001$ on day 2], but no significant group effects or tone intensity × group interactions. Therefore, there was no residual effect or direct effect of the drug on tone intensity threshold.

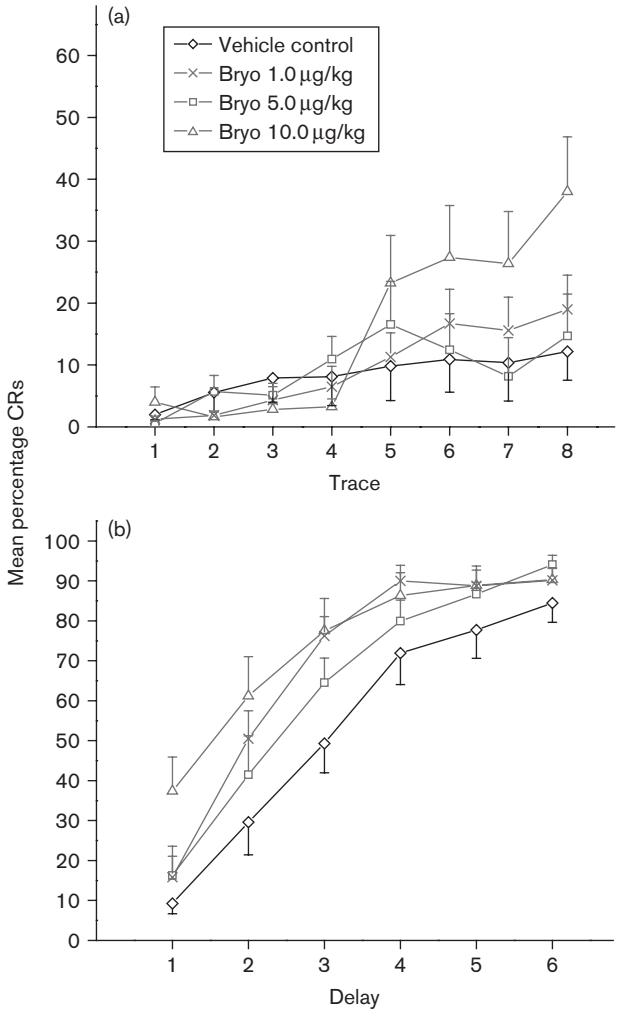
Bryostatin-1 posttreatment facilitates classical conditioning of the rabbit nictitating membrane response

Two sources of Bryo (from The National Cancer Institute and from L.C. Laboratories) were used in Bryo

posttreatment; a comparison of responding to Bryo revealed no statistically significant differences between the sources and the data were combined.

Figure 3 depicts mean percent CRs for the rabbits treated with different doses of Bryo after trace conditioning across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b). Inspection of Fig. 3a reveals a dose-specific facilitation of CR acquisition – rabbits injected with10 μg/kg Bryo demonstrated higher percent CRs by the end of trace conditioning than those given either a lower dose of Bryo (5 and 1 μg/kg) or vehicle. A repeated-measures analysis of percent CRs during trace conditioning indicated a significant main effect of days [$F(7,259) = 14.92$, $P < 0.001$] and a significant day × group interaction [$F(21,259) = 2.54$, $P < 0.001$]. The day × group interaction was attributable to a significant difference between 10 μg/kg Bryo and vehicle [$F(1,38) = 33.25$, $P < 0.001$]. When shifted to delay conditioning (Fig. 3b), rabbits treated with 10 μg/kg Bryo showed a continued higher level of responding and more rapid acquisition of CRs than those given vehicle. Analysis of percent CRs during delay conditioning indicated a significant main effect of days [$F(5,185) = 197.53$, $P < 0.001$] and day × group interaction [$F(15,185) = 2.91$, $P < 0.01$]. As there was no drug administration during delay conditioning, the facilitation induced by Bryo given during trace conditioning may have carried over to the easier delay task, or it was a long-term cumulative effect of Bryo-induced PKC activation and its subsequent increase synthesis of proteins. Taken together, these data suggest 10 μg/kg Bryo had long-term enhancing effects on classical conditioning of the rabbit NMR.

Fig. 3



Mean percent (±SEM) conditioned responses (CRs) across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b) for rabbits posttreated with bryostatin-1 (Bryo)-alone or vehicle. Trace conditioning consisted of eight 60-trial sessions in which a 100-ms, 82-dB, 1-kHz tone-conditioned stimulus (CS) was presented 600 ms before a 100-ms, 4-psi corneal air puff (AP) unconditioned stimulus. Delay conditioning consisted of six 60-trial sessions in which a 400-ms, 82-dB, 1-kHz tone CS coterminated with a 100-ms, 4-psi AP.

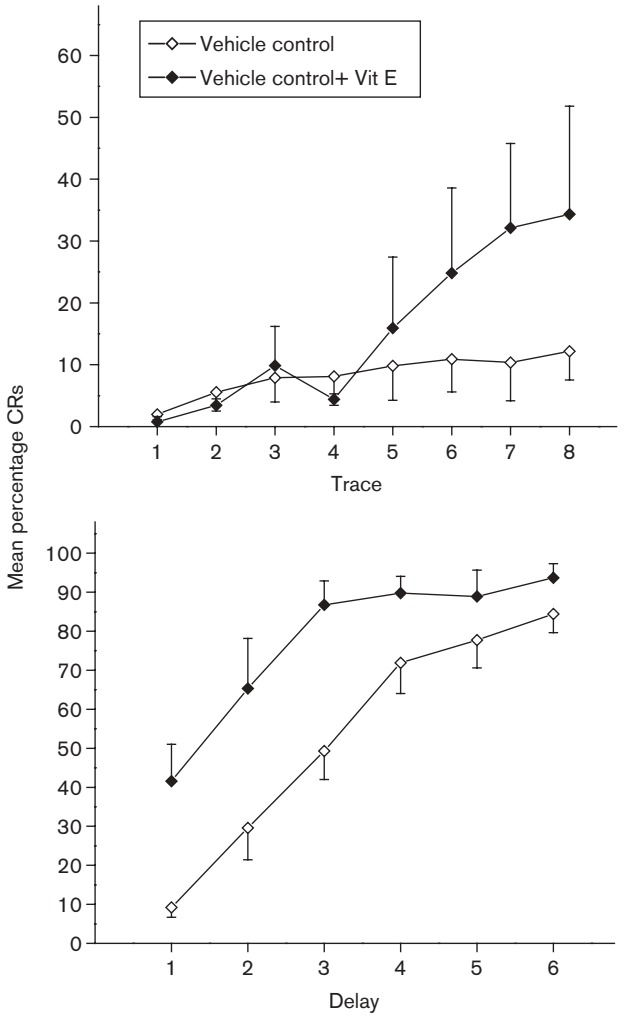
Vitamin E pretreatment facilitates classical conditioning of the rabbit nictitating membrane response

Figure 4 depicts mean percent CRs of the rabbits treated with vehicle and vehicle + Vit E across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b). Compared with rabbits given the vehicle (Fig. 4a), rabbits given Vit E had a higher level of CRs. A repeated measures analysis of percent CRs during trace conditioning indicated a significant main effect of days [$F(7,98) = 19.08$, $P < 0.001$] and day × group interaction [$F(7,98) = 3.94$, $P < 0.01$]. This day × group interaction was attributable to a significant difference between vehicle + Vit E and vehicle [$F(1,14) = 8.59$, $P < 0.05$]. When rabbits were shifted to delay conditioning (Fig. 4b), rabbits receiving vehicle + Vit E showed a continued higher level of conditioning than rabbits given the vehicle. Analysis of percent CRs during delay conditioning indicated significant main effects of days [$F(5,75) = 63.96$, $P < 0.001$] and group [$F(1,15) = 10.04$, $P < 0.01$] and day × group interaction [$F(5,75) = 4.93$, $P < 0.01$]. These results indicate that Vit E administered before classical conditioning enhanced the acquisition of CRs and also facilitated the transition between the two training protocols in our experiment.

Bryostatin-1 posttreatment combined with vitamin E pretreatment markedly enhances classical conditioning of the rabbit nictitating membrane response

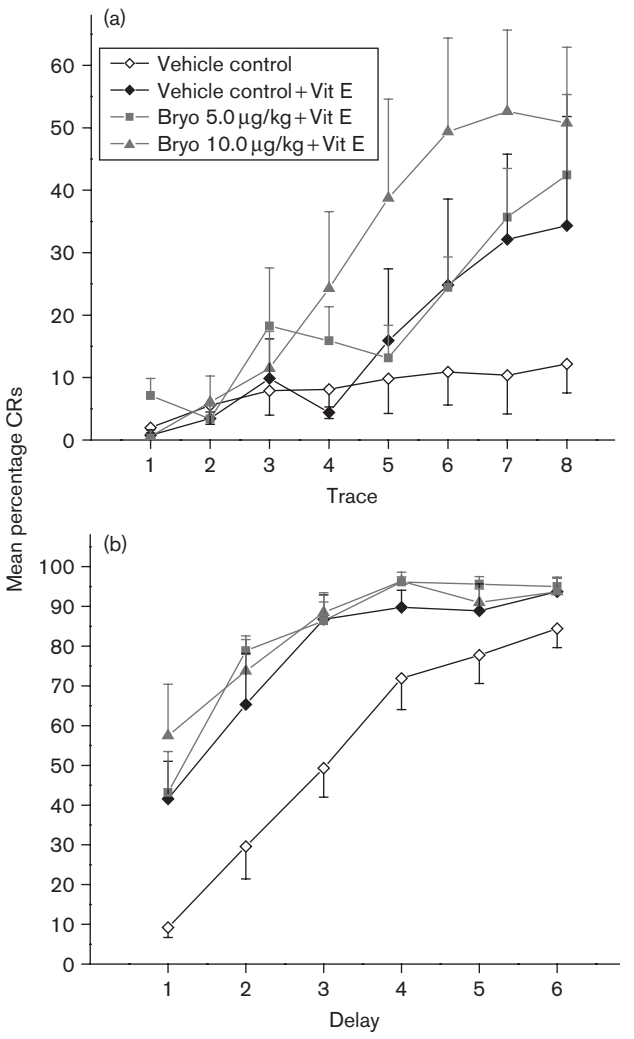
Figure 5 depicts mean percent CRs for rabbits treated with Bryo and Vit E across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b). The most interesting feature of the data is that Bryo combined with Vit E markedly enhanced the acquisition of CRs over the vehicle control group. In fact, Fig. 5a shows that rabbits in 10 μg/kg Bryo + Vit E group demonstrated greater percent CRs than those in the vehicle control group, the vehicle control + Vit E group, or the 5 μg/kg Bryo + Vit E

Fig. 4



Mean percent (±SEM) conditioned responses (CRs) across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b) for rabbits posttreated with vehicle or posttreated with vehicle and pretreated with vitamin E (Vit E). Trace conditioning consisted of eight 60-trial sessions in which a 100-ms, 82-dB, 1-kHz tone-conditioned stimulus (CS) was presented 600 ms before a 100-ms, 4-psi corneal air puff (AP) unconditioned stimulus. Delay conditioning consisted of six 60-trial sessions in which a 400-ms, 82-dB, 1-kHz tone CS coterminated with a 100-ms, 4-psi AP.

Fig. 5



Mean percent (±SEM) conditioned responses (CRs) across the 8 days of trace conditioning (a) and 6 days of delay conditioning (b) for rabbits posttreated with vehicle, posttreated with vehicle and pretreated with vitamin E (Vit E), posttreated with bryostatin-1 (Bryo) and pretreated with Vit E. Trace conditioning consisted of eight 60-trial sessions in which a 100-ms, 82-dB, 1-kHz tone-conditioned stimulus (CS) was presented 600 ms before a 100-ms, 4-psi corneal air puff (AP). Delay conditioning consisted of six 60-trial sessions in which a 400-ms, 82-dB, 1-kHz tone CS coterminated with a 100-ms, 4-psi AP.

group. A repeated measures analysis of percent CRs during trace conditioning indicated a significant main effect of days [$F(7,182) = 22.78$, $P < 0.001$] and day × group interaction [$F(21,182) = 3.06$, $P < 0.001$]. The day × group interaction was attributable to a significant difference between Bryo + Vit E and vehicle [$F(1,19) = 721.66$, $P < 0.001$]. Further analysis of percent CRs across Bryo treatments showed treatment with Bryo 10.0 µg/kg + Vit E resulted in a significantly higher level of responding than treatment with Bryo at lower doses (0, 1.0, and 5.0 µg/kg) on trace conditioning days 6, 7, and 8 (all $P < 0.05$). These data indicate Vit E facilitated Bryo's long-term memory enhancement of rabbit classical conditioning.

When rabbits were shifted to delay conditioning (Fig. 5b), all rabbits given Bryo + Vit E showed a continued higher level of responding and a more rapid acquisition of CRs than those in vehicle control group. Analysis of percent CRs during delay conditioning indicated significant main effects of group [$F(3,27) = 11.34$, $P < 0.001$] and days [$F(5,135) = 82.68$, $P < 0.001$] and day × group interaction [$F(15,135) = 4.05$, $P < 0.001$]. Further comparison of percent CRs across Bryo treatments revealed treatment

with 10.0 μg/kg Bryo + Vit E demonstrated higher percentages of CRs than those treated with Bryo at lower doses (0, 1.0 μg/kg) on delay conditioning days 1, 2, and 3 (all $P < 0.05$). These data suggest Bryo has a strong long-term cumulative facilitating effect on classical conditioning of the rabbit NMR and indicate that Vit E added to Bryo's long-term memory enhancement.

Neither bryostatin-1 posttreatment nor vitamin E pretreatment sensitizes the rabbit to air puff

Comparison of percent URs for all unpaired groups across the eight sessions of unpaired stimulus presentations indicated considerable variability in responding but no systematic differences between groups (data not shown). A repeated measures analysis of percent URs during unpaired stimulus presentations indicated a significant main effect of days [$F(7,196) = 7.69$, $P < 0.001$], but there was no significant group effect or day × group interaction ($F < 1.3$). Variability in responding during day 1 of unpaired stimulus presentations was attributable to two rabbits in 10.0 μg/kg Bryo group and one in 10.0 μg/kg Bryo + Vit E group that responded to AP at relatively low levels (percent URs were 35.2, 48.3, and 55%, respectively). As there were no significant group differences or day × group interaction in this analysis, the data suggest that neither Bryo, Vit E, nor the combination of Bryo + Vit E sensitized the rabbits to AP.

Comparisons of several other UR dependent variable measures across the eight sessions of unpaired stimulus presentations reflected daily fluctuations in some of these dependent variables (data not shown). A repeated-measures analysis of UR amplitude and area indicated significant day × group interactions [$F(28,196) = 2.14$, $P < 0.01$; $F(28,196) = 2.01$, $P < 0.01$, respectively], but no significant main effect of days [$F(7,196) = 0.77$, NS; $F(7,196) = 0.38$, NS, respectively] or group [$F(4,28) = 1.24$, NS; $F(4,28) = 0.76$, NS, respectively]. The day × group interactions were probably attributable to daily fluctuations rather than any systematic effects of the drug. An analysis of UR onset latency and peak latency yielded significant main effects of days [$F(7,196) = 8.41$, $P < 0.01$; $F(7,196) = 4.19$, $P < 0.001$, respectively], but no significant group effect [$F(4,28) = 0.30$, NS; $F(4,28) = 1.34$, NS, respectively] or day × group interactions [$F(28,196) = 1.33$, NS; $F(28,196) = 1.50$, $P = 0.06$, respectively]. Taken together, these data indicate that facilitation of classical conditioning of the rabbit NMR by Bryo, Vit E, and the combination of Bryo + Vit E was not due to a systematic alteration in motor activity.

Neither bryostatin-1 posttreatment nor vitamin E pretreatment sensitizes the rabbit to tone

Comparison of mean percent responding to the tone for all unpaired groups across the eight sessions of unpaired stimulus presentations and 6 days of paired delay conditioning reflected no systematic changes in the very low levels of responding to the tone during the unpaired stimulus presentations. A repeated-measures analysis of percent responses indicated a significant main effect of days [$F(7,196) = 5.97$, $P < 0.001$], but no significant group effect [$F(4,28) = 0.67$, NS] or day × group interaction [$F(28,196) = 1.32$, NS]. As a result, it seems that neither Bryo, nor Vit E, nor the combination of Bryo + Vit E sensitized responding to the tone CS.

Analysis of amplitude, area, onset latency, and peak latency of responses to the tone during unpaired stimulus presentations yielded no significant main effects of days, group, or day × group interaction. These data further support the conclusion that neither Bryo nor Vit E had an effect on the baseline levels of responding to the tone.

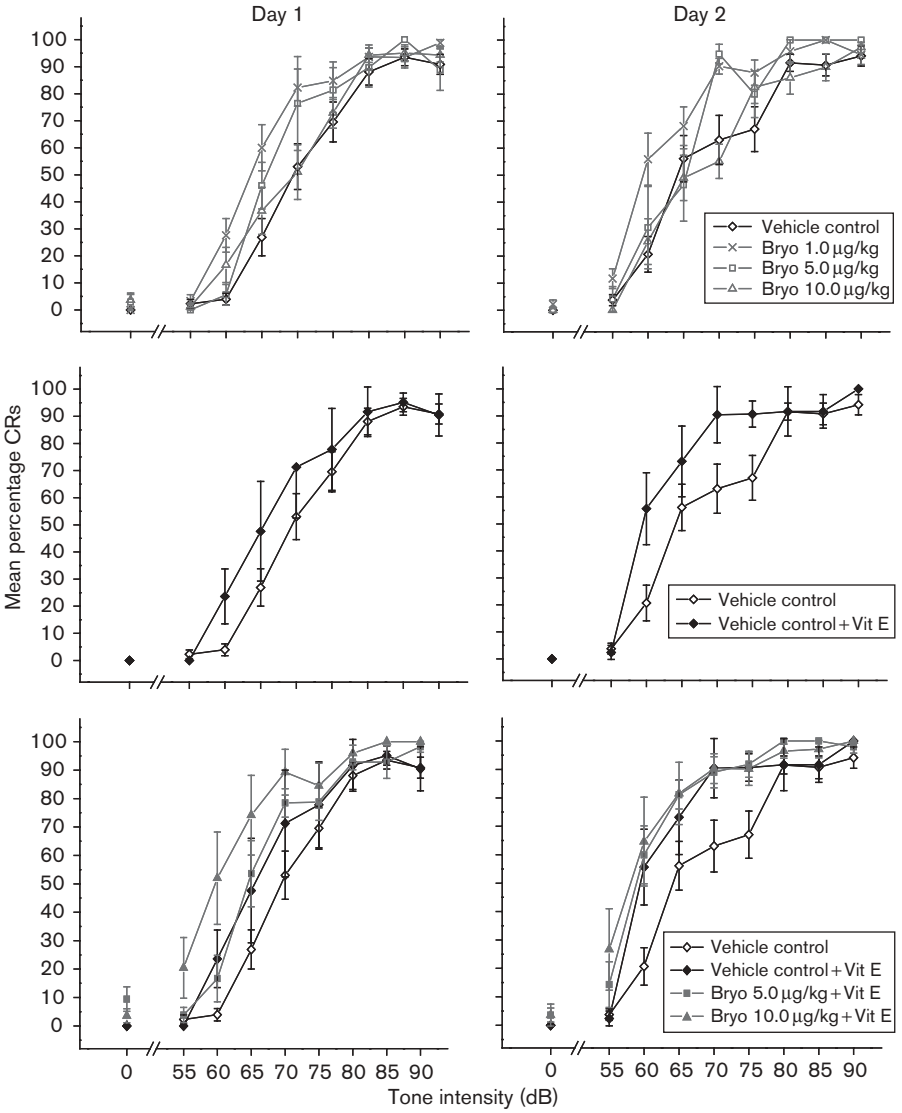
When these unpaired rabbits were shifted to delay conditioning, all rabbits showed rapid acquisition of CRs. Analysis of percent CRs during delay conditioning indicated a significant main effect of days [$F(5,140) = 159.05$, $P < 0.001$], but no significant group effect [$F(4,28) = 1.37$, NS] or day × group interaction [$F(20,140) = 1.14$, NS]. This indicates those rabbits previously given unpaired trace conditioning were all able to learn the delay conditioning task regardless of drug treatment.

Even though no sensitivity changes were found among groups receiving unpaired presentations of CS and US, it is still possible that the enhanced learning was attributed, at least partly, to the drug's enhancement of the sensory processing of the CS during classical conditioning (Gormezano and Harvey, 1980; Wang *et al.*, 2006). To confirm whether there were drug-induced changes in CS processing, we administered tones of different intensities during 2 additional days of delay conditioning. Day 1 of tone intensity testing in this case was used to address any long-term residual effects of the drug, whereas day 2 of testing addressed any potential effects on tone responding of the drug administered on the previous day.

Examination of mean percent CRs elicited by eight tone intensities in rabbits from all previously unpaired groups showed that the level of CRs increased as a function of tone intensity but that there were no differences among these groups on either day 1 or day 2 of tone intensity testing. These observations were corroborated by an analysis of variance that yielded a significant main effect of tone intensity [$F(8,208) = 146.80$, $P < 0.001$ on day 1, $F(8,208) = 145.83$, $P < 0.001$ on day 2], but no significant group effects or tone intensity × group interactions. Therefore, there was no residual effect or direct effect of the drug on tone sensitivity.

Figure 6 illustrates mean percent CRs elicited by eight tone intensities in rabbits from the previously paired

Fig. 6



Mean percent (±SEM) conditioned responses (CRs) elicited by eight tone intensities (55, 60, 65, 70, 75, 80, 85, 90 dB) or 0 dB, in rabbits from paired groups on day 1 and day 2 of tone intensity testing. Top panel is from posttreated with bryostatin-1 (Bryo)-alone, middle panel is from pretreated with vitamin E (Vit E)-alone; and bottom panel is from posttreatment with Bryo + pretreatment with Vit E groups. Tone intensity testing consisted of the presentation of one of the eight 400-ms tone intensities or zero intensity that coterminated with a 100 ms, 4 psi corneal air puff (AP). Each tone intensity-AP pairing was presented eight times as a randomized sequence with each trial delivered, on average, every 60 s (50–70 s range).

groups. Inspection of the panels in Fig. 6 shows that the level of responding for all rabbits that previously received paired trace conditioning increased as a function of tone intensity and there tended to be a number of between-group differences in responding to tones of lower intensities on both day 1 and day 2. Interestingly, there appeared to be no differences in the levels of responding at or above the CS training intensity on either day.

An analysis of variance for the rabbits treated with Bryo or vehicle control on day 1 of tone intensity testing revealed significant main effects of CS intensity [$F(8,280) = 215.80$, $P < 0.001$], group [$F(3,35) = 3.12$, $P < 0.05$], and a significant tone intensity × group interaction [$F(24,280) = 2.08$, $P < 0.01$]. The same results were found among the rabbits treated with Bryo + Vit E and the vehicle, with significant main effects of CS intensity [$F(8,216) = 169.07$, $P < 0.001$] and group [$F(3,27) = 4.38$, $P < 0.05$], and a significant tone intensity × group interaction [$F(24,216) = 2.16$, $P < 0.01$]. When responding of rabbits given the vehicle was compared with that of rabbits given vehicle + Vit E, there was a significant main effect of CS intensity [$F(8,120) = 90.16$, $P < 0.001$], but there was no

significant main effect of group [$F(1,15) = 1.53$, NS] or tone intensity × group interaction [$F(8,120) = 1.29$, NS]. The same statistical results were obtained when the day 2 data were analyzed.

The tone intensity data for rabbits previously given paired trace conditioning suggest that there might be both residual and direct drug-induced changes in tone threshold. The results of tone intensity testing from previously unpaired groups, however, show that there are no drug effects on auditory responding or thresholds. The significant differences in tone intensity responding for the paired groups are probably attributable to lower levels of tone intensity responding in the vehicle control group that resulted from a lower terminal level of responding during delay conditioning.

Side effects of bryostatin-1 posttreatment and vitamin E pretreatment do not affect facilitation classical conditioning of the rabbit nictitating membrane response

As a new approach to cancer therapy currently in phases I and II clinical trials, Bryo has side effects including myalgia, nausea, and vomiting (Winegarden *et al.*, 2003). In our study, body weights were monitored during training to assess the effects of daily administration of Bryo. Data from both paired and unpaired groups revealed reversible weight loss among groups of rabbits treated with 10 μg/kg Bryo, 10 μg/kg Bryo + Vit E, and 5 μg/kg Bryo + Vit E, whereas rabbits given the lower doses of Bryo and vehicle did not exhibit weight loss. These data suggest Bryo exerts significant reversible anorectic effects and Vit E did not ameliorate this side effect. Nevertheless, the anorectic effects did not influence learning because there was no significant relationship between the amount of weight loss and any of the CR measures.

Discussion

The principal findings of the present experiment were: (i) Bryo facilitated the acquisition of and memory retention for classical conditioning of the rabbit NMR, presumably through PKC modulation; (ii) Vit E added to the memory-enhancing efficacy of Bryo; and (iii) both drugs exhibited a net action on associative learning not attributable to a sensitivity change to AP or tone.

Regardless of whether the conditioning task had a long or short CS–US interval, this study shows that 10 μg/kg Bryo, given either before training or after training, enhanced the acquisition of classical conditioning of the rabbit NMR. These data are consistent with recent reports from spatial maze learning and memory in rats (Sun and Alkon, 2005), recall in *Hermissenda* (Alkon *et al.*, 2005; Kuzirian *et al.*, 2006), and behavioral improvements in Alzheimer's disease transgenic mice (Etcheberrigaray *et al.*, 2004). As control animals from the Bryo posttreatment did not show much learning during the 8 days trace conditioning, it seems that the trace conditioning task was very difficult for rabbits to learn. In some ways, this may mimic the difficulty Alzheimer's patients have in acquiring new memories. Daily injection of Bryo after training enhanced classical conditioning of the rabbit NMR with this difficult task suggests that Bryo improved learning and memory and had a long-term enhancing effect on classical conditioning of rabbit NMR.

As rabbits pretreated with Bryo showed markedly increased classical conditioning during the first 10 trials rather than the total 30 trials of trace conditioning, a time course similar to maximal PKC activation induced by Bryo, these data support our hypothesis that enhanced learning may be partly mediated by a short-term effect of Bryo-induced PKC activation. As Bryo was given every other day before trace conditioning, it is highly likely that a long-term effect of increased protein synthesis induced by PKC activation also made a contribution to the enhanced learning. In addition, because significantly increased responding on the first trial of each trace conditioning session occurred on day 4 of trace conditioning in rabbits pretreated with Bryo, and all rabbits posttreated with Bryo, showed comparably low CR levels during the first 4 days of trace conditioning, these data suggest that Bryo took several days to become effective in aiding the rabbit's ability to acquire and consolidate the memory trace. This supports the conclusion that the increased synthesis of proteins required for long-term memory by Bryo-induced PKC activation may underlie at least part of the improved behavioral performance (Alkon *et al.*, 1988, 2005; Olds *et al.*, 1989). It is also possible that the enhanced learning was produced by both the short-term and long-term effect of activated PKC. Earlier reports suggest initial PKC upregulation after Bryo administration is always followed by subsequent PKC downregulation, which is maintained at least through QJ;the duration of drug administration or even longer (Varterasian *et al.*, 1998; Marshall *et al.*, 2002). This, however, only happens after prolonged exposure and at higher doses (Varterasian *et al.*, 1998; Kuzirian *et al.*, 2006) and may be a PKC isoform-dependent effect (Marshall *et al.*, 2002) because Bryo does not produce downregulation of the PKC-α isozyme if administered at a low dose over a prolonged infusion time. On the basis of our data, it is conceivable that either there may be transient or minimal PKC downregulation after the initial PKC activation with our current Bryo dose regimen or PKC downregulation happened only in the specific PKC isoform(s) not responsible for learning and memory in the rabbit.

The binding of Bryo to PKC results in PKC activation and is then followed by rapid PKC translocation from cytosol to the plasma membrane and phosphorylation (Alkon *et al.*, 2005). These initial steps subsequently trigger

enhanced learning and memory via increased intrinsic membrane excitability by direct inhibition of 115 ± 6 pS potassium channels or 64-pS potassium channel in *Hermissenda* neurons (Alkon *et al.*, 1982,1988; Etcheberrigaray *et al.*, 1992; Nelson *et al.*, 1996) or inhibition of the potassium channel-mediated slow after hyperpolarization (Nelson *et al.*, 1996). In addition, this enhanced membrane excitability caused by long-lasting depolarization has been shown to produce positive feedback during memory consolidation (Alkon *et al.*, 2007).

PKC comprises a family of isozymes (α, ζ I, β II, γ, δ, ε, θ, η, λ/ι (mouse/human), and ζ) with different patterns of tissue and subcellular distribution. The exact function of each PKC isozyme is not yet known. Recent studies indicate that the present Bryo-induced facilitation of classical conditioning of the rabbit NMR may be mediated by PKC-α (Pascale *et al.*, 2005; Alkon *et al.*, 2007), PKC-ε (Alkon *et al.*, 2007), PKC-β (Lallemend *et al.*, 2005), and PKC-δ (Wender *et al.*, 2004).

Earlier studies have shown that associative effects of a drug on conditioning may arise from drug's enhancement of sensory processing of the CS (Gormezano and Harvey, 1980; Wang *et al.*, 2006) or US (Romano *et al.*, 2000). For example, 4-aminopyridine enhances classical conditioning as a result, at least in part, of drug-induced CS threshold alterations (Wang *et al.*, 2006). Similarly, lysergic acid diethylamide facilitated conditioning through modulating the threshold of CS processing for eliciting CRs (Gormezano and Harvey, 1980). In our experiments, we did not find a consistent drug-related change in the sensitivity to AP and tone, nor was there Bryo-induced alteration in tone-intensity responding. These findings suggest that the systematic effects of Bryo on acquisition of CRs reflect the net action of Bryo on associative learning rather than on sensory processing. To date, these are the first systematic in-vivo data showing the long-term cognitive effects of Bryo and its net action on associative learning.

It is now recognized that low levels of oxidants can modify cell-signaling proteins with functional consequences and that PKC signaling is a target for oxidative stress (Egger *et al.*, 2003; Lin and Takemoto, 2005). As it can block PKC in many tissues, Vit E has been clinically tested as a way of reducing PKC-mediated microvascular complications of diabetes through inhibition of PKC-α (Venugopal *et al.*, 2002) and PKC-β (Ganz and Seftel, 2000). Moreover, because it cannot readily cross the blood–brain barrier and does not accumulate in the cerebrospinal fluid (Pappert *et al.*, 1996), Vit E was originally introduced in our study to reduce PKC toxicity produced by Bryo in non-CNS tissues, especially the peripheral nervous system. Vit E, however, did not ameliorate Bryo-related side effects of weight loss and loss of appetite. The dose range of bryostatin likely to be used to treat neurological disorders in humans (20–40 μg/m^2/week) only has one consistent side effect – myalgia, which can be largely controlled by administering a nonsteroidal antiinflammatory. The other side effects, nausea and vomiting, occurred only at higher doses.

Unexpectedly, Vit E was found to facilitate classical conditioning of NMR in healthy adult rabbits. To our knowledge, this is the first demonstration of learning facilitation by systemic administration of Vit E in normal, young, and intact animals. These data are consistent with earlier studies that have shown Vit E can significantly overcome learning and memory deficits induced by physiological manipulations, disease, or aging (Yamada *et al.*, 1999; Fukui *et al.*, 2002; Tuzcu and Baydas, 2006). Vit E was also found to add to the memory-enhancing effects of Bryo on classical conditioning of the rabbit NMR (Fig. 5). Moreover, if compared with either the Bryo or Vit E groups, the increase in percent CRs occurred earlier in the Bryo + Vit E group, suggesting stronger CR acquisition. Therefore, Vit E, a traditional antioxidant, may enhance learning and memory by acting as an exogenous modulator of PKC effects such as free radical generation (Lin and Takemoto, 2005). It is possible that Vit E may produce different effects on the central and peripheral nervous systems because the partition of Vit E across the blood–brain barrier may be unfavorable for brain access (Pappert *et al.*, 1996). If there are centrally mediated behavioral effects induced by Vit E, they may be partly attributable to the modification by Vit E of different PKC isozymes in the CNS (Egger *et al.*, 2003). For example, Vit E may enhance learning and memory by affecting neuronal plasticity through PKC-δ inhibition (Ferri *et al.*, 2006) or through PKC-γ inhibition (Lin and Takemoto, 2005) by disassembling connection 43 gap junction plaques and increasing the activity of gap junctions (Lin and Takemoto, 2005).

In summary, we have found that Bryo and Vit E produced an enhancement of classical conditioning of the rabbit NMR and that there was an additive effect when rabbits were given Bryo and Vit E. It seems that Bryo and Vit E produce long-term centrally mediated behavioral effects consistent with modifying different PKC isozymes and with the protein synthesis required for memory formation and consolidation.

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