**In vitro** extinction learning in *Hermissenda*: involvement of conditioned inhibition molecules

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Extinction of a conditioned association is typically viewed as the establishment of new learning rather than the erasure of the original memory. However, recent research in the nudibranch, *Hermissenda crassicornis* (*H.c.*) demonstrated that extinction training (using repeated light-alone presentations) given 15 min, but not 23 h, after memory acquisition reversed both the cellular correlates of learning (enhanced Type B cell excitability) and the behavioral changes (reduced phototaxis) produced by associative conditioning (pairings of light, CS, and rotation, US). Here, we investigated the putative molecular signaling pathways that underlie this extinction in *H.c.* by using a novel *in vitro* protocol combined with pharmacological manipulations. After intact *H.c.* received either light-rotation pairings (Paired), random presentations of light and rotation (Random), or no stimulation (Untrained), B cells from isolated CNSs were recorded from during exposure to extinction training consisting of two series of 15 consecutive light-steps (LSs). When *in vitro* extinction was administered shortly (2 h, but not 24 h) after paired training, B cells from Paired animals showed progressive and robust declines in spike frequency by the 30th LS, while control cells (Random and Untrained) did not. We found that several molecules implicated in *H.c.* conditioned inhibitory (CI) learning, protein phosphatase 1 (PP1) and arachidonic acid (AA)/12-lipoxygenase (12-LOX) metabolites, also contributed to the spike frequency decreases produced by *in vitro* extinction. Protein phosphatase 2B (PP2B) also appeared to play a role. Calyculin A (PP1 inhibitor), cyclosporin A (PP2B inhibitor), and baicalein (a 12-LOX inhibitor) all blocked the spike frequency declines in Paired B cells produced by 30 LSs. Conversely, injection of catalytically-active PP1 (caPP1) or PP2B (caPP2B) into Untrained B cells partially mimicked the spike frequency declines observed in Paired cells, as did bath-applied AA, and occluded additional LS-produced reductions in spiking in Paired cells.

Keywords: extinction, memory erasure, *Hermissenda crassicornis*, phosphatases, PP1, PP2B, arachidonic acid, 12(S)-HPETE

**INTRODUCTION**

After an association has been formed between a conditioned stimulus (CS) and an unconditioned stimulus (US), repeated presentations of the CS without the US result in the reduction of the conditioned response (CR), a process known as extinction (Pavlov, 1927). The reduction of the CR produced by extinction training is often only temporary, as evidenced by spontaneous recovery (Reviewed by Myers and Davis, 2002; see Sangha et al., 2003 for spontaneous recovery in the snail *Lymnaea*), or US-induced reinstatement of the extinguished CR (Rescorla and Heth, 1975). The fact that the original associative memory can reappear after extinction training has led to a pervasive view that extinction involves the formation of new fragile, context-dependent learning that counteracts or inhibits the original learning (Rescorla and Cunningham, 1978; Robbins, 1990; Bouton, 1994).

However, recent research has indicated that, under certain conditions, extinction can “erase” the original associative memory, without reappearance of the original CR (Monfils et al., 2009; Schiller et al., 2010). Apparent erasure has also been observed on the cellular level, where extinction training abolished the behavioral effects of associative fear conditioning and reversed conditioning-produced insertion of AMPA GluR1 receptors in mouse (Clem and Huganir, 2010) and rat (Mao et al., 2006) amygdala neurons. In Mao et al. (2006), extinction-produced cellular erasure effects were only observed when extinction was given shortly (1 h, but not 24 h) after the end of learning acquisition, suggesting that erasure might be sensitive to specific acquisition-extinction intervals.

Research in the invertebrate model system *Hermissenda crassicornis* (*H.c.*) has also demonstrated extinction-produced erasure of associative memories on both the behavioral and cellular levels (Richards et al., 1984; Cavallo et al., 2014). Associative memories in *H.c.* are formed using repeated pairings of light (CS) and high-speed rotation (US) (see Farley, 1988b; Crow, 2004; Blackwell and Farley, 2009 for review). Rotation stimulates the *H.c.* vestibular system (statocyst hair cells) and elicits a natural “clinging” response that inhibits locomotion toward...
light (phototaxis) (Lederhendler et al., 1986). Paired training using light and rotation produces marked suppression of phototactic behavior (CR), which was extinguished using repeat light-alone presentations without any evidence of spontaneous recovery (Richards et al., 1984; Cavallo et al., 2014) or reinstatement (using additional US presentations) (Cavallo et al., 2014) of the CR. Additional neurophysiological data supported the extinction-produced erasure hypothesis and found that extinction reversed conditioning-produced increases in Type B photoreceptor excitability, both in terms of the light response generator potential (Richards et al., 1984) and light-evoked spike frequencies (Cavallo et al., 2014). Because B cells are a principal site of memory storage (Farley and Alkon, 1980, 1982; Richards and Farley, 1987) that are causally related to suppressed phototaxis (Farley et al., 1983), this suggests that the extinction-produced reversal of conditioned behavior results from a corresponding attenuation of enhanced B cell excitability. The goal of the present research was to identify the molecular signaling pathways that mediate extinction-produced alterations in B cell excitability.

Associative conditioning (paired training) increases H.c. Type B cell excitability through reductions in somatic K+ currents (Alkon et al., 1985; Farley, 1988a; Jin et al., 2009). These alterations are mediated, in part, by training-produced persistent activation of protein kinase C (PKC) (Farley and Auerbach, 1986; Farley and Schuman, 1991). Because PKC-mediated inhibition of K+ channels underlies the increased excitability produced by associative conditioning, we hypothesized that extinction training would reverse this process by dephosphorylating K+ channels (or channel-associated proteins) through the activation of protein phosphatase 1 (PP1). PP1 constrains learning-produced increases in Type B cell excitability in vitro (Huang and Farley, 2001) and has also been implicated as a principal molecule mediating extinction of conditioned taste aversion in mice (Stafstrom-Davis et al., 2001) and rats (Oberbeck et al., 2010). Protein phosphatase 2B (PP2B, aka calcineurin) is an upstream regulator of PP1 (Mulkey et al., 1994) that limits the expression of long-term memory in Aplysia (Sharma et al., 2003), constrains contextual fear learning in mice and mediates its extinction (Havekes et al., 2008). PP2B activity is also implicated in the extinction of fear potentiated startle responses in rats (Lin et al., 2003) and in extinction of conditioned taste aversion in mice (Baumgärtel et al., 2008). Therefore, we also examined whether the PP2B-PP1 signaling pathway participated in the extinction changes in B cell excitability. Additionally, because prior H.c. work has identified arachidonic acid (AA) and its metabolite 12(S)-hydroperoxy-eicosatetraenoic acid [12(S)-HPETE] as molecules that reduce B cell excitability and enhance K+ currents (Walker et al., 2010), we suspected that these molecules might also participate in extinction and decrease B cell excitability, as they do in the related phenomenon of conditioned inhibition (CI) learning (Walker et al., 2010).

To ascertain which molecular mechanisms mediate this process, we developed an in vitro protocol. Animals first received paired training (Paired), random presentations of light and rotation (Random), or no stimulation (Untrained). Next, B cells from isolated CNSs were recorded from during exposure to two series of 15 consecutive light-steps (LSs). When this extinction method was administered 2 h (but not 24 h) after paired training, B cells from Paired animals showed large and progressive decreases in spike frequency by the 30th LS, while control cells did not. We then combined this protocol with pharmacological manipulations and found that several molecules involved in H.c. CI learning also contributed to the spiking decreases produced by in vitro extinction, including PP1, PP2B, and AA/12-LOX metabolites.

Finally, these data were incorporated into a conceptual framework to create a molecular model of extinction learning in H.c. (Figure 13). The key assumptions of this model are: (1) Paired conditioning increases B cell excitability through phosphorylation of somatic K+ channels (or associated proteins), (2) in vitro extinction (repeated LSs) produces large increases in cytosolic Ca2+, but only in paired-trained cells, (3) Large intracellular Ca2+ levels preferentially activate PP2B, (4) PP2B inhibits PP1, (5) PP1 dephosphorylates somatic K+ channels (or associated proteins), which reduces B cell excitability, and (6) in vitro extinction further reduces B cell excitability through the activation of a parallel AA/12-LOX pathway, which also interacts with somatic K+ channels.

METHODS

ANIMALS

Adult H.c. were provided by Monterey Abalone Co. (Monterey, CA) and individually housed in perforated 50-ml plastic tubes in aquaria containing artificial seawater (ASW, Bio-sea Marine Mix, AquaCraft, Hayward, California, pH 7.8–8.2) at 15°C on a 6.5/17.5-h light/dark cycle (4 μW/cm² radiant intensity), as in Richards et al. (1984). Animals were fed with scallops (Mytilus edulis) twice weekly, but food was removed 48 h prior to behavioral testing.

ANIMAL TRAINING

The methods and apparatus used for behavioral training have been described previously (Farley, 1987a) and methods for extinction training were designed after Richards et al. (1984). Animals were placed into clear plastic tubes (20 cm) filled with ASW and mounted on a turntable in a refrigerator (11°C). Vestibular stimulation consisted of high-speed rotation (100 RPM), resulting in a 2.24 g centrifugal force stimulation of the statocytes. Photoreceptors were stimulated using whole-field illumination provided by a 60 W incandescent light source (56 μW/cm² intensity) located above the turntable. Timing and duration of stimuli were controlled automatically using an IBM DOS computer connected to digital/analog controllers made by department engineers. Animals were randomly assigned to one of three treatment conditions: Untrained, Paired, and Random. The Untrained animals received no behavioral training and remained in the home aquarium during scheduled training sessions. The Paired animals received two consecutive daily sessions of paired conditioning (50 trials each). One trial consisted of paired light and rotation (both 30 s) presentations (simultaneous onsets and offsets), with an inter-trial interval (ITI) of 2 min (variable). Random animals received the same type of training as paired conditioning (same amount, stimulus duration, and ITI), but had stimuli presented randomly.
NERVOUS-SYSTEM PREPARATION AND INTRACELLULAR RECORDING

General methods for preparing and performing electrophysiological recordings from \textit{H.c.} nervous systems have been described extensively (e.g., Farley and Alkon, 1982, 1987; Farley, 1987a,b, 1988a). Dissected \textit{H.c.} circumesophageal nervous systems (Figure 1) were secured on a microscope slide within a 495 μL well of ASW (15°C). Each isolated nervous system was incubated in protease (1 mg/ml; Subtilisin A, Sigma P5380) for ~8–9 min at room temperature (~18–20°C) to facilitate cell impalement. After protease exposure, nervous systems were rinsed with ten volumes of cold (4°C) standard ASW.

**IN VITRO EXTINCTION PROTOCOL**

Methods for \textit{in vitro} conditioning using isolated \textit{H.c.} nervous systems have been previously developed for simulating paired conditioning (Farley, 1987b; Farley and Alkon, 1987; Grover and Farley, 1987; Huang and Farley, 2001; Jin et al., 2009). Based on these studies, we developed an \textit{in vitro} extinction protocol that delivered two sets of 15 LSs to isolated \textit{H.c.} CNSs from conditioned or \textit{Untrained} animals. Animals were dissected and standard electrophysiological experiments were performed on Type B photoreceptors (Farley and Alkon, 1982; Farley, 1987b, 1988a). B cells from \textit{Random} animals were recorded from ~1–2 h after training concluded, while \textit{Paired} cells were recorded from ~1–2 h or 24 h after associative conditioning. B cells were impaled and dark-adapted for 15 min, while continuously monitoring spontaneous spiking and synaptic activity. Following dark adaptation, cells were exposed to 15 presentations of a 30 s LS (2 min ISI). These 15 LSs were designed as a replacement for whole-animal extinction training (Richards et al., 1984), which comprises 25 light-alone presentations following paired conditioning. Thus, our \textit{in vitro} extinction method entailed repeated sequential 30 s LS presentations of the same intensity, duration, and frequency as those used during extinction training given to intact animals. After the first 15 LSs, cells were re-dark adapted for 10 min, and given another 15 LSs. The intervening dark adaptation period

**FIGURE 1 | The \textit{Hermissenda} (\textit{H.c.}) circumesophageal nervous system (CNS): entire CNS, eye, and statocyst.** (A) Low resolution macrophotograph of an \textit{in situ} toluidine-blue stained CNS. Note that the eye and statocyst lie on the dorsal surface of the CNS, in between the cerebropleural (cpg) and pedal (ped) ganglia. Pedal nerves (e.g., p3) project from the pedal ganglia down to the pedal musculature. (B) Photomicrograph of a \textit{H.c.} eye (top, dark structure) and statocyst (bottom, ST, crystalline structure). Non-excitable, and non-photoresponsive pigmented cells are responsible for the black appearance of the eye, and obscure the photoreceptors. Portions of the photoreceptors are visible along the bottom of the eye. (C) Schematic drawing of a right eye, illustrating the relative positions of the three Type B photoreceptors (medial, intermediate, and lateral, colored here as green, purple, and magenta, respectively) and the two Type A photoreceptors (medial and lateral, colored here as red and yellow, respectively). Note the black rhodaminic portions of the photoreceptors, where phototransduction occurs. The rhodamines are oriented toward the lens. Note also that each photoreceptor has an axo-dendritic process extending from its base. The one extending from the (red) medial A photoreceptor is shown here in gray, running over the soma of the medial Type B photoreceptor. The axo-dendritic processes collect and fasciculate at the base of the eye, to form the optic nerve, which projects underneath the optic ganglion (see B) into the cerebropleural ganglia, where it extensively ramifies. Action potentials are generated at the axon hillock region. The majority of fast synaptic potentials (EPSPs and IPSPs) originate distal to the axon hillock at the axo-dendritic ramifications. A variety of slow synaptic responses (e.g., originating from GABA B, muscarinic Ach, and 5-HT GPCRs) are located on the axo-dendritic process and soma. The soma is non-spiking. An appropriately located razor lesion of the axon (optic nerve), close to the base of the eye, eliminates action potentials and fast synaptic potentials, without affecting the light-induced generator potentials, light adaptation, or slow synaptic responses from exogenously applied neurotransmitters. In the synaptically-intact preparation, action potentials recorded from the soma propagate passively and in a retrograde fashion from the axon hillock. Sharp electrode intracellular recordings (and two microelectrode voltage clamp) from the soma allow one to readily record light-induced generator potentials, superimposed action potentials, and synaptic potentials (fast and slow).
was administered to minimize light-adaptation effects on B cell excitability that might have accumulated as a result of the first 15 LSs (Farley, 1987a). Light adaptation in invertebrate photoreceptors is a molecular desensitization process that is mechanistically distinct from extinction (Lisman and Brown, 1972; Bader et al., 1976).

PHOSPHATASE ENZYMES AND INHIBITORS
Activity of catalytically active protein phosphatase 1 (caPP1) (α-isofrom, derived from rabbit skeletal muscle, produced via recombinant E. coli, Calbiochem, cat. No. 539493) was 2500 units/mL and was dissolved in buffer to obtain 500 activity units/mL. caPP1 buffer was composed of (in mM): 50 HEPES, 200 NaCl, 1 MnCl2, 0.1 EGTA, 2.5 DTT, 0.025% Tween 20. Catalytically active protein phosphatase 1 (caPP1) (α-isofrom, derived from rabbit skeletal muscle, produced via recombinant E. coli, Calbiochem, cat. No. 539493) was 2500 units/mL and was dissolved in buffer to obtain 500 activity units/mL. CaPP1 buffer was composed of (in mM): 50 HEPES, 200 NaCl, 1 MnCl2, 0.1 EGTA, 2.5 DTT, 0.025% Tween 20 (Polyoxyethylene sorbitan Monolaurate, Sigma P-1379), and 50% glycerol. CaPP1 was further diluted in buffer (661.7 nM final concentration), backfilled into microelectrodes, and filled with 1.5 M KCl. CaPP1 was introduced into a single B cell via leakage/ionophoresis through the recording electrode using five, 30 s – 0.5 nA current injections during the first dark adaptation period.

Catalytically active protein phosphatase 2B (caPP2B) was obtained as a gift from Dr. Claude Klee. Recombinant caPP2B (rCaNAB45) (14 μg/ml concentration) was suspended in buffer (in mM): 40 Tris, 100 GuHCl, 100 NaCl, 0.1 CaCl2, 2 DTT, and 100 μg/ml soybean inhibitor. CaPP2B activity was 15–20 nmol/min, in the assay with 1 nM rCnA5B45, in a buffer of (in mM): 40 Tris, 100 NaCl, 0.4 mg/ml BSA, 0.6 CaCl2, 0.5 MnCl2, and 1 DTT. CaPP2B was backfilled into electrodes, filled with 1.5 M KCl, and introduced into B cells using the same methods described for CaPP1.

Calyculin A (Sigma-Aldrich #C5552) was dissolved in dimethyl sulfoxide (DMSO) (0.1 mM concentration), then diluted with D2O to a stock concentration of 2 μM. Five microliter of stock solution was added to the ASW bath (20 nM final concentration). Cyclosporin A (Sigma-Aldrich #C3662) was dissolved in 3 M KCl (0.01% DMSO) (100 nM final concentration). Concentrations of calyculin and cyclosporin were 10–20 higher than IC50 values reported for inhibition of PP1 (Resjö et al., 1999) and PP2B (Fruman et al., 1992), and identical to concentrations used in prior H. c. research (Huang and Farley, 2001).

ARACHIDONIC ACID AND METABOLITE INHIBITORS
AA (Sigma-Aldrich #A8798) was stored at a stock concentration of 0.1 M in 100% ethanol at –70°C. Daily AA solutions were prepared by diluting stock solutions to 10 mM with D2O. Baicalin (Sigma #465119) was stored in powder form at 4°C and 10 mM solutions were prepared daily in D2O. Final bath concentrations of AA and baicalin were 100 μM, and ethanol concentrations never exceeded 0.1%.

DATA AND STATISTICAL ANALYSIS
Several measures of B cell excitability were obtained, including: resting membrane potential (V_m), input resistance (R_in), and light-evoked spike frequency (number of action potentials, in Hz, during the last 10 s of LS). Input resistance was calculated using Ohms law. Brief (200 ms) current pulses of –0.25 nA were given and voltage drops were recorded. B cells with a V_m more positive than –39 mV and a R_in of less than 30 MΩ were considered damaged and were discarded.

Differences in B cell spike frequency during LSs 1–2 (averaged) were determined using One-Way ANOVA and Bonferroni post-hoc tests. Analysis of LSs 6–30 and LSs 16–30 were done using repeated measures ANOVA comparisons and Bonferroni post-hoc pairwise tests. All statistics were performed using the SPSS 20.0 software suite. In cases where a Mauchly’s test of sphericity revealed a violation of sphericity (p < 0.05), a Greenhouse-Geisser correction was used. Two-tailed significance tests were used (unless otherwise noted), and significant p-values are < 0.05.

RESULTS
Learning-produced alterations in phototactic behavior are correlated with increased light-evoked frequencies in Type B cells (Farley and Alkon, 1982) that drive downstream changes in sensory-interneuron-motorneuron circuits that control locomotor behavior (Goh et al., 1985; Richards and Farley, 1987; Crow, 2004). Behavioral extinction training of intact animals using short acquisition-extinction intervals (15 min) appeared to reverse this cellular correlate of paired conditioning and abolished the learning-produced increases in B cell light-evoked spike frequencies when measured 24 h after conditioning (Cavallo et al., 2014). To investigate the cellular changes that occur during extinction training in real-time, we developed an in vitro extinction protocol. Isolated CNSs from associatively trained (Paired), or control animals (Untrained and Random) were exposed to two sets of 15 successive light presentations (light steps, LSs), commencing ~2 h after the conclusion of conditioning. Light-evoked spike activity of Type B photoreceptors was tabulated during each LS. LSs were the same duration (30 s), frequency (2 min ISI), and intensity as LSs given to intact animals during behavioral extinction experiments. Associative conditioning can be simulated in semi-intact preparations or in isolated CNSs using several procedures [e.g., light-B cell depolarization (Farley et al., 1983), light-stato-cov hair cell stimulation (Farley and Alkon, 1982, 1987; Farley, 1987b; Grover and Farley, 1987)]. Because these methods produce many of the same learning-produced alterations in B cell excitability and phototactic behavior elicited by paired training in vivo, the current procedure consisting of LSs presented to isolated CNSs was used to act as a substitute for whole-animal extinction training (e.g., Richards et al., 1984). We then combined this in vitro extinction protocol with selective pharmacological inhibition of the PP1/PP2B phosphatase and AA/12(S)-HPETE fatty acid-signaling pathways, injection of purified constitutively-active phosphatases, and stimulation with AA. Our results indicated that both phosphatase- and lipid-signaling pathways appeared to be involved in the reductions in B cell excitability produced by in vitro extinction.

