Research

Comparison of the ionic currents modulated during activity-dependent and normal presynaptic facilitation

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One of the major questions in psychology is whether associative and nonassociative learning are fundamentally different or whether they involve similar processes and mechanisms. We have addressed this question by comparing mechanisms of a nonassociative form of learning, sensitization, and an associative form of learning, classical conditioning of the siphon-withdrawal reflex of hermaphroditic *Aplysia*. In an analog of differential conditioning, action potentials in one siphon sensory neuron (SN) were paired with shock to the pedal nerves, producing activity-dependent presynaptic facilitation, and action potentials in another SN were unpaired with the shock as a control. The difference between paired and unpaired training is a measure of associative plasticity. Before and after this training, we voltage clamped each SN and measured the outward current during depolarizing pulses. There was a significantly greater decrease in the net outward current in the paired SN than in the unpaired SN. We obtained similar results when we substituted the depolarizing voltage clamp pulse for action potentials during training. We then bathed the ganglion in serotonin as a measure of nonassociative plasticity. The current that was modulated differentially (paired−unpaired) had time and voltage dependencies similar to the current that was modulated by serotonin (I_S). These results suggest that an associative form of plasticity, activity-dependent presynaptic facilitation underlying conditioning, involves enhanced modulation of the same ionic current as a nonassociative form, normal presynaptic facilitation underlying sensitization.

One of the major questions in psychology is whether associative and nonassociative learning are fundamentally different or whether they involve similar processes and mechanisms. We have taken a reductionist approach to this question by comparing mechanisms of a nonassociative form of learning, sensitization, and an associative form of learning, classical conditioning of the siphon-withdrawal reflex of *Aplysia*, using in vivo, semi-intact, and in vitro preparations (for reviews, see Hawkins and Byrne 2015; Hawkins et al. 2017). The gill- and siphon-withdrawal reflex of *Aplysia* exhibits sensitization in response to the tail shock, and classical conditioning when a weak stimulus to the siphon or mantle (the conditioned stimulus or CS) is paired with tail shock (the unconditioned stimulus or US). Sensitization involves serotonin- and cAMP-dependent presynaptic facilitation at the synapses from siphon sensory neurons (SNs) to gill- and siphon-motor neurons, due in part to broadening of action potentials in the SNs and enhanced transmitter release. Classical conditioning involves activity-dependent amplification of that mechanism when action potentials in the SN are paired with the shock.

The first experiments to demonstrate this mechanism used the preparation illustrated in Figure 1A, which consists of the isolated nervous system attached to the tail by the posterior pedal nerves. A training procedure based on that used to produce differential conditioning of the withdrawal reflex behaviorally also produced differential facilitation of the EPSPs from two siphon SNs to a siphon motor neuron (Hawkins et al. 1983). During training, action potentials in one SN were paired with shock to the tail or pedal nerves, and action potentials in the other SN were unpaired. This procedure produced significantly greater facilitation of the EPSP from the paired than from the unpaired SN, which we referred to as activity-dependent facilitation. In similar experiments, this training procedure also produced greater broadening of the action potential in the paired than the unpaired SN, either in a TEA or in normal seawater. These results suggested that a mechanism of conditioning was an amplification of the mechanism of sensitization.

However, subsequent experiments have revealed additional pre- and postsynaptic mechanisms, some of which are shared by sensitization and conditioning and some of which are unique to conditioning, raising questions about their relationship (Hawkins and Byrne 2015; Hawkins et al. 2017). Furthermore, broadening of action potentials in the SN might be due to changes in several different ionic currents. If conditioning involves amplification of a mechanism of sensitization, then activity-dependent facilitation should involve modulation of the same ionic current as normal presynaptic facilitation. Normal presynaptic facilitation involves a decrease in two K+ currents, a voltage-dependent current, I_{KV}, and a serotonin- and cyclic AMP-sensitive current, I_S, which is also important for an increase in SN excitability during facilitation (Klein et al. 1982; Shuster et al. 1985; Baxter and Byrne 1989; Goldsmith and Abrams 1992; Hochner and Kandel 1992; Byrne and Kandel 1996). We have now investigated whether activity-dependent facilitation involves modulation of the same currents. Because there are pairing-specific increases in SN excitability as well as facilitation of the EPSP during behavioral conditioning (Antonov et al. 2001, 2003, 2007), we have focused on I_S.

Results

The training procedure was identical to that used in the experiments demonstrating activity-dependent facilitation of the EPSP

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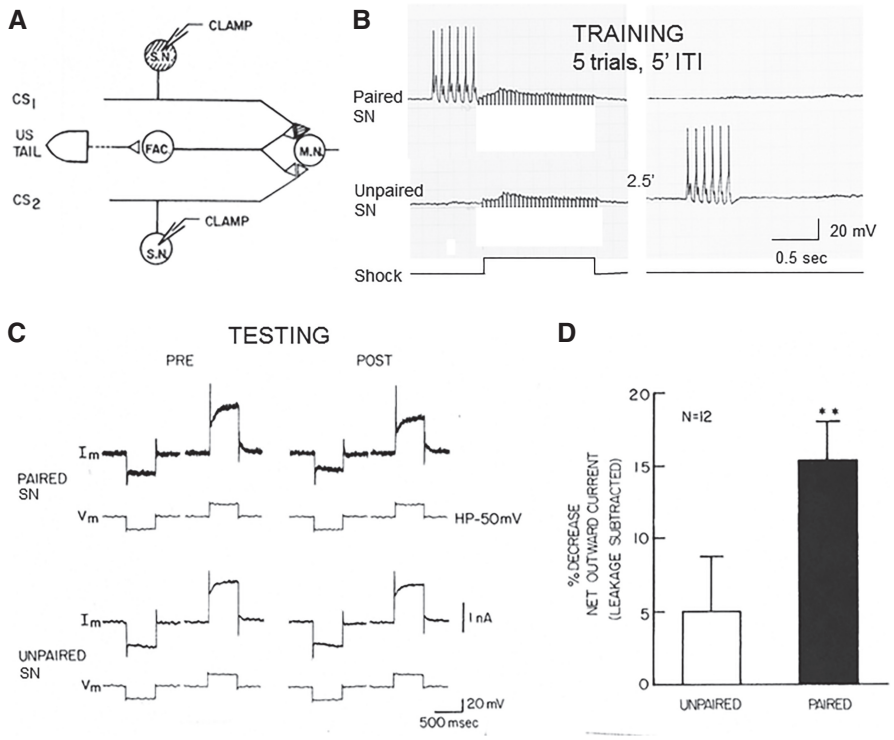

Figure 1. A training procedure for differential facilitation in *Aplysia* produces a differential decrease in an outward current in the SNs. (*A*) The experimental preparation. (*B*) An example of one training trial in which a brief train of action potentials in the paired SN started 0.5 sec before shock to the posterior pedal nerves (indicated by the shock artifacts) and a train of action potentials in the unpaired SN started 2.5 min later. There were five training trials with a 5 min ITI. (*C*) An example of testing 5 min before (Pre) and after (Post) training. We measured the outward current in each SN during a 500 msec, 20 mV depolarizing voltage clamp pulse from a holding potential of −50 mV. We also measured the inward current during a 20 mV hyperpolarizing pulse as an estimate of the leakage current, which was subtracted from the outward current to give an estimate of the net voltage-dependent current. (*D*) Average results from 12 experiments like the one shown in *C*. There was a significantly greater decrease in the net outward current in the paired than the unpaired SN after training.

(Fig. 1A,B; Hawkins et al. 1983). There were five training trials with a 5 min intertrial interval. On each trial, we produced a train of six action potentials at 10 Hz in the paired SN starting 0.5 sec before shock to the posterior pedal nerves, and a train of action potentials in the unpaired SN starting 2.5 min later. The train of action potentials was similar to the firing of the SN during behavioral training in a semi-intact preparation (Antonov et al. 2001, 2003, 2007). However, now instead of measuring the amplitudes of the EPSPs from each SN to a motor neuron, we voltage clamped each SN with a Dagan single electrode clamp and measured the outward current during 500 msec, 20 mV depolarizing pulses from a holding potential of −50 mV, 5 min before and after training (Fig. 1C). Training and testing were both carried out in normal artificial seawater. We also measured the inward current during hyperpolarizing voltage clamp pulses as an estimate of leakage current, which was subtracted from the outward current to give an estimate of the net voltage-dependent current. In the example shown in Figure 1C, there was a substantial decrease in the outward current in the paired SN, and a much smaller decrease in the unpaired SN. In 12 experiments like that one, there was a significantly greater decrease in the net outward current in the paired than the unpaired SNs ($t_{(11)} = 3.15$, $P < 0.01$) (Fig. 1D). As controls, there was no significant difference in the baseline resting potential (average = −44 mV), drift in the holding potential as measured at the end of the experiment (average = −54 mV), pretest depolarizing and leakage currents, or the change in leakage current following training.

The parameters of the depolarizing voltage clamp pulse, which was relatively small and long lasting, were chosen on the basis of previous experiments (Klein et al. 1982; Shuster et al. 1985; Baxter and Byrne 1989; Goldsmith and Abrams 1992; Hochner and Kandel 1992) so that most of the outward current would be carried by I_S. We further tested that hypothesis by comparing the time and voltage dependence of the current that is modulated differentially to the time and voltage dependence of I_S. At the end of each experiment, we exposed the ganglion to 10^{-4} M serotonin (which produces a near maximal decrease in I_S) and measured the currents again. We then compared that current to the current that was modulated by paired and unpaired training in the same cells. To do this, we looked at the "difference" current, or the Pre–Post change in current produced by the training procedure. The results from the experiment illustrated in Figure 1C are plotted in Figure 2A, which shows the difference current in the paired cell, the unpaired cell, and the average response to serotonin in both cells as a function of time during the 500 msec depolarizing pulse. The magnitude of the difference current is different in the three cases but the curves have similar shapes, indicating that the currents have similar time dependence.

Figure 2B shows the current that is modulated differentially, that is, the current in the paired cell minus the current in the unpaired cell, which is a measure of associative plasticity, plotted on a different scale to facilitate comparison with the current that is modulated by serotonin, which is a measure of nonassociative plasticity. The time dependence of these two currents is very similar. In particular, neither shows appreciable inactivation within 500 msec, which is a distinguishing feature of I_S. These results support the idea that the differential training procedure produces a differential decrease in I_S.

As the second test of that idea, we have begun to examine the voltage dependence of the current that is modulated differentially in these experiments by measuring that current during 40 mV as well as 20 mV depolarizing pulses before and after training and also after serotonin in each of the experiments shown in Figure 1. The average results from those experiments are illustrated in Figure 3. Figure 3A shows the Pre–Post difference current (corrected for changes in leakage) at the two levels of depolarization from a holding potential of −50 mV in paired cells, unpaired cells, and all cells following exposure to serotonin. There were significant effects of training ($F_{(2,22)} = 37.45$, $P < 0.001$), voltage step ($F_{(1,11)} = 19.62$, $P < 0.001$) and the training by voltage step interaction ($F_{(2,22)} = 27.88$, $P < 0.001$) in a repeated-measures ANOVA. Furthermore, the three curves have similar shapes, and the interaction became nonsignificant ($F = 0.00$, $P > 0.99$) when the curves were scaled to the same overall mean. These results indicate that the currents have similar voltage dependence in this range.

Figure 3B shows the current that is modulated associatively or differentially in these experiments, that is the current in the paired cell minus the current in the unpaired cell. Again, compared with

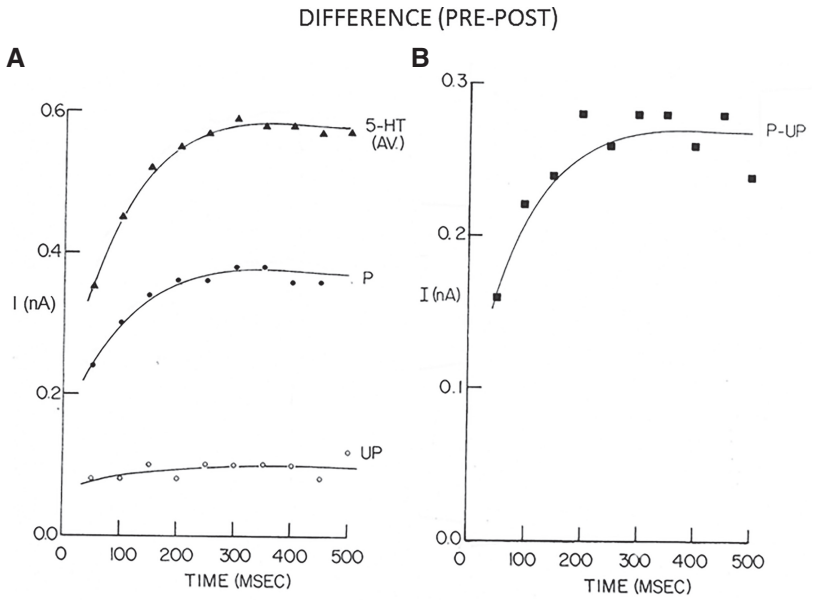

Figure 2. Time dependence of the current that is modulated associatively or differentially, compared with the current that is modulated nonassociatively by serotonin. (*A*) Graph of the Pre–Post difference current as a function of time during the 500 msec depolarizing pulse in the paired and unpaired SN, and then measured again in both neurons after adding 10^{-4} M serotonin to the bath. The response to serotonin would be expected to have similar properties in both neurons, which were, therefore, averaged to provide a better estimate. (*B*) Graph of the current that is modulated differentially (Paired–Unpaired) as a function of time. The shape of that curve is very similar to the curve for the current that is modulated by serotonin, indicating that those currents have similar time dependence in this range.

the current that is modulated nonassociatively by serotonin there were significant effects of training ($F_{(1,11)} = 33.38$, $P < 0.001$), voltage step ($F_{(1,11)} = 26.39$, $P < 0.001$) and the training by voltage step interaction ($F_{(1,11)} = 23.91$, $P < 0.001$). Furthermore, the two curves have similar shapes, and the interaction became nonsignificant ($F = 0.00$, $P > 0.99$) when they were scaled to the same overall mean. These results indicate that the voltage dependence of the current that is modulated associatively (P-UP) is very similar to that of the current that is modulated nonassociatively by serotonin. In particular, both currents are turned on in the range of −50 to −30 mV, meaning that they are turned on at the resting potential, and both have modest nonlinearity with increasing depolarization. There was a trend for the current that is modulated by serotonin (Pre-5HT) to be more occluded (Post-5HT) following paired than unpaired training. However, the two were not significantly different and would be expected to have the same voltage dependence, and therefore have been pooled. These results provide further support for the idea that an associative form of plasticity, activity-dependent presynaptic facilitation, involves modulation of the same ionic current as a nonassociative form, normal presynaptic facilitation.

Another question concerning the relationship between associative and nonassociative plasticity is what properties of the CS are necessary to produce associative plasticity? In these and previous experiments on activity-dependent facilitation the CS was a brief train of action potentials in the SN (Hawkins et al. 1983; Walters and Byrne 1983; Clark et al. 1994; Eliot et al. 1994; Schacher et al. 1997; Bao et al. 1998), designed to mimic what happens during behavioral conditioning (Antonov et al. 2001, 2003). We asked whether the same depolarizing voltage clamp pulse that we used to measure the current before and after training might also act as the CS during training. The protocol was identical to that shown in Figure 1B except that now, instead of a train of six action potentials in the SN on each training trial, there was a 500 msec, 20 or 40 mV depolarizing voltage-clamp current pulse in the paired SN starting 0.5 sec before pedal nerve shock, and a pulse in the unpaired SN 2.5 min later (Fig. 4A). In 12 experiments with this protocol, there was a significantly greater decrease in the net outward current in the paired than in the unpaired SN following training ($t_{(11)} = 3.46$, $P < 0.01$) (Fig. 4B). This differential decrease was significant with either a 20 mV ($P < 0.05$) or 40 mV ($P < 0.05$ one-tail) pulse as the CS, although there was a trend for a greater differential decrease with the 40 mV pulse (12.8 ± 4.7% vs. 21.3 ± 8.8%). These results replicate the results shown in Figure 1 and indicate that action potentials are not necessary, but rather a 20 or 40 mV depolarizing voltage clamp pulse can act as the CS during activity-dependent facilitation.

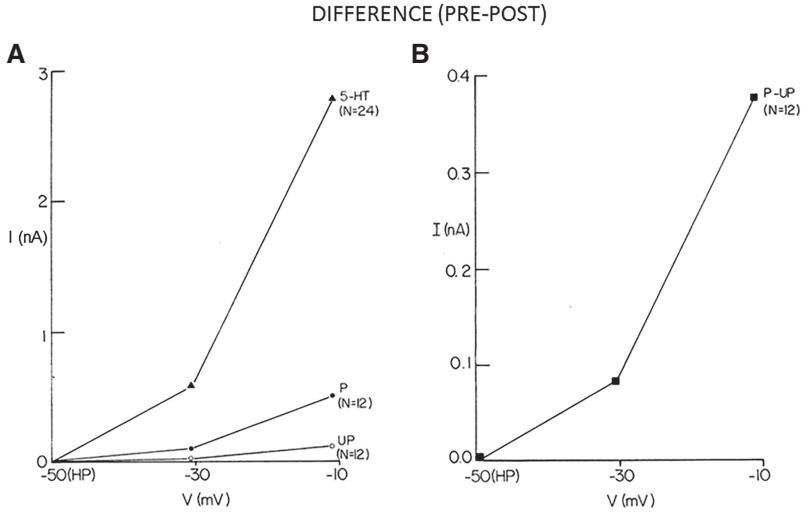

Figure 3. Voltage dependence of the current that is modulated associatively or differentially, compared with the current that is modulated nonassociatively by serotonin. (*A*) Graph of the Pre–Post difference current (corrected for leakage) during 20 and 40 mV depolarizing pulses in the paired and unpaired SN, and then measured again in all neurons after adding 10^{-4} M serotonin to the bath. (*B*) Graph of the current that is modulated differentially (Paired–Unpaired) as a function of voltage. The shape of that curve is very similar to the curve for the current that is modulated by serotonin, indicating that those currents have similar voltage dependence in this range.

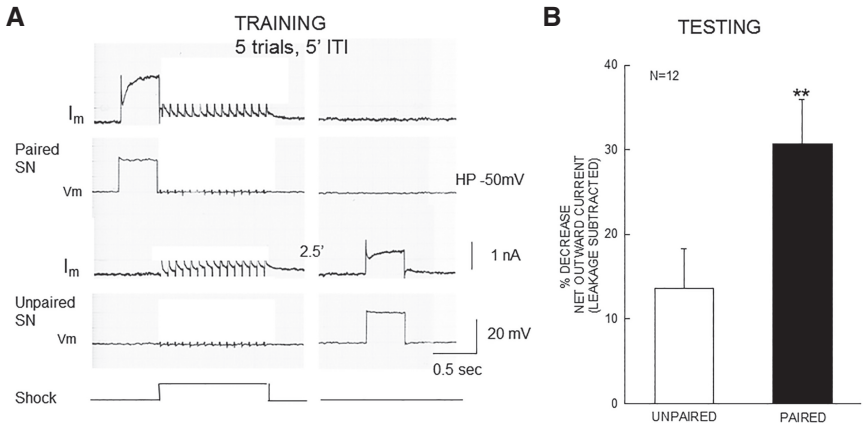

Figure 4. The depolarizing voltage clamp pulse can also act as the CS during training. (*A*) Example of a training trial with a 500 msec, 20 mV depolarizing voltage clamp pulse as the paired and unpaired CS instead of a brief train of action potentials. (*B*) Average testing results from 12 experiments like the one shown in *A*. There was a significantly greater decrease in the net outward current in the paired than the unpaired SN after training with a depolarizing voltage clamp pulse as the CS.

Discussion

Our results support the idea that associative and nonassociative plasticity share cellular and molecular mechanisms, and are therefore not fundamentally different. More specifically, the results support the hypothesis that activity-dependent presynaptic facilitation, an associative form of plasticity that contributes to classical conditioning in *Aplysia*, involves amplification of the mechanisms of normal presynaptic facilitation, a nonassociative form that contributes to sensitization, and that they both involve modulation of the same ionic current in the SNs. Some of the early studies of associative and nonassociative plasticity in *Aplysia* found that they both involve serotonin, cAMP, