Effect of Interstimulus Interval on Pairing-Induced LTP of Aplysia Sensorimotor Synapses in Cell Culture

Xiang Y. Lin and David L. Glanzman

1 Department of Physiological Science, 2 Brain Research Institute, University of California, Los Angeles, California 90095-1568

Lin, Xiang Y. and David L. Glanzman. Effect of interstimulus interval on pairing-induced LTP of Aplysia sensorimotor synapses can also exhibit Hebbian LTP (Lin and Glanzman 1994a,b). This form of invertebrate LTP (apLTP), like LTP of synapses in the CA1 region of the mammalian hippocampus (Collingridge et al. 1983; Kelso et al. 1986; Lynch et al. 1983; Malinow and Miller 1986; Wigström et al. 1986), depends on postsynaptic depolarization, postsynaptic elevation of intracellular Ca²⁺, and activation of N-methyl-D-aspartate (NMDA) or NMDA-like receptors (for related data see Clark et al. 1995; Cui and Walters 1994; Dale and Kandel 1993). A central issue for evaluating the potential role of NMDA-receptor-dependent LTP in learning and memory is its sensitivity to the temporal order of stimuli (see Bourne and Nicoll 1993). This is because the order in which two stimuli are presented is often critical for associative learning. Many types of classical conditioning, for example, exhibit an optimal interval for the presentation of the conditioned stimulus (CS) and unconditioned stimulus (US). Conditioning is typically best when the onset of the CS precedes the onset of the US by a relatively short interval. Conditioning typically weakens as the CS-US interval lengthens and is often slight, or nonexistent, if the CS and US occur simultaneously or if the US occurs before the CS (Mackintosh 1983) (although see DISCUSSION).

Classical conditioning of the siphon-withdrawal reflex of Aplysia (Carew et al. 1981, 1983) exhibits such “temporal specificity” of CS and US occurrence. Thus behavioral conditioning of the withdrawal reflex is optimal when the onset of the CS (weak tactile or electrical stimulation of the siphon or mantle skin) precedes the onset of the US (strong electrical shocks applied to the animal’s tail) by ~0.5 s (Hawkins et al. 1986). We have proposed that Hebbian apLTP, induced by the conjoint activation of sensory neurons and siphon motor neurons, might play a critical role in classical conditioning of the siphon-withdrawal reflex in Aplysia (Glanzman 1994, 1995; Lin and Glanzman 1994b). Herefore, however, simultaneous pre- and postsynaptic stimulation has been employed to induce apLTP. It is therefore crucial to determine whether apLTP can be induced when the stimulation of the sensory neuron and motor neuron are temporally offset. For this reason we varied the temporal interval between the onset of presynaptic stimulation and the onset of postsynaptic depolarization, and measured the resulting apLTP of in vitro synapses.

METHODS

Cell cultures

The cell cultures consisted of sensory neurons taken from pleural ganglia, and small siphon motor neurons (Frost et al. 1988) taken...
from abdominal ganglia of adult *Aplysia californica* (80–120 g). The neurons were dissociated, cultured, and maintained as described previously (Lin and Glanzman 1994a; Rayport and Schacher 1986). Each motor neuron was cocultured with a single presynaptic sensory neuron. The cultures were housed in an incubator (Forma Scientific No. 3919, Marietta, OH) at 18°C until the start of the experiments. The cell cultures used in these experiments were 2–4 days old.