IN VITRO EXTINCTION PRODUCES A PROGRESSIVE DECREASE IN B CELL SPIKE FREQUENCY, BUT ONLY IN PAIRED-CONDITIONED CELLS
As reported in Cavallo et al. (2014, Figure 7) and summarized again here in Figures 2A,B, Type B cells from Paired (n = 11) animals exhibited greater light-evoked spike frequencies during LSs 1–2 (7.44 ± 0.31 Hz) than cells from control animals (Random,
n = 5, 5.67 ± 0.33 Hz; Untrained, n = 16, 5.96 ± 0.26 Hz) 2 h after the end of associative conditioning. This increased excitability was followed by a large and progressive decline in spiking during later LSs that was unique to Paired cells and did not occur in control cells (Figures 2A,C). The initial differences in spike frequencies between Paired and control groups diminished with successive LSs (Figure 2C), as observed previously (Farley, 1987a). Spike frequencies were similar for the Paired and control groups throughout LSs 4–15. After a re-dark adaptation period (10 min), spike frequencies of these groups began to diverge with successive LSs. Paired cells showed a steady decline in spiking during LSs 16–30. A repeated measures ANOVA compared the three groups over LSs 16–30 and revealed that Paired (n = 12) cells spiked significantly less frequently than Untrained (n = 15) [F(1, 25) = 7.26, p = 0.012], and Random [F(1, 16) = 6.73, p = 0.02] cells (Figure 2C), while control cells failed to differ [F(1, 19) = 0.20, p = 0.659].

Because cells from Paired animals initially spiked at higher rates than controls, the decreases in spike frequencies might be more conspicuous for Paired cells because of the greater room for decline. Therefore, we also analyzed the relative changes in spike frequency by calculating a percent change score for each
cell. The spike frequency of a cell during each LS 6–30 was compared to the spiking during LS 5 for that same cell, when spike frequencies had stabilized and initial training-associated differences were on average no longer apparent. This analysis yielded qualitatively similar results to those reported for absolute spike frequencies. Around LS 6–7, Paired cells began to show a progressive decrease in spike frequency that was not evident in control cells (Figure 2D). By the 30th LS, cells from the Paired group showed a 33.5 ± 6.5% decrease in spike frequency, which was much larger than the small changes evident in Untrained (2.6 ± 6.0% decrease) and Random (4.2 ± 7.7% increase) control cells. A repeated measures ANOVA compared the three conditions across LSs 6–30 and revealed that the spike frequency decrease in Paired cells was significantly larger than in Untrained [F(1, 25) = 10.06, p = 0.004] and Random cells [F(1, 16) = 15.08, p = 0.001]. Untrained and Random control cells failed to differ [F(1, 19) = 0.26, p = 0.618] (Figure 2D). These results indicate that repetitive LSs produced a reduction in B cell spike frequency that was contingent upon prior associative conditioning. These findings paralleled the behavioral in vivo extinction results (Figure 7B from Cavallo et al., 2014) and suggest that 30 successive LSs given to isolated H.c. CNSs was an effective in vitro extinction protocol for producing extinction-correlated reductions in B cell excitability.

OMISSION OF THE DARK ADAPTATION PERIOD BETWEEN LSs 15 AND 16 DOES NOT AFFECT IN VITRO EXTINCTION

The previous in vitro extinction experiments included a 10 min re-dark adaptation period between the two sets of 15 LSs to ensure that any changes in B cell excitability produced by in vitro extinction resulted from the extinction process rather than from changes due to light adaptation (see Methods). The question arises as to whether the emergence of the spike frequency differences during LSs 16–30 for Paired vs. control cells was predominantly due to: (1) the additional 10 min per se, (2) the additional dark adaptation that occurred, (3) the additional LSs, or (4) some combination/interaction of these factors. To address this question, we repeated the in vitro extinction experiments for different groups of Paired and Untrained cells, but omitted the intervening re-dark adaptation period. Thus, cells in each group (Paired no dark adaptation, NDA, and Untrained NDA) were given 30 consecutive LSs with the same LS interval, duration, and intensity used in the prior experiments, but without additional dark adaptation between LSs 15 and 16. Even without the re-dark adaptation period, the same general pattern emerged. Thirty successive LSs produced a progressive reduction in spike frequencies in Paired, but not in Untrained cells (Figure 3).

Paired NDA cells (n = 6) showed significantly greater levels of light-evoked spike frequencies than Untrained NDA (n = 5) cells during LSs 1–2 (8.45 ± 0.27 Hz vs. 7.38 ± 0.34 Hz, respectively) [t(1, 9) = 2.48, p = 0.035]. During later LSs 16–30, a repeated measures ANOVA found that Paired cells (n = 5) exhibited a progressive and significant decrease in spike frequency with successive LSs when compared to Untrained NDA cells (Figure 3A) [significant interaction between LS and condition: F(14, 112) = 4.97, p = 0.000]. This indicated that repeated LSs produced a reduction in spike frequency, but only in Paired cells.

We next compared Untrained NDA and Paired NDA groups with their re-dark adapted counterparts and found that they generally did not differ. Spike frequencies of Untrained NDA cells during LSs 16–30 did not significantly differ from Untrained re-dark adapted cells (Untrained group from previous experiment) [F(1, 18) = 1.72, p = 0.206]. Although a significant interaction between LS and condition was found [F(2,93, 52.79) = 2.91, p = 0.044, corrected for sphericity], pairwise comparisons of each LS indicated that Untrained NDA cells spiked more frequently than Untrained re-dark adapted cells, but only at LS 16 (p = 0.023). This transient difference in spike frequency was a result of the 10 min dark adaptation period and was gone by LS 17 (see Figure 2C). No other differences were detected during LSs 17–30. Spike frequencies of Paired NDA and Paired
re-dark adapted cells did not differ significantly \(F(1, 15) = 0.07, p = 0.790\).

The same pattern and conclusions were obtained when spike frequencies were analyzed as a percent change from the 5th to 30th LS (Figure 3B). Paired NDA cells showed a large 37.3 ± 8.7% reduction in spike frequency by the 30th LS that was significantly greater than Untrained NDA cells (0.9 ± 6.6% decrease) \[main effect of treatment condition, \(F(1, 8) = 6.86, p = 0.031\] (Figure 3B). Spike frequencies of Untrained NDA and Paired NDA groups were no different from their re-dark-adapted counterparts \(F(1, 18) = 0.10, p = 0.761,\) and \(F(1, 15) = 1.06, p = 0.320,\) respectively. These data confirmed that in vitro extinction led to a progressive reduction in Paired B cell spike frequency that took time and repetitive LSs to develop, regardless of the middle re-dark adaptation period.

CALYCLIN A PARTIALLY BLOCKS THE EFFECTS OF IN VITRO EXTINCTION

Prior research found that inhibition of PP1 mimicked and occluded further increases in Type B cell excitability produced by in vitro paired conditioning, suggesting that PP1 might limit B cell excitability and oppose the pairing-produced effects of PKC activation (Huang and Farley, 2001). Because inhibitory conditioning (Britton and Farley, 1999) and extinction training produce directionally similar behavioral and electrophysiological effects (enhanced phototaxis, reduced B cell excitability), we hypothesized that PP1 activity might also be recruited during in vitro extinction. To test this, we exposed B cells to the PP1 inhibitor, calyculin A, for 15 min prior to in vitro extinction training. We predicted that inhibition of PP1 during the course of in vitro extinction would block the progressive decrease in spike frequency observed in Paired cells, but have little effect in control cells. Consistent with this expectation, Paired cells incubated in calyculin showed a much smaller reduction in spike frequency compared to their Paired (no drug) counterparts (Figure 4).

Incubation in calyculin did not affect the average spike frequencies of Paired (Paired + Calyculin, \(n = 8, 6.34 ± 0.47\) Hz) or Untrained (Untrained + Calyculin, \(n = 7, 5.79 ± 0.69\) Hz) cells during LSs 1–2 compared to their no drug counterparts. At later LSs 16–30, Paired + Calyculin cells (\(n = 7\)) showed calyculin and was no different than the spike frequencies of Untrained cells incubated in calyculin (Unt + Calyculin). (C) Summary data of the relative percent change in B cell spike frequency from the 5th to 30th LS. The incubation of Paired cells in calyculin significantly attenuated the large decrease in spike frequency over the course of 30 LSs normally exhibited by Paired cells without drug. Paired + Calyculin cells spiked at frequencies not statistically different from Unt + Calyculin cells, but frequencies significantly greater than Paired (no drug) cells. Error bars are ± s.e.m. and significant \(p\)-values are < 0.05. Note that one cell in the Paired + Calyculin group was omitted from spike frequency analysis during LSs 5–30 and LSs 16–30 because impalement was lost after LS 25.

![Diagram](image-url)
a small reduction in spike frequencies that was not different from Untrained + Calyculin cells \([F(1, 12) = 0.52, p = 0.485]\), nor from Untrained (no drug) cells \([F(1, 20) = 0.25, p = 0.624]\) (Figures 4A,B), indicating that calyculin blocked the large decrease in spike frequency and maintained spike frequencies at control levels. Paired + Calyculin cells exhibited spike frequencies that were not significantly greater than Paired (no drug) cells during this period \([F(1, 17) = 2.73, p = 0.117]\).

The effects of calyculin were more apparent when analyzed as a percent change in spike frequency from the 5th to 30th LS (Figure 4C). Paired + Calyculin cells showed a negligible 5.6 ± 7.4% decrease in spike frequency over the course of 30 LSs that was not different from Untrained + Calyculin cells (10.8 ± 12.5% increase) \([F(1, 12) = 2.21, p = 0.163]\) (Figure 4C), nor from Untrained (no drug) cells (2.6 ± 6.0% decrease) \([F(1, 20) = 0.02, p = 0.893]\). In contrast, the 5.6 ± 7.4% decrease in spike frequency observed in Paired + Calyculin cells was significantly different from Paired (no drug) cells (33.5 ± 6.5% decrease) \([F(1, 17) = 11.08, p = 0.004]\) (Figure 4C). Thus, pre-incubation of B cells in calyculin prevented the large spike frequency decrease produced by in vitro extinction in Paired cells.

**PROTEIN PHOSPHATASE 1 OCCUMES FURTHER SPIKE FREQUENCY REDUCTIONS IN PAIRED CELLS AND PARTIALLY MIMICS THE EFFECTS OF IN VITRO EXTINCTION IN UNTRAINED CELLS**

To further investigate the involvement of PP1 in the in vitro extinction process, we injected the catalytically active subunit of PP1 (caPP1) into B cells prior to the administration of 30 LSs. If in vitro extinction activates endogenous PP1, and thereby contributes to the progressive decrease in B cell spike frequencies, then injection of exogenous caPP1 into Paired cells should also reduce spike frequencies and occlude any further reductions that result from repetitive LSs (i.e., in vitro extinction). Injections of caPP1 into Untrained cells might also reduce spike frequencies due to dephosphorylation of spike-regulating K⁺ channels. These reductions might not be apparent immediately, but could develop over time, owing to delays associated with intracellular enzyme diffusion, dephosphorylation rates, etc.

In support of the PP1-involvement hypothesis, Untrained and Paired cells injected with caPP1 exhibited reductions in spike frequency during later LSs 16–30, (Figures 5A,C), an effect more pronounced when measured as a percent change in spike frequency (Figure 5D). The reduction in spike frequency seen in Paired cells injected with caPP1 was not greater than what was observed in Paired (no drug) cells. Thus, caPP1 injection appeared to partially mimic and occlude the effects of in vitro extinction on B cell spike frequency.

An ANOVA of LSs 1–2 compared the average spike frequency of Untrained and Paired cells with their caPP1-injected counterparts and the Buffer control group, and found a main effect of treatment condition \([F(4, 47) = 4.63, p = 0.003]\). Post-hoc analysis revealed that injection of caPP1 into Untrained cells (Untrained + PP1; \(n = 11\)) significantly increased spike frequencies compared to Untrained (no drug) cells (7.60 ± 0.29 Hz vs. 5.96 ± 0.26 Hz, respectively) \((p = 0.005)\), but not compared to PP1 Buffer controls \((n = 7)\) (6.74 ± 0.64 Hz) \((p = 1.0)\). This indicated that the increased spike frequencies observed in Untrained + PP1 cells were partly due to non-specific effects of the PP1 buffer. Paired + PP1 cells \((n = 7)\) were no different than Paired (no drug) cells during LSs 1–2 (7.19 ± 0.39 Hz vs. 7.44 ± 0.31 Hz, respectively) \((p = 1.0)\).

Spike frequencies of Untrained + PP1 and Paired + PP1 cells remained approximately at control levels from LSs 4–15 (Figure 5C). During LSs 16–30, Untrained + PP1 cells showed slight (non-significant) decreases in spike frequencies below Untrained \([F(1, 24) = 0.05, p = 0.829]\) and Buffer control \([F(1, 16) = 2.44, p = 0.138]\) levels, and were significantly higher than Paired (no drug) cells \([F(1, 21) = 6.74, p = 0.017]\) (Figure 5C). Buffer control cells showed slight (non-significant) increases in spike frequencies compared to Untrained cells \([F(1, 20) = 3.70, p = 0.069]\) (Figure 5B). This suggests that the small excitatory effects of the PP1 buffer opposed reductions in spike frequencies produced by caPP1 injection, and thereby prevented caPP1 from reducing spike frequencies below Buffer or Untrained control levels.

Paired + PP1 cells showed progressive declines in spike frequencies during LSs 16–30 that were no different than the decreases observed in Paired (no drug) cells \([F(1, 17) = 0.61, p = 0.446]\), and were significantly below Buffer \([F(1, 12) = 13.90, p = 0.003]\), but not Untrained \([F(1, 20) = 2.48, p = 0.131]\) cells (Figure 5C). Thus, caPP1 injection into Paired cells produced decreases in spike frequencies that were not larger than the decreases observed in Paired (no drug) cells, and appeared to occlude the effects of repeated LSs and any further reductions in spike frequency.

To further evaluate the effects of caPP1 injections, we analyzed the relative changes in spike frequencies, measured as a percent change from the 5th to 30th LS. Untrained + PP1 cells showed a 20.0 ± 3.1% reduction in spike frequency by the 30th LS that was nominally less, but not significantly different, than Paired (no drug) cells (33.5 ± 6.5% reduction) \([F(1, 21) = 2.77, p = 0.111]\) (Figure 5D). This decreased spike frequency of Untrained + PP1 cells was significantly greater than Buffer control cells (1.1 ± 3.0% decrease) \([F(1, 16) = 11.34, p = 0.004]\), and qualitatively greater than Untrained control cells (2.6 ± 6.0% decrease) \([F(1, 24) = 3.97, p = 0.058]\). Paired + PP1 cells showed a significant 21.6 ± 2.4% reduction in spike frequency from the 5th to 30th LS compared to Buffer \([F(1, 12) = 21.55, p = 0.001]\) and Untrained \([F(1, 20) = 5.11, p = 0.035]\) control cells, that occluded any further decreases produced by in vitro extinction, and was not significantly different than Paired (no drug) cells \([F(1, 17) = 0.57, p = 0.462]\) (Figure 5D).

In summary, Untrained cells injected with caPP1 showed spike frequency reductions that mimicked those observed in Paired cells (without caPP1) during in vitro extinction. The declines in spike frequencies due to in vitro extinction in Paired cells injected with caPP1 were no greater than those observed in Paired cells (without caPP1), suggesting that caPP1 occluded the spike frequency reductions produced by repeated LSs during in vitro extinction. Combined with the calyculin data reported above, these results are consistent with the hypothesis that endogenous PP1 is involved in the in vitro extinction-produced changes in B cell excitability.
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**FIGURE 5** | Injection of catalytically active protein phosphatase 1 (caPP1) reduced spike frequencies in *Untrained* cells by the 30th LS and occluded any additional spike frequency reductions in *Paired* cells. (A) Representative light responses recorded from an *Untrained* B cell injected with PP1 buffer (*Unt + PP1 Buffer*; top trace) or caPP1 (*Unt + PP1*; bottom trace). The *Unt + PP1 Buffer* control cell showed a slight increase in spike frequency from the 5th to 30th LS, while the *Unt + PP1* cell showed a progressive 23% decrease in spike frequency that mimicked the decreases observed in *Paired* (no drug) cells, i.e., in vitro extinction. Inset traces show last 10 s of LS. (B) Summary spike frequency data for *Untrained* and *Untrained + PP1 Buffer* control cells across 30 LSs. Note the slight excitatory effect of the buffer. (C) Summary spike frequency data for 30 successive LSs indicated that *Unt + PP1* cells showed qualitatively lower spike frequencies during LSs 16–30 compared to *Unt + PP1 Buffer* and *Untrained* controls, and were no different than *Paired* (no drug) cells, thereby producing spike frequency reductions similar to those observed during in vitro extinction. *Paired* cells injected with caPP1 (*Paired + PP1*) showed significant decreases in spike frequency below *Unt + PP1 Buffer* control cells. These reductions were no larger than those observed in *Paired* (no drug) cells, suggesting that caPP1 injection occluded any further spike frequency reductions produced by repetitive LSs. (D) Summary data of the relative percent change in B cell spike frequency from the 5th to 30th LS. *Unt + PP1* cells exhibited significant declines in spike frequency from the 5th to 30th LS (∼20%) compared to *Unt + PP1 Buffer* (1% increase) control cells. These decreases observed in *Unt + PP1* cells were not statistically different than the spike frequency reductions observed in *Paired* (no drug) cells, suggesting that caPP1 is able to produce decreases in spike frequency that are similar in magnitude and time course to the reductions produced by in vitro extinction. caPP1 injection into *Paired* cells produced decreases in spike frequency that occluded any further decreases produced by repeated LSs in *Paired* (no drug) cells (∼22% vs. ∼34%, respectively), suggestive of an occlusion effect. Error bars are ± s.e.m. and significant *p*-values are < 0.05.

**CYCLOSPORIN A BLOCKS THE EFFECTS OF IN VITRO EXTINCTION**

To further probe the link between repeated LSs and PP1 activation, we investigated an upstream regulator of PP1, protein phosphatase 2B (PP2B, aka calcineurin). PP2B is a Ca\(^{2+}\)/calmodulin-dependent phosphatase (Stemmer and Klee, 1994) that positively regulates PP1 in many cells through the dephosphorylation of inhibitor-1 and the subsequent disinhibition of PP1 (Mulkey et al., 1994). Because repeated LSs can lead to an accumulation of intracellular Ca\(^{2+}\) in (untrained) Type B cells, which constrains associative learning in a phosphatase-dependent manner (Muzzio et al., 1999), we hypothesized that the repeated LSs used during in vitro extinction would also activate PP2B in *Paired* cells, leading to the disinhibition of PP1 and reductions in B cell spike frequency. To test this hypothesis, B cells from *Paired* or *Untrained* animals were injected with cyclosporin A, a PP2B-specific inhibitor, and then exposed to in vitro extinction. If the extinction-produced reduction in B cell spike frequency requires PP2B activity, then the addition of cyclosporin to *Paired* cells prior to in vitro extinction should block the decrease. In support of this hypothesis, cyclosporin injection into *Paired* cells blocked the progressive decrease in spike frequency observed during in vitro extinction, but had no effect on spike frequency in *Untrained* cells (Figure 6).

Cyclosporin had small (non-significant) facilitatory effects on spike frequencies during LSs 1–2 for both *Untrained* (*Untrained + Cyclosporin*; *n* = 5; 6.48 ± 0.36 Hz) and *Paired* (*Paired +
Cyclosporin; \( n = 7; \ 7.87 \pm 0.23 \text{ Hz} \) cells. This suggests that constitutive activity of endogenous PP2B, and off-target effects of cyclosporin on B cell excitability, were low-to-negligible. During LSs 16–30, cyclosporin blocked the progressive decrease in spike frequencies produced by in vitro extinction in Paired cells. Paired + Cyclosporin cells spiked significantly more frequently than Paired (no drug) cells \([F_{1, 17} = 18.35, p = 0.001]\) (Figures 6A,B). The spike frequency of Paired + Cyclosporin cells was greater than Untrained cells \([F_{1, 20} = 5.12, p = 0.035]\), but no different than Untrained + Cyclosporin control cells \([F_{1, 10} = 0.52, p = 0.486]\) (Figure 6B). Untrained + Cyclosporin cells were not different from Untrained (no drug) cells \([F_{1, 18} = 1.23, p = 0.282]\).

Similar conclusions concerning the effects of cyclosporin followed from analysis of the relative changes in spike frequency from the 5th to 30th LS (Figure 6C). Paired + Cyclosporin cells failed to show the progressive declines in spike frequency normally produced without drug, and instead showed a small 0.2 ± 3.8% decline compared to Paired (no drug) cells (33.5 ± 6.5% decrease) \([F_{1, 17} = 13.82, p = 0.002]\) (Figure 6C). The negligible spike frequency decrease of Paired + Cyclosporin cells was not significantly different from Untrained (no drug) cells (2.6 ± 5.98% decrease) \([F_{1, 20} = 0.02, p = 0.878]\), or Untrained + Cyclosporin cells (7.6 ± 2.83% increase) \([F_{1, 10} = 3.29, p = 0.10]\) (Figure 6C).

In summary, the injection of cyclosporin into B cells prior to in vitro extinction blocked the progressive reduction in B cell spike frequencies produced by repeated LSs, suggesting that PP2B participates in the extinction-produced spike frequency decreases in B cells.

**Protein Phosphatase 2B Prevents the Spike Frequency Decreases in Paired Cells Due to In Vitro Extinction but Mimics the Effects of In Vitro Extinction in Untrained Cells**

If endogenous PP2B mediates the decreased B cell spike frequency observed during in vitro extinction, the injection of

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**FIGURE 6** | Injection of cyclosporin A (100 nM in electrode) into Paired Type B cells at the start of in vitro extinction blocked the progressive reduction in spike frequency produced by repeated LSs in Paired cells. (A) Representative light responses recorded from a Paired Type B cell (top trace) and a Paired cell incubated in cyclosporin A (Paired + Cyclosporin; bottom trace). The Paired cell showed a clear decrease in spike frequency from the 5th to 30th LS, while the Paired + Cyclosporin cell exhibited no change. (B) Summary spike frequency data over 30 LSs of Type B cells injected with cyclosporin. The progressive reduction in spike frequency from the 5th to 30th LS, while the Paired + Cyclosporin cell exhibited no change. (C) Summary data of the relative percent change in B cell spike frequencies from the 5th to 30th LS. Injection of Paired cells with cyclosporin prevented the large decrease in spike frequency over the course of 30 LSs observed in Paired cells without drug (0% change vs. ~34% decrease, respectively). The spike frequency changes of Paired + Cyclosporin cells over the course of 30 LSs were indistinguishable from those observed in Unt + Cyclosporin cells. Error bars are ± S.E.M.
exogenous caPP2B into Paired cells would be expected to produce a decrease in spiking that occludes any further decreases elicited by the repeated LSs. Prediction of the effects of caPP2B injection into Untrained cells is less clear. If PP2B acts exclusively through disinhibition of PP1, but PP1 is either not part of the K+ signaling complex and/or the K+ channels are not appreciably serine/threonine-phosphorylated in the basal (untrained) state, then injection of caPP2B might not significantly affect spike frequency. Alternatively, if endogenous PP1 is targeted to K+ channel signaling complexes and the K+ channels are serine/threonine-phosphorylated in Untrained cells, then injection of the caPP2B could activate PP1, dephosphorylate K+ channels (or associated proteins), enhance K+ currents, and decrease spike frequencies similar to that produced by in vitro extinction.

To test these hypotheses, we injected caPP2B into B cells from Untrained or Paired-trained animals prior to in vitro extinction. Counter to our expectations, caPP2B injection into Paired cells did not decrease spike frequencies, but instead prevented the reduction in spiking normally produced by in vitro extinction (Figure 7). In contrast, the injection of caPP2B into Untrained cells reduced spike frequencies over the course of 30 LSs, which was very similar to the decreases observed during in vitro extinction for Paired cells (without caPP2B).

cPP2B injection had no appreciable effect on spike frequency during LSs 1–2 for either Untrained (Untrained + PP2B; n = 11; 6.04 ± 0.33 Hz) or Paired cells (Paired + PP2B; n = 7; 7.50 ± 0.28 Hz). PP2B buffer (Buffer control; n = 6; 5.83 ± 0.35 Hz) also had no effect on spike frequency during LSs 1–2. During LSs 16–30, Paired + PP2B cells showed spike frequencies that were significantly elevated compared to Paired (no drug) cells [F(1, 17) = 16.71, p = 0.001], and slightly (non-significantly) above Buffer control levels [F(1, 11) = 4.37,
p = 0.06] (Figure 7B). When measured as a percent change in spike frequencies from the 5th to 30th LS, Paired + PP2B cells exhibited a 8.7 ± 4.1% decrease in spiking that was significantly less than Paired (no drug) cells (33.5 ± 6.5% decrease) \([F_{1, 17} = 8.11, p = 0.011]\), and no different from Buffer cells (2.6 ± 6.0% increase) \([F_{1, 11} = 0.46, p = 0.514]\) (Figure 7C).

In contrast, Untrained + PP2B cells showed a progressive reduction in spike frequencies throughout LSs 16–30 that was no different from the decreases observed in Paired (no drug) cells \([F_{1, 21} = 2.71, p = 0.114]\), but was significantly greater than Untrained \([F_{1, 24} = 20.03, p = 0.000]\) and Buffer \([F_{1, 13} = 14.75, p = 0.002]\) cells (Figure 7B). Analysis of the percent change in spike frequencies from the 5th to 30th LS yielded similar results. Untrained + PP2B cells showed a large 33.6 ± 3.6% reduction in spiking that was no different than Paired (no drug) cells (33.5 ± 6.5% decrease) \([F_{1, 17} = 0.89, p = 0.358]\) (Figure 7C).

In summary, these results partially support the hypothesis that PP2B participates in the reduction in spike frequencies produced by in vitro extinction. caPP2B injection into Untrained cells decreased spike frequencies, mimicking the effects of in vitro extinction. Unexpectedly, injection of caPP2B into Paired cells did not decrease spike frequencies and blocked the spiking reductions produced by in vitro extinction.

**CALCULIN A PREVENTS THE DECREASE IN SPIKE FREQUENCY PRODUCED BY PROTEIN PHOSPHATASE 2B IN UNTRAINED CELLS**

The progressive reduction of spike frequencies produced by in vitro extinction in Paired cells appears to depend upon both PP2B and PP1, because inhibitors of either phosphatase blocked the spiking reductions. To further probe possible interactions between the two phosphatases, we used calyculin A to block endogenous PP1 activity and injected caPP2B into cells from Untrained animals prior to in vitro extinction. If the reduction of spike frequency produced by caPP2B occurs through the activation of PP1, then prior incubation in calyculin should block this spiking decrease. This is what we observed (Figures 8B–D).

The combination of caPP2B and calyculin appeared to produce a general facilitatory effect on spike frequencies. An ANOVA of LSs 1–2 showed a significant main effect of treatment condition for Untrained + PP2B + Calyculin (n = 8), Untrained + Calyculin (n = 7), Untrained + PP2B (n = 11), and Paired (n = 11) groups \([F_{3, 33} = 5.74, p = 0.003]\) (Figure 8A). Post-hoc analysis revealed that spike frequencies of Untrained + PP2B + Calyculin cells (7.97 ± 0.47 Hz) were significantly greater than Untrained + PP2B cells (6.04 ± 0.33 Hz) \((p = 0.018)\) and Untrained + Calyculin cells (5.79 ± 0.69 Hz) \((p = 0.016)\), but were not different from Paired (no drug) cells (7.44 ± 0.31 Hz) \((p = 1.0)\) (Figure 8A). These data indicated that Untrained + PP2B + Calyculin cells enhanced excitability during LSs 1–2 to levels characteristic of Paired cells.

During LSs 16–30, calyculin blocked the reduction in spike frequencies produced by caPP2B injection, and spiking of Untrained + PP2B + Calyculin cells was not different than Untrained + Calyculin cells \([F_{1, 13} = 3.28, p = 0.093]\), but was significantly greater than Untrained + PP2B cells \([F_{1, 17} = 40.27, p = 0.000]\) (Figures 8B,C). This block of caPP2B’s effects was not a result of general increases in spiking, as similar results were found when spike frequencies were analyzed as a relative percent change from the 5th to 30th LS (Figure 8D). Untrained + PP2B + Calyculin cells showed a 5.3 ± 4.6% decrease in spike frequency by the 30th LS that was not significantly different from Untrained + Calyculin cells (10.8 ± 12.5% increase) \([F_{1, 13} = 3.35, p = 0.090]\), but was significantly different from Untrained + PP2B cells (33.6 ± 3.6% decrease) \([F_{1, 17} = 23.61, p = 0.000]\) (Figure 8D).

In summary, caPP2B did not reduce B cell spike frequencies when endogenous PP1 was inhibited. These results support the view that the reduction in Untrained cell spike frequencies produced by caPP2B injection was mediated (at least partly) through endogenous PP1. The combination of calyculin and caPP2B also enhanced spike frequencies of Untrained cells during LSs 1–2 to levels comparable to Paired cells, suggesting that caPP2B may have both excitatory and inhibitory effects on B cell spike frequency. When the latter effects were blocked by calyculin, the excitatory effects were unmasked. Our results indicate that the PP1 and PP2B pathways interact to reduce spike frequencies in Untrained cells to levels observed during in vitro extinction in Paired cells.

**ARACHIDONIC ACID OCCLUDES ADDITIONAL SPIKE FREQUENCY DECREASES PRODUCED BY EXTINCTION IN PAIRED CELLS AND MIMICS THE EFFECTS OF IN VITRO EXTINCTION IN UNTRAINED CELLS**

Previous data from our lab indicates that AA and one of its principle metabolites, 12(S)-HPETE, contribute to the decrease in Type B cell excitability produced by inhibitory conditioning (Walker et al., 2010). Because AA and 12(S)-HPETE both elicit decreases in B cell light-evoked spike frequencies (similar to the effect of in vitro extinction), we hypothesized that these molecules might also be recruited during in vitro extinction. To test this, we bath applied AA onto Untrained and Paired cells during administration of in vitro extinction. AA was added to the bath solution after the 5th LS, once spike frequencies had stabilized (as in Walker et al., 2010). If AA is involved in the spike frequency reduction observed during in vitro extinction, then the decreases in spiking produced by exogenous AA should occlude further decreases in spike frequencies by repeated LSs in Paired cells. In support of this idea, the addition of AA to Paired cells produced a reduction in spike frequency that was no larger than the decreases observed in Paired (no drug) cells (Figure 9).

Because AA was added after LS 5, an analysis of AA’s effects on LSs 1–2 was precluded. During LSs 16–30, application of AA to Paired cells (Paired + AA; n = 13) produced a reduction in spike frequencies that was below Solvent control cell (n = 7) levels, and became larger over time [significant interaction between LS and condition, corrected for sphericity: \(F_{3,86, 51.42} = 5.78, p = 0.002\)] (Figure 9B). The reduction in spike frequency observed in Paired + AA cells was slightly (non-significant) less than the reductions produced in Paired (no drug) cells \([F_{1, 23} = 3.55, p = 0.072]\) (Figure 9B). Analysis of the percent change in spike frequencies from the 5th to 30th LS further supported these conclusions. Paired + AA cells exhibited a 22.3 ± 2.7% decrease in spike frequencies that was significantly greater than Solvent control cells (3.0 ± 3.2% increase) \([F_{1, 18} = 11.70, p = 0.003]\), but...
not significantly different from Paired (no drug) cells (33.5 ± 6.5% decrease) \(F_{(1, 23)} = 2.07, p = 0.163\) (Figure 9C).

As expected (Walker et al., 2010), the addition of AA to Untrained cells (Untrained + AA; \(n = 7\)) produced a decrease in spike frequency that mimicked the reductions produced by in vitro extinction. Compared to Solvent controls, Untrained + AA cells showed progressive reductions in spike frequencies during LSs 16–30 [significant interaction between LS and condition: \(F_{(14, 168)} = 3.72, p = 0.000\)] (Figures 9A,B). The reduced spike frequencies observed in Untrained + AA cells were indistinguishable from Paired (no drug) cells \(F_{(1, 17)} = 2.02, p = 0.174\) (Figure 9B). The effects of AA were more pronounced when considered as a percent change in spiking from the 5th to 30th LS. Untrained + AA cells exhibited a 23.0 ± 4.1% decrease in spike frequency by the 30th LS, which was significantly greater than Solvent control cells (3.0 ± 3.2% increase) \(F_{(1, 12)} = 14.25, p = 0.003\), and not significantly different from Paired (no drug) cells (33.5 ± 6.5% decrease) \(F_{(1, 17)} = 1.86, p = 0.191\) (Figure 9C).

These findings suggest that AA might be one molecule responsible for the in vitro extinction-produced decreases in spike frequency. Not only did AA addition to Untrained cells mimic the effects of in vitro extinction, but the addition of AA to Paired cells also occluded any further declines in spike frequency produced by in vitro extinction, suggesting that they are part of a common signaling pathway.

**BAICALIN, AN INHIBITOR OF 12(S)-HPETE PRODUCTION, BLOCKS IN VITRO EXTINCTION**

Our previous research (Walker et al., 2010) found that AA-produced reductions in B cell spike frequency were mainly a result of 12(S)-HPETE, an AA metabolite produced by the 12-lipoxygenase (12-LOX) enzyme. We therefore hypothesized that...
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12(S)-HPETE might also be a critical metabolite for in vitro extinction. We predicted that baikalein, a selective inhibitor of the 12-LOX enzyme, would block the decrease in spike frequency produced by in vitro extinction. To test this idea, we incubated Untrained and Paired cells in baikalein for 10 min prior to the administration of in vitro extinction. Consistent with our hypothesis, baikalein significantly blunted the decreases in B cell spike frequency observed in Paired cells, but had little impact on Untrained cells (Figure 10).

Incubation of Untrained and Paired cells in baikalein (Untrained + Baicalein, n = 7, 7.06 ± 0.36 Hz; Paired + Baicalein, n = 7, 6.89 ± 0.61 Hz) did not alter spike frequencies during LSs 1–2. In contrast, baikalein had a profound effect during LSs 16–30, but only for Paired cells. Paired (no drug) cells failed to show the progressive decline in spike frequency during LSs 16–30 and spiked at rates significantly greater than Paired (no drug) cells [F(1, 17) = 9.17, p = 0.008] (Figure 10A), but were no different than Untrained [F(1, 20) = 0.46, p = 0.504] or Untrained + Baicalein cells [F(1, 12) = 0.02, p = 0.897] (Figure 10B). This blockade of the reduction in spike frequency was not a result of non-specific increases in spiking, as spike levels of Untrained + Baicalein cells during LSs 16–30 were not different from levels exhibited by Untrained (no drug) cells [F(1, 20) = 0.65, p = 0.428]. Similar conclusions concerning the effects of baikalein were reached when considered as a percent change in spike frequencies from the 5th to 30th LS. Paired + Baicalein cells showed a 6.8 ± 0.7% reduction in spike frequency, which was significantly less than Paired (no drug) cells (33.5 ± 6.8% increase).

FIGURE 9 Bath application of arachidonic acid (AA; 100 μM) onto Paired Type B cells reduced B cell spike frequency and occluded any further decreases produced by in vitro extinction. AA applied to Untrained B cells produced reductions in spike frequency that mimicked the decreases observed in Paired (no drug) cells. AA or vehicle solvent (ethanol, 0.1% final bath concentration) was bath applied to exposed H.c. nervous systems immediately after the 5th LS. (A) Representative light responses recorded from an Untrained Type B cell exposed to solvent (Unt + Solvent; top trace) or AA (Unt + AA; bottom trace). (B) Summary spike frequency data of Type B cells exposed to AA or solvent over 30 LSs. Paired + AA cells exhibited a decrease in spike frequency compared to Unt + Solvent control cells (significant interaction between LS and condition). The significant reduction in spike frequencies produced in Paired + AA cells was as large, but no larger, than the decrease produced in Paired (no drug) cells, which suggests that AA occluded the spike frequency reducing effects of repeated LSs in Paired (no drug) cells. The application of AA to Untrained cells mimicked the effects of in vitro extinction and produced reductions in spike frequency that were significantly below levels observed in Unt + Solvent control cells. (C) Summary data of the relative percent change in B cell spike frequency from the 5th to 30th LS. Paired + AA B cells exhibited a significant decline (~22%) in spike frequency over the course of 30 LSs, which was significantly larger than the small changes observed in Unt + Solvent control cells (3% increase), but not statistically different from the decreases seen in Paired (no drug) cells (~34%). Unt + AA cells also showed significantly larger declines in spike frequency (~23%) compared to Unt + Solvent controls, and these decreases were not statistically different from Paired (no drug) cells. Error bars are ± s.e.m. and significant p-values are < 0.05.
In summary, baicalein blocked the reduction in spike frequency produced by repeated LSs in Paired cells, yet had no effect in Untrained cells. These findings support the hypothesis that 12(S)-HPETE is involved in mediating the reduction of B cell spike frequency observed during in vitro extinction.

**Calyculin Does Not Block the Effects of Arachidonic Acid**

Precedents exist for an interaction between AA/lipoxygenase metabolites, protein phosphatases, and K⁺ channels (e.g., Duerson et al., 1996). Because the addition of exogenous cPP1 and AA both separately reduced B cell spike frequencies over the course of 30 LSs in Untrained cells, we wondered if they did so by participation in the same, or different, signaling pathway(s). To investigate these possibilities, we exposed B cells to AA in the presence of the PP1 inhibitor, calyculin A. B cells were first incubated in calyculin for 15 min, followed by the bath application of AA after the 5th LS. If the spike frequency decreases produced by AA are mediated through a PP1-dependent pathway, then incubation of B cells in calyculin prior to AA addition should prevent/attenuate the reduction in spike frequency. Our results failed to support this view. We found instead that calyculin did not block the reductions in spike frequency produced by AA and actually led to greater reductions in spiking (Figure 11) than AA-alone.

Untrained cells incubated in calyculin and exposed to AA (Untrained + Calyculin + AA; n = 6) showed marked reductions in spike frequencies during LSs 16–30 that were significantly below levels observed in Untrained + Calyculin [F(1, 11) = 10.14, p = 0.009], and AA vehicle Solvent control cells [F(1, 11) = 11.69, p = 0.006] (Figure 11B). This reduction was significantly greater than that observed in Untrained + AA cells [F(1, 11) = 6.75, p = 0.025] (Figures 11A,B), and was no different than the reductions observed in Paired (no drug) cells [F(1, 15) = 1.06, p = 0.318].
Similar effects were observed when spike frequencies were analyzed as a percent change in spiking from the 5th to 30th LS. *Untrained* + *Calyculin* + *AA* cells exhibited a $37.0 \pm 6.2\%$ decrease in spike frequency that was significantly greater than *Untrained* + *Calyculin* cells ($10.8 \pm 12.5\%$ increase) $[F_{(1, 11)} = 9.39, \ p = 0.011]$ (Figure 11C), and Solvent controls ($3.0 \pm 3.2\%$ increase) $[F_{(1, 11)} = 20.60, \ p = 0.001]$. The $37.0 \pm 6.2\%$ decrease in spike frequency observed in *Untrained* + *Calyculin* + *AA* cells was qualitatively, though not statistically, greater than *Untrained* + *AA* cells ($23.0 \pm 4.1\%$ decrease) $[F_{(1, 11)} = 3.82, \ p = 0.077]$, and was not significantly different from *Paired* (no drug) cells ($33.5 \pm 6.5\%$ reduction) $[F_{(1, 16)} = 0.46, \ p = 0.508]$.

In summary, calyculin failed to block the reduction in spike frequencies produced by *AA*, and instead enhanced the inhibitory effect of *AA* on spike frequency. These results indicate that the effects of *AA* are not mediated by PP1, and it seems unlikely that PP1 lies downstream from *AA* in B cell signaling pathways. However, it is possible that the reverse is true: activation of PP1 (by LSs and PP2B in *Paired* cells) might be critical for synthesis/release of *AA* (and/or activity of 12-LOX). Our results suggest that there may also be an inhibitory interaction between the PP1- and *AA*-signaling pathways in B cells, because the combination of calyculin and *AA* led to greater decreases in spike frequencies than *AA* alone.

**DELAYED IN VITRO EXTINCTION FAILS TO PRODUCE REDUCTIONS IN SPIKE FREQUENCY**

Prior research has demonstrated that the interval between the end of learning acquisition and the beginning of extinction training can affect the outcome of extinction (Maren and Chang, 2006; Myers et al., 2006; Cavallo et al., 2014). Extinction training conducted shortly after associative conditioning can improve the effectiveness of extinction, and may even abolish the original associative memory (Maren and Chang, 2006; Myers et al., 2006; Monfils et al., 2009). In other circumstances, delayed extinction is more effective at producing lasting CR reduction without detectable evidence of the original memory (i.e., no spontaneous recovery) (Maren and Chang, 2006).
To identify whether similar differences existed for extinction effectiveness in H.c., we repeated the in vitro extinction experiments using a prolonged (24 h) interval between original acquisition (light-rotation pairings) and the beginning of in vitro extinction. We hypothesized that memory consolidation processes would strengthen the memory for paired conditioning during the 24 h period after conditioning. Consequently, we expected that delayed in vitro extinction was ineffective at altering B cell excitability when delayed by 24 h.

During LSs 1–2, Paired 24-h cells exhibited spike frequencies similar to that of Paired 2-h cells. Thereafter, however, Paired 24-h cells failed to exhibit the characteristic decrease in spiking observed in Paired 2-h cells (Figures 12A–C) and had higher levels of spike frequencies during LSs 23–30 than Paired 2-h cells. These results indicate that in vitro extinction was ineffective at altering B cell excitability when delayed by 24 h.

During LSs 1–2, spike frequencies of Paired 24-h cells (n = 7, 7.23 ± 0.27 Hz) were greater than Untrained (n = 16, 5.96 ± 0.26 Hz) control cells, and comparable to Paired 2-h cells (n = 11, 7.44 ± 0.31; see Figure 2B), indicative of the enhanced excitability produced by associative learning (Farley and Alkon, 1982, 1987). An ANOVA compared the average spike frequencies during LSs 1–2 for Paired 24-h, Paired 2-h, and Untrained cells, and found a significant main effect of treatment condition [F(2, 31) = 8.66, p = 0.001]. Post-hoc analysis revealed that both Paired 24-h and Paired 2-h cells spiked significantly more frequently than Untrained cells (p’s = 0.023, 0.002, respectively), but did not differ from each other.

FIGURE 12 | Delayed in vitro extinction failed to reduce Type B cell spike frequencies. (A) Representative light responses recorded from Type B photoreceptors from paired-trained animals either 2 h (Paired 2-h; top trace) or 24 h (Paired 24-h; bottom trace) after the end of paired conditioning. The Paired 24-h cell did not show any appreciable decline in spike frequency over the course of 30 LSs, while the Paired 2-h cell showed a significant decline. (B) Summary spike frequency data of Type B cells across 30 LSs from Untrained or Paired animals, both 2 and 24 h after conditioning. Paired 24-h cells showed increased spike frequencies compared to Untrained control and Paired 2-h cells during LSs 1–30, and failed to show the progressive decrease in spike frequency exhibited by Paired 2-h cells. (C) Summary data of the percent change in B cell spike frequency from the 5th to 30th LS. Paired 24-h cells showed no change (~0%) in spike frequency over the course of 30 LSs, which was indistinguishable from control cells (~3% decrease), yet significantly different from the ~34% spike frequency decrease exhibited by Paired 2-h cells. Error bars are ± s.e.m. and significant p-values are < 0.05. Note that one cell from each of the Paired 2-h and Paired 24-h groups was excluded from analysis during LSs 1–2 due to missing spike frequency data as a result of electrode plugging.
other ($p = 1.0$). After the first few LSs, spike frequencies of *Paired* 24-h cells were relatively constant and did not diminish with successive LSs, unlike *Paired* 2-h cells (Figures 12A–C). *Paired* 24-h cells showed enhanced rates of spiking throughout LSs 1–30 that were significantly greater than *Untrained* cells [$F_{(1, 21)} = 5.38$, $p = 0.030$] (Figure 12B). *Paired* 24-h ($n = 8$) cells also showed significantly greater spike frequencies than *Paired* 2-h ($n = 12$) cells over LSs 1–30 [$F_{(1, 18)} = 10.45$, $p = 0.005$], and failed to show the progressive decline in spike frequencies observed in *Paired* 2-h cells as a result of repetitive LSs (Figure 12B).

Similar results were found when spike frequencies were analyzed as a percent change from the 5th to 30th LS. *Paired* 24-h cells showed little change in spike frequency during LSs 5–30 (0.4 ± 6.1% decrease), which was significantly different from *Paired* 2-h cells (33.5 ± 6.5% decrease) [$F_{(1, 18)} = 9.61$, $p = 0.006$], but not different from *Untrained* cells (2.6 ± 6.0% decrease) [$F_{(1, 21)} = 0.00$, $p = 0.968$] (Figure 12C).

These results indicate that exposure of B cells to repeated LSs produced a large decrease in spike frequency when given to *Paired* cells relatively soon (1–2 h) after conditioning. Delaying this presentation by 24 h abolished the decrease in spike frequency produced by *in vitro* extinction.

**DISCUSSION**

**CELLULAR AND MOLECULAR CORRELATES OF EXTINCTION TRAINING**

The use of an *in vitro* extinction protocol allowed us to simulate whole-animal extinction in isolated *H. c. nervus* systems and investigate the cellular and molecular processes that occur during extinction training. Type B cells from paired-trained animals exposed to thirty successive LSs showed large (33.5%) and progressive reductions in spike frequencies that were significantly below *Untrained* and non-associative control (*Random*) levels. These spike frequency reductions provided a striking correlate of whole-animal behavioral extinction (Cavallo et al., 2014), because they were contingent upon prior associative conditioning and only occurred when *in vitro* extinction was administered 1–2 h, but not 24 h, after paired conditioning. Our results implicated three molecules as participating in mediating the effects of *in vitro* extinction: PP1, PP2B, and AA12(S)-HPETE.

**Protein phosphatase 2B**

Two findings argue for the involvement of PP2B signaling in the decreased excitation produced by *in vitro* extinction. First, incubation of B cells in the PP2B-specific inhibitor, cyclosporin A, prior to *in vitro* extinction completely blocked the large reduction in spiking elicited in *Paired* cells without drug, but did not significantly affect *Untrained* cells. Second, injection of caPP2B into *Untrained* cells elicited a 33.6% decrease in spiking that was essentially identical to the 33.5% decline observed during *in vitro* extinction in *Paired* cells. The caPP2B-produced spiking reduction in *Untrained* cells was dependent on PP1 activity, as it was blocked by the PP1 inhibitor calyculin A. This finding, combined with the fact that calyculin produced a similar block of spike frequency reductions in *Paired* cells, suggests that in *Paired* cells, PP2B activation acts upstream of PP1 to reduce B cell spiking during *in vitro* extinction.

One unexpected finding concerned the failure of caPP2B to reduce spiking when injected into *Paired* cells and to occlude any further declines in spike frequencies produced by repetitive LSs. This failure might be a consequence of injecting untargeted caPP2B into B cells, which might fail to dephosphorylate the substrates normally exposed to PP2B during *in vitro* extinction. We propose that endogenous PP2B is likely targeted to subcellular A-type K⁺-channel signaling complexes, through its associated regulatory subunit (Cohen, 1989), where it acts to...
FIGURE 13 | Molecular model of a Paired and Untrained Type B cell given repeated light steps. (A) A cartoon illustration of a single Type B photoreceptor with expanded view of the rhabdomere and soma subcellular regions. Note the three classes of voltage-dependent ion channels (sodium, potassium, and calcium) located in the axon hillock region (indicated by the dotted line). (B) Expanded view of a Type B cell 1–2 h after paired training. During light steps (LSs) 1–2 (left), many (∼30–50%) of the somatic somatic IA  
K^+ channels are shut (indicated by an X in the channel pore) due to PKC-mediated phosphorylation of the channel itself or channel-associated proteins (P indicates a phosphate group). Paired conditioning also mobilizes inhibitor-1/PP1 complexes and PP2B to K^+ channel signaling complexes, where both inactive phosphatases are localized via anchoring proteins such as yotiao/AKAP. Paired conditioning also activates AA, which facilitates the (Continued)
disinhibit PP1 (through inactivation of inhibitor-1) and reduce B cell excitability. In contrast, injection of exogenous caPP2B (without a regulatory subunit), might not preferentially associate with these K⁺-channel signaling complexes, and thus would fail to activate PP1. caPP2B might even dephosphorylate other targets that endogenous PP2B would not normally encounter [e.g., voltage-gated Ca²⁺ channels (Chad and Eckert, 1986)], and thereby oppose the reductions in B cell excitability produced by PP2B-mediated disinhibition of PP1.

In summary, our results suggest that PP2B is critically involved in the spike frequency reduction produced by in vitro extinction. PP2B may reduce B cell excitability through the disinhibition of PP1, and the subsequent dephosphorylation and opening of somatic K⁺ channels, reversing learning-produced increases in excitability. This finding is reminiscent of work in rats indicating that PP2B mediates the extinction of conditioned fear responses and reverses the molecular processes (e.g., Akt kinase phosphorylation) associated with fear conditioning (Lin et al., 2003).

Specificity of phosphatase inhibitors calycin and cyclosporin A

A legitimate issue in our use of calycin and cyclosporin concerns the cross-species similarity of their intended protein phosphatase targets, and whether these inhibitors have similar substrate specificity in H.c. neurons. We believe the likelihood that these molecules exhibit similar functionality in H.c. is high for two reasons, (1) Biochemical analysis of phosphatases in invertebrate preparations, including starfish (Pondhaven and Cohen, 1987), Drosophila (Orgad et al., 1987), and Aplysia (Ichinose et al., 1990; Endo et al., 1995), indicate that their properties are remarkably similar to corresponding proteins in mammals (e.g., dephosphorylation of the beta subunit of phosphorylase kinase, IC₅₀ values for inhibitors-1 and -2, etc.), and (2) Genomic and crystallographic studies suggest striking conservation of phosphatase structures across species. For instance, genomic analyses indicate that the catalytic subunit of PP1 is one of the most conserved eukaryotic proteins (Ceulemans and Bollen, 2004; Shi, 2009), with ~70% or greater protein sequence identity. Crystallographic studies of PP1 indicate that the active site is comprised of multiple calycin-interaction regions, which prevent binding to downstream substrates (Lindvall et al., 1997; Kita et al., 2002).

Thus, the highly conserved amino acid sequence and structure of PP1's active site make similar pharmacological and functional profiles for calycin-produced inhibition of PP1 in H.c. neurons likely.

Cyclosporin has been used as an inhibitor of PP2B in numerous species (see Rühlmann and Nordheim, 1997 for review), ranging from yeast (Marton et al., 1998) to humans. The mechanism for cyclosporin's inhibition of PP2B has been extensively studied (Huai et al., 2002), and there appears to be strict conservation throughout all eukaryotes, such that all PP2B A genes encode for a polypeptide that distinguishes PP2B from other phosphatases. As with calycin, it therefore seems unlikely that cyclosporin would have reliable effects in organisms as distant as yeast and humans, but not have noticeable effects in H.c. Although molecular identification and biochemical characterization of phosphatases from H.c. has not yet been accomplished, it seems highly likely that PP1 and PP2B were the major targets affected by calycin and cyclosporin, respectively.

Arachidonic acid and 12(S)-HPETE

Three lines of evidence point to AA and 12(S)-HPETE as important contributors to the spike frequency decrease produced by in vitro extinction. First, baicalein, a specific 12-LOX enzyme inhibitor, blocked the spike frequency reduction observed in Paired cells, yet had no effect in Untrained cells. The 12-LOX enzyme is one of several intermediary enzymes that metabolize AA into second messenger molecules (Marks and Furstenberger, 1999). Our prior work in H.c. (Walker et al., 2010) found 12(S)-HPETE to be a main AA metabolite mediating the B cell spiking decreases produced by both AA application and inhibitory conditioning.

The block of extinction-produced reductions in spike frequency by baicalein was somewhat surprising given the evident involvement of phosphatase-signaling pathways in H.c. extinction. Baicalein would not be expected to directly impact the activity of PP1 or PP2B, leaving the phosphatase-signaling pathway capable of activating K⁺ channels and reducing spike frequencies. One possible explanation might be that baicalein indirectly interferes with the PP2B-PP1 pathway through the
disruption of intracellular Ca2+ signaling that relies on AA/12(S)-HPETE to activate Ca2+ entry pathways, such as the transient receptor potential-like (TRP-L and/or TRP) (Chyb et al., 1999) and arachidonate-regulated Ca2+ (ARC) channels (Shuttleworth et al., 2004; Meves, 2008). Although the precise mechanism of this AA/12(S)-HPETE/phosphatase relationship is unclear, our novel finding that calculin enhanced the reduction of spiking produced by AA in Untrained B cells supports the idea that both signaling pathways interact in a restrictive way.

The second finding that implicates AA/12(S)-HPETE involvement during in vitro extinction is that application of AA to Untrained cells elicited a progressive 23.0% reduction in spiking that mimicked the effects of in vitro extinction in Paired cells. This decrease was slightly (though not significantly) less than the 33.5% decrease produced in Paired (no drug) cells during in vitro extinction, which might indicate that AA/12(S)-HPETE is not the only molecule utilized during in vitro extinction, and/or that the pharmacokinetics of AA/12(S)-HPETE production are different for exogenous AA application vs. physiological stimulation. The AA-produced spiking decrease replicates prior research in H.c., which showed that application of AA or 12(S)-HPETE reduced spiking in Untrained B cells (Walker et al., 2010). Walker et al. (2010) also found that AA enhanced the transient IA K+ current, the same current that is reduced following paired conditioning (Alkon et al., 1985; Farley, 1988a) and increased following inhibitory conditioning (Farley et al., submitted). Based on these findings, AA/12(S)-HPETE-produced increases in IA K+ currents are likely one mechanism that contributes to the reduction in B cell spiking during in vitro extinction (see Figure 13).

The third result that suggests AA/12(S)-HPETE is utilized during in vitro extinction is that the reduction in spike frequency produced by AA application to Paired cells occluded any further declines as a result of repeated LSs. This suggests that AA signaling and in vitro extinction share a common molecular pathway. Our previous experiments with AA (Walker et al., 2010) suggest that the AA-produced occlusion of additional spike frequency reductions observed here for Paired + AA cells is unlikely to represent a “floor” effect in spike frequency reduction. Walker et al. (2010) found that the application of AA to B cells from inhibitory-conditioned animals produced an additional ~22% reduction in spike frequency beyond that elicited by inhibitory conditioning alone. The somewhat smaller spiking reduction observed presently in Paired + AA cells compared to Paired (no drug) cells might be a result of AA-produced enhancements of PKC activity (Lester et al., 1991), which could temporarily increase B cell excitability. This possibility was raised by Walker et al. (2010) to explain a brief elevation in B cell spike frequency produced by inhibitory conditioning, which dissipated 24h later. We suggested that although AA metabolites like 12(S)-HPETE might reduce spiking over time, AA itself might transiently activate PKC and temporarily increase, rather than decrease, spiking (Walker et al., 2010). The present application of AA to Paired cells could have created a large fraction of non-metabolized AA that partially elevated B cell spiking through its facilitation of PKC and the resulting PKC-mediated reduction of IA. Our baicalein results indicate, however, that in vitro extinction favors AA metabolism and the activity of 12(S)-HPETE.

**MOLECULAR MODEL OF EXTINCTION IN H.c.**

Collectively, our results support the conclusion that PP1, PP2B, and AA/12(S)-HPETE mediate reductions in B cell spike frequency produced by in vitro extinction. Here we propose a molecular model of extinction that incorporates this evidence into the broader body literature in H.c. (Figure 13). As previously proposed (Farley and Auerbach, 1986), paired conditioning activates PKC through the simultaneous release of two PKC cofactors, Ca2+ and diacylglycerol (DAG). Ca2+ release results primarily from a phototransduction cascade involving rhodopsin, phospholipase C, and the production of inositol trisphosphate (IP3) (Sakakibara et al., 1994), which activates IP3 receptors and releases Ca2+ from the endoplasmic reticulum into the cytosol, a process thought to be critical for learning-produced alterations of K+ conductances and B cell excitability (Sakakibara et al., 1986). DAG production results from rotation-induced activation of G-protein coupled GABA_{B} and 5-HT receptors (see Blackwell and Farley, 2009 for review). Activated PKC is mobilized to the plasmamembrane where it reduces the activity of multiple classes of somatic K+ channels (e.g., IA and IK-Ca), causing enhanced B cell excitability (Farley and Auerbach, 1986; Farley and Schuman, 1991). We suggest that endogenous PP1 is also localized to somatic K+ channel signaling complexes, where it remains inactive due to inhibition by inhibitor-1. Rotation given during associative conditioning also stimulates the cleavage of AA from membrane phospholipids (via GABA_{B} activation), which has been hypothesized to augment PKC effects and further enhance B cell excitability (Lester et al., 1991; Talk et al., 1997; Muzzio et al., 2001).

Shortly (1–2 h) after paired conditioning (Figure 13A), in vitro extinction (repeated LSs) elicits larger than normal increases in cytosolic Ca2+ (Ito et al., 1994). We suggest that elevated intracellular Ca2+ preferentially activates PP2B due to PP2B’s high affinity for Ca2+ compared to PKC (Lisman, 1989), and saturation of PKC signaling. Activated PP2B aggregates near PP1-containing K+ channel complexes through regulatory subunit targeting (e.g., yotiao/AKAP), where it dephosphorylates inhibitor-1 and relieves PP1 inhibition (Mulkey et al., 1994). Enzymatically active PP1 is now able to enhance the activity of A-type K+ channels and reduce B cell spiking. Exposure to cyclosporin or calculin after paired conditioning blocks this phosphatase-signaling pathway and prevents in vitro extinction from reducing B cell spike frequencies. Concurrent with the PP2B-PP1 signaling pathway, AA is metabolized by the 12-LOX enzyme into 12(S)-HPETE, which further reduces B cell excitability through enhancements of IA. The mechanism by which 12-LOX is activated by repeated LSs in Paired cells is presently unknown.

Our experiments using catalytically active phosphatases (caPP1 and caPP2B) into Untrained B cells are congruent with this model (Figure 13B). Injection of caPP2B into Untrained cells might dephosphorylate a large fraction of cytosolic inhibitor-1/PP1 complexes that have not been mobilized to the membrane. Once dephosphorylated, activated PP1 anchors to stabilizing proteins (e.g., yotiao) and dephosphorylates K+ channel signaling-complexes (or associated proteins). Injection of caPP1 into Untrained B cells has similar, but less robust, effects on K+ channel activity. Although caPP1 is able to dephosphorylate a
certain percentage of K+ channel signaling-complexes (or associated proteins), we suggest that the lack of a regulatory subunit prevents localization to K+ channel signaling-complexes, and therefore results in less dephosphorylation.

In contrast to in vitro extinction given shortly after associative conditioning, delayed in vitro extinction did not decrease B cell spike frequencies. Two possible explanations present themselves. First, 24 h after associative conditioning, repeated LSs might activate PP2B, PP1, and AA/12(S)-HPETE, but their ability to reduce spike frequencies (through enhancements of IA), is counteracted and overshadowed by persistent, constitutive PKC activity (Farley and Schuman, 1991), which suppresses IA and increases cell excitability, thus preventing reductions in spike frequencies. Second, perhaps phosphatases are unable to significantly impact B cell spiking during delayed in vitro extinction because phosphatase-signaling effects are restricted to a relatively brief critical period after paired conditioning. Although numerous mechanisms could create the conditions for such a critical period, one possibility is that phosphatase activation shortly after paired conditioning disrupts the consolidation of associative memories by regulating the activity of cAMP response element-binding protein (CREB) (Silva et al., 1998), which could inhibit additional synthesis of PKC necessary for producing the behavioral changes indicative of long-term associative memories in H.c. (Alkon et al., 2005).

**RELATIONSHIP TO EXTINCTION IN OTHER SYSTEMS**

Our evidence indicates that PP1, PP2B, and AA/12(S)-HPETE all participate in the in vitro extinction-produced reduction of B cell spiking. Therefore, these molecules are likely candidates for mediating the cellular changes in excitability that accompany the extinction-produced reversal of phototactic suppression. Not surprisingly, there are precedents for involvement of these same (or closely-related) signaling pathways in extinction of memories in other systems.

For example, a similar bi-directional modification of K+ channels by PKC/PKA and PP1 is believed to underlie short-term facilitation and depression of sensory-motor neuron synaptic transmission in *Aplysia* (Brahala et al., 1990; Sugita et al., 1994; Endo et al., 1995). Additionally, in rat hippocampal CA1 neurons, PP1 reduces synaptic transmission during the induction of some forms of long-term depression (LTD) of synaptic transmission (Morishita et al., 2001). This could be one mechanism by which PP1 facilitates extinction of conditioned taste aversion in rats (Oberbeck et al., 2010) and promotes memory decline during water maze tasks in mice (Genoux et al., 2002). PP1 also constrains learning during short-interval (i.e., massed) associative training in mice (Genoux et al., 2002) and H.c. (Muzzio et al., 1999). To our knowledge, neither AA nor 12-LOX metabolites have been implicated in extinction learning in other (vertebrate) systems. However, related fatty acids in the endocannabinoid signaling system are believed to be important contributors to extinction. Cannabinoid (CB) receptors and their endogenous ligands, endocannabinoids, have been reported to participate in extinction of memory for aversive events in rats (Marsicano et al., 2002; Cannich et al., 2004). Despite the detection of endocannabinoids in *Aplysia* ganglia (Di Marzo et al., 1999), and other invertebrates (e.g., leech, Stefano et al., 1997), their existence in other invertebrate species is still debated (McPartland et al., 2006), and their role in learning has yet to be determined. Nevertheless, it is possible that AA/LOX metabolites and associated ion channels (TRP and K+ channels) may be invertebrate evolutionary precursors to the endocannabinoid system of vertebrates, and play similar functional roles.

A final point concerns the fact that in vitro extinction reduced Paired B cell spiking below the levels of Untrained and Random control cells. If a simple reversal/erasure of the pairing-produced changes in cell excitability were occurring, then one might expect the spike frequencies of Paired cells to be indistinguishable from control cells during later LSs. However, in vitro extinction produced a pairing-specific decrease in spiking that was clearly lower than control cells by the 30th LS. The lower spike frequency of Paired cells is reminiscent of the persistent reductions in B cell spiking produced by CI learning (Britton and Farley, 1999). In conjunction with the common involvement of PP1 and AA/12(S)-HPETE in both CI and extinction, the in vitro results suggest that extinction might reflect “new” CI-like learning, although it is unclear whether the in vitro-produced reductions in spike frequency persist for 24 h. The view that the original molecular substrates of associative memories are completely “erased,” and the nervous system returned to its original dé novo state by extinction training, may be overly simplistic. A more nuanced view of cellular and behavioral erasure effects by extinction might be that a new, possibly dynamic, equilibrium (between opposing original acquisition, “new” extinction learning, and erasure processes) is established following extinction training. Therefore, the original associatively-acquired behavioral changes could appear absent due to overall circuit level alterations, but changes at the scale of individual neurons (and subcellular alterations) might persist and reflect varying combinations of old and new learning mechanisms. Whether extinction in H.c. produces a complete erasure of the original associative memory might depend on the level of analysis (i.e., behavior, circuit, or individual neuron). Although it is a convenient heuristic to organize research efforts around mutual exclusive categories of extinction learning (i.e., erasure or new learning), this tendency could be overly simplistic and should not blind us to the inherent complexity of extinction learning.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the National Institute of Neurological Disorders and Stroke (NIH NINDS NS48156). We thank Claude Klee for her gift of PP2B, and Katie Babcock and Sally Miller for their invaluable assistance with this research. Finally we would like to thank Tony Walker for his intellectual contributions to this project and for his general support.

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Conflict of Interest Statement: The authors declare that the research was con- ducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 May 2014; accepted: 23 September 2014; published online: 21 October 2014.

Citation: Cavallo JS, Hamilton BN and Farley J (2014) In vitro extinction in Hermissenda. Frontiers in Behavioral Neuroscience October 2014 | Volume 8 | Article 354 | 24