**Electrophysiology**

During experiments the cultures were perfused with a solution consisting of 50% sterile artificial seawater and 50% Leibowitz-15 (Sigma, St. Louis, MO), modified as described previously (Lin and Glanzman 1994a). All experiments were carried out at 18–21°C. The identity of a postsynaptic neuron as a small siphon cell was confirmed electrophysiologically before an experiment was begun. This was done by strongly hyperpolarizing the neuron with the intracellular recording electrode and then suddenly releasing it from hyperpolarization. The membrane potential of small siphon cells exhibits a distinctive “notch,” possibly due to activation of A current, when these cells are suddenly released from strong hyperpolarization (Eliot 1991). The mean resting membrane potential of the motor neurons was 38.5 ± 0.4 (SE) mV (range 31–51 mV). There were no significant differences in the membrane potentials of the motor neurons among the various experimental groups. The electrophysiological methods have been described previously (Lin and Glanzman 1994a,b). Briefly, the sensory neurons were stimulated with extracellular electrodes and the resulting excitatory postsynaptic potentials (EPSPs) were recorded from the motor neurons with intracellular electrodes. Extracellular, rather than intracellular, electrodes are commonly used for presynaptic stimulation of in vitro *Aplysia* synapses (e.g., Ghirardi et al. 1992), because intracellular impalement of sensory neurons has previously been shown to disrupt posttetanic potentiation of these synapses (Eliot 1991), probably by abnormally elevating intracellular Ca$^{2+}$.

We wished to avoid any potential disruption of mechanisms of plasticity in our experiments. The motor neuron was held at 50 mV below its resting potential to prevent the motor neuron from spontaneous or stimulus-evoked firing during testing. This was achieved by passing negative current (−0.3–0.5 nA) into the cell via the bridge circuit of the intracellular amplifier. The paired stimulation consisted of 0.5 s of 25-Hz extracellular stimulation and 0.5 s of postsynaptic depolarization. A total of 2 nA of intracellularly injected current was used to depolarize the motor neuron during pairing; this depolarizing current was superimposed on the constant hyperpolarizing current used for testing the strength of the EPSP. The mean initial amplitude of the sensorimotor EPSP in these experiments, measured with the motor neuron hyperpolarized, was 31.1 ± 1.8 mV (range 7–72 mV). There were no systematic differences among the various experimental groups with respect to the sizes of initial evoked EPSPs.

**Statistical analyses**

The peak amplitudes of the EPSPs evoked on the trials were measured and then normalized to the amplitude of the EPSP on the second (0-min) trial. The normalized data are expressed as means ± SE. The normalized EPSPs were used for all statistical comparisons, which were performed by a computer program (IN-STAT, GraphPad, San Diego, CA). Nonparametric tests were used for between-group comparisons whenever the SDs of the group data, as indicated by Bartlett’s tests for homogeneity of variances, were sufficiently different to invalidate the use of parametric tests.

**RESULTS**

Pairing-induced apLTP exhibits an ISI function that is symmetrical about the zero ISI

The basic paired stimulation protocol of Lin and Glanzman (1994b) was utilized. The interstimulus interval (ISI) between pre- and postsynaptic stimulation was systematically varied from 5.0-s backward pairing (−5.0 s ISI), in which the onset of postsynaptic stimulation preceded the onset of presynaptic stimulation by 5.0 s, to 5.0-s forward pairing (5.0 s ISI), in which the onset of presynaptic stimulation preceded the onset of postsynaptic stimulation by 5.0 s. *Aplysia* sensorimotor synapses, both in the CNS (Castel-lucci and Kandel 1974) and in cell culture (Rayport and Schacher 1986), depress significantly when stimulated at low rates. LTP of the sensorimotor synapses is therefore indicated by a significant increase in the mean size of the sensorimotor EPSP compared with the mean size of the EPSP for synapses that receive only the test stimuli (Fig. 1 and Lin and Glanzman 1994a,b). In the present experiments, whether or not synapses in a specific ISI group exhibited significant apLTP was determined by comparing the mean size of the EPSP, averaged across the 60- to 80-min trials, for the synapses that received paired stimulation with the mean size of the EPSP for synapses that received only the test stimuli (test-alone group). A Kruskal-Wallis nonparametric analysis of variance indicated that the various ISIs produced significantly different amounts of potentiation, as indicated by the overall differences among the mean amplitudes of the group EPSPs ($KW = -4.208, P < 0.0001$). Subsequent pairwise statistical comparisons of the averaged 60- to 80-min EPSPs with the use of the Dunn’s test, a nonparametric test that corrects for multiple comparisons,
indicated that paired stimulations with ISIs of 0.0, -0.5, and 0.5 s all produced significant apLTP compared with the test-alone protocol (Figs. 2 and 3). The mean test-alone EPSP for the 60- to 80-min trials was 69.3 ± 4% of the EPSP on the 0-min trial. By comparison, the mean 60- to 80-min EPSP was 92.4 ± 4% for ISI = −0.5 s (P < 0.01); 111 ± 5% (P < 0.001) for ISI = 0.0 s; and 90.6 ± 5% for ISI = 0.5 s (P < 0.05). (The P values are for the comparison with the mean test-alone EPSP and represent 2-tailed values.) The 60- to 80-min test-alone EPSP was not significantly different from that of any of the other ISI groups (P > 0.05 for each comparison). Although simultaneous onset of the presynaptic tetanus and postsynaptic depolarization produced the most apLTP, there were no statistically significant differences among the EPSPs for the −0.5-, 0.0-, and 0.5-s groups (P > 0.05 for each comparison, Dunn’s tests). These results indicate that the temporal specificity of Hebbian apLTP is symmetrical about an ISI of zero. Moreover, because the duration of both the presynaptic stimulus and postsynaptic stimulus lasted ~0.5 s, our results indicate that apLTP can be induced by paired stimulation even when the pre- and postsynaptic stimuli do not temporally overlap. However, apLTP induced by an ISI of 0.5 s is insensitive to the order in which the presynaptic tetanus and postsynaptic depolarization occur. The temporal specificity profile for pairing-induced apLTP is depicted in Fig. 4, which presents the net enhancement of the sensorimotor EPSP (mean normalized EPSP minus the value for the normalized test-alone EPSP, data from the 60- to 80-min trials) for each of the ISIs in our experiments.

Our finding that pairing-induced apLTP exhibits no temporal asymmetry represents a potential problem for the hypothesis that apLTP-related plasticity plays a significant role in classical conditioning in Aplysia (Glanzman 1994, 1995; Lin and Glanzman 1994a). This is because the temporal specificity for Hebbian apLTP, as represented in Fig. 4, differs from that reported for classical conditioning of the reflex (Hawkins et al. 1986; see their Fig. 3). In particular, Hawkins et al. obtained significantly less conditioning of the withdrawal reflex with simultaneous pairing of the CS and US than with forward pairing, and no conditioning at all with backward pairing. This difference may reflect a fundamental distinction between apLTP and behavioral conditioning of the reflex, or it may simply be due to differences between the training protocols used in the cellular and behavioral studies. In the conditioning experiments there were typically...
several CS-US pairings at a rate of one per 5 min, whereas in our LTP experiments there was only a single pairing of pre- and postsynaptic stimulation. In addition, 1.0-s tail shocks were used for the US in behavioral experiments, whereas the motor neurons were only depolarized for 0.5 s in the LTP experiments. To determine whether the differences in the ISI functions reflect a fundamental difference between apLTP and behavioral conditioning in Aplysia, we carried out additional experiments with the use of a protocol designed to more closely parallel that used for the behavioral training (Hawkins et al. 1986). In these experiments we compared the relative efficacy of 0.5-s backward pairing and 0.5-s forward pairing in inducing apLTP of cultured synapses.

**Forward pairing and backward pairing are equally effective in inducing apLTP with a conditioning-type training protocol**

In this series of experiments there were seven test trials with an intertrial interval of 5 min. Paired stimulation consisted of five pairings of presynaptic tetanic stimulation and postsynaptic depolarization (Fig. 5A). The paired stimulation was delivered at a rate of one per 5 min, with the first pairing occurring 1 min after the second trial. In addition, there were two posttraining trials, one at 15 min (PT15), and the other at 60 min (PT60), after the fifth (last) bout of paired stimulation. There were three treatment groups: a group that received paired stimulation in which the onset of the presynaptic tetanus occurred 0.5 s before the onset of postsynaptic depolarization (forward pairing, n = 11); a group that received paired stimulation in which the onset of the postsynaptic depolarization occurred 0.5 s before the onset of the presynaptic tetanus (backward pairing, n = 9); and, finally, a group that received only the test stimuli (test alone, n = 16). Figure 5B presents the group data from these experiments and Fig. 5C presents representative results. A nonparametric analysis of variance (Kruskal-Wallis test) performed on the mean group EPSPs from each of the trials T3–T7 indicated that the differences among the three experimental groups were highly significant on all five of the trials (P < 0.0001 for each trial). Pairwise comparisons indicated that both types of paired stimulation produced significant enhancement of the EPSP on T3–T7 compared with the test-alone protocol (P < 0.05 for each comparison, Dunn’s tests). However, the EPSPs in the forward pairing group did not differ from those in the backward pairing group on these trials (P > 0.05 for each comparison). We used parametric tests to analyze the posttest data. This is warranted because Bartlett’s tests for homogeneity of variances indicated that the differences among the SDs for the posttest data from the three groups were not statistically significant (P > 0.05 for each Bartlett’s test on the data for PT15 and PT60). One-way analyses of variance on the group means for PT15 and PT60 indicated that the variation among the means was highly significant on both posttests [F(2,33) = 7.24, P < 0.0025 for PT15, and F(2,33) = 5.07, P < 0.02 for PT60]. Tukey-Kramer tests, which correct for multiple comparisons, were used for pairwise comparisons of the group means for PT15 and PT60. These tests indicated that the forward pairing and backward pairing EPSPs were both significantly potentiated compared with the test-alone EPSP on PT15 (q = 4.67 for the comparison between forward pairing and test-alone EPSPs, P < 0.01; q = 4.27 for the comparison between the backward pairing and test-alone EPSPs, P < 0.05), as well as on PT60 (q = 3.92 for the comparison between forward pairing and test-alone EPSPs, P < 0.05; q = 3.56 for the comparison between backward pairing and test-alone EPSPs, P < 0.05). But the forward pairing and backward pairing EPSPs did not differ significantly from each other on either posttest (P > 0.05 for both comparisons).

**DISCUSSION**

**Temporal properties of pairing-induced apLTP: comparison with hippocampal LTP**

Pairing-induced apLTP of synapses in cell culture exhibits an approximately Gaussian ISI profile centered about the zero ISI (Fig. 4). The closer the presynaptic and postsynaptic stimuli are together in time, the greater the potentiation. In this series of experiments there were seven test trials...
ditioning of the siphon-withdrawal reflex in *Aplysia* requires elevation of Ca\(^{2+}\) in the postsynaptic motor neuron and activation of NMDA or NMDA-related receptors. By demonstrating that Hebbian apLTP does not require simultaneous presynaptic and postsynaptic stimulation, the present results provide some support for the hypothesis (Glanzman 1994, 1995; Lin and Glanzman 1994b) that this form of plasticity contributes to classical conditioning of the siphon-withdrawal reflex in *Aplysia*. In particular, because significant potentiation results when tetanic stimulation of the sensory neuron precedes the depolarization of the motor neuron by as much as 0.5 s, the training involved in classical conditioning of siphon withdrawal—in which the onset of the CS precedes the onset of the US by 0.5 s (Carew et al. 1981, 1983)—could, in principle, result in significant LTP of sensorimotor connections. Nevertheless, our findings raise the question: what is the reason for the blockade of conditioning to backward pairing of the CS and US if classical conditioning in *Aplysia* is mediated by apLTP-related plasticity?

Another form of synaptic enhancement prominently implicated in classical conditioning in *Aplysia* is activity-dependent enhancement of presynaptic facilitation (ADPF) (Hawkins et al. 1983; Walters and Byrne 1983a). According to current models (Abrams and Kandel 1988; Byrne 1987), ADPF is induced by the conjunctive firing of central siphon sensory neurons activated by the CS and of facilitatory interneurons, some of which are serotonergic (Glanzman et al. 1989; Mackey et al. 1989), activated by the US. The conjoint activity of the sensory neurons and facilitatory interneurons is thought to produce two intracellular signals in the presynaptic terminals of the sensory neurons: elevation of intracellular Ca\(^{2+}\) (due to the CS) and stimulation of adenylate cyclase (due to release of serotonin by facilitatory interneurons). The elevation of Ca\(^{2+}\) in the sensory neurons is hypothesized to somehow “prime” the cyclase, thereby increasing its production of adenosine 3',5'-cyclic monophosphate (cAMP), which is known to mediate presynaptic facilitation of transmitter release by the sensory neurons (Baxter and Byrne 1990; Braha et al. 1990; Brunelli et al. 1976; Castellucci et al. 1980; Ghirardi et al. 1992; Klein 1993; Klein and Kandel 1978). Thus ADPF is ascribed to a Ca\(^{2+}\)/calmodulin-triggered enhancement of the cAMP cascade within the sensory neurons, an idea supported by biochemical data (Abrams et al. 1991; Ocorr et al. 1985) and by data from cell culture experiments (Eliot et al. 1994). A still-unresolved question, however, is whether ADPF can account for the temporal specificity exhibited by classical conditioning. Abrams and Kandel (1988) have suggested that the behavioral ISI function arises from the temporal requirements for the priming of the adenylate cyclase by Ca\(^{2+}\)/calmodulin. According to their model, the increase in intracellular Ca\(^{2+}\) must precede the binding of serotonin to its postsynaptic receptors on the sensory neuron by ~0.5 s for enhancement of the cAMP cascade; if serotonin binds...

**FIG. 5.** Results from the comparison of forward and backward pairing in conditioning-type experiments. A: representative examples of the motor neuron’s response during paired stimulation for forward pairing and backward pairing experiments. Thin upward deflections in the records represent stimulus artifacts produced by the presynaptic tetanus. B: mean EPSPs in forward pairing, backward pairing, and test-alone experiments, normalized to the amplitude of the EPSPs on trial 2 (T2). Both types of pairing regimens produced significant potentiation of the sensorimotor synapse, as indicated by comparisons with the test-alone data for the 15- and 60-min posttests. Arrows below abscissa: occurrence of paired stimulation. Error bars: means ± SE; C: representative EPSPs from the experiments in forward pairing and backward pairing experiments on T2, which immediately preceded the 1st bout of paired stimulation, trial 3 (T3), which followed the 1st bout of paired stimulation, and the posttest trial that occurred 15 min after the 5th (last) bout of paired stimulation (PT\(_{15}\)) (see RESULTS and Fig. 6B). Also shown are the corresponding EPSPs from a test-alone experiment. Dashed horizontal lines: peak amplitudes of the EPSPs in T2. Vertical scale: 10 mV for the –0.5-s EPSPs, 15 mV for the other EPSPs.
to its receptors before, or at the same time as, the increase in intracellular Ca\(^{2+}\), substantially less enhancement of cAMP production results (Abrams et al. 1991; Yovell and Abrams 1992).

Another cellular mechanism that might contribute to the effect of ISI in classical conditioning in Aplysia is suggested by the finding that tail shock initially produces transient inhibition of the siphon-withdrawal reflex as well as of the monosynaptic sensorimotor connections within the abdominal ganglion (Mackey et al. 1987; Marcus et al. 1988). Mackey et al. hypothesized that this transient inhibition could underlie the blockade of classical conditioning when the CS and US are backwardly paired (see also Walters and Byrne 1983b). Recent experiments by Clark et al. (1994) have provided data regarding the validity of the hypothesis of Mackey et al. Clark et al. have shown that enhancement of the monosynaptic sensorimotor EPSP due to sensory neuron activity and tail nerve shock is greater when the sensory neuron activity (the CS for the cellular analogue of classical conditioning) precedes the nerve shock (the US) than when the US precedes the CS. In their study, Clark et al. measured the US-produced hyperpolarization of the sensory neuron’s membrane potential during training. They found that 0.5-s backward pairing produced significantly greater hyperpolarization of the sensory neuron membrane potential than did 0.5-s forward pairing (for related data see Walters and Byrne 1983b). These results represent the strongest evidence to date in support of the hypothesis of Mackey et al. that the block of backward conditioning in Aplysia is due, at least in part, to US-elicited inhibition. But Clark et al. also found that forward pairing of sensory neuron activity with puffs of serotonin onto sensory neurons produced greater broadening of the sensory neuron action potential (in tetraethylammonium chloride) than did backward pairing. Because such spike broadening is thought to contribute to facilitation of sensorimotor synapses (Byrne and Kandel 1996; Eliot et al. 1993; Gingrich and Byrne 1985; Hochner et al. 1986; Klein and Kandel 1978; although see Klein 1994, 1995), the data of Clark et al. would seem to indicate that ADPF also contributes to the temporal specificity of Aplysia conditioning (but see Eliot 1991 for contradictory data). It is difficult, however, to convincingly rule out the effects of US-elicited heterosynaptic inhibition (Fitzgerald and Carew 1991; Mackey et al. 1987) from conditioning experiments in the CNS of Aplysia. For this reason, the issue of whether ADPF is intrinsically sensitive to the order of presynaptic activity and serotonin must ultimately be resolved by experiments on cultured neurons.

**Does the requirement for asynchronous presentation of stimuli that characterizes many instances of classical conditioning result from the properties of molecules or circuits?**

Although, as described above, the most efficacious procedure for many types of classical conditioning is to have the onset of the CS precede that of the US, psychologists have long recognized that forward pairing of stimuli is not required for Pavlovian learning. The behavioral literature contains convincing examples of classical conditioning due to simultaneous pairing (Heth and Rescorla 1973; Mahoney and Ayres 1976) as well as to backward pairing (Heth and Rescorla 1973; Mower and Aiken 1954; Shurtleff and Ayres 1981). Indeed, for certain types of higher-order conditioning, simultaneous pairing is as effective, and in some cases more effective, than forward pairing (Barnet et al. 1991, 1993; Rescorla 1980). Models of associative synaptic enhancement that depend on temporal asynchrony of stimuli (Abrams and Kandel 1988; Holmes and Levy 1990) cannot account for such behavioral results. By contrast, the type of associative long-term synaptic change we have described here, which is sensitive only to the amount of temporal contingency between stimuli, could mediate simultaneous, as well as backward, conditioning. Possibly, the various optimal temporal parameters that characterize different classical conditioning paradigms result from underlying, intrinsic differences among different molecular mechanisms of synaptic change. According to this view, some of these molecular mechanisms are sensitive to stimulus order, whereas others are not. Alternatively, all types of associative synaptic enhancement might be intrinsically sensitive only to the temporal correlation between stimuli, not to stimulus order. According to this second view, the order specificity that characterizes some forms of classical conditioning is due to input from modulatory pathways extrinsic to the site of synaptic change or (possibly in some cases) to delays in the time required for neuronal signals produced by conditioning stimuli to travel from peripheral receptors to critical synaptic sites within the CNS. Classical conditioning of the siphon-withdrawal reflex in Aplysia provides a model system for determining which of these two opposing views of learning-related synaptic modulation is correct.

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Address for reprints requests: D. L. Glanzman, Dept. of Physiological Science, University of California, 405 Hilgard Ave., Los Angeles, CA 90095-1568.

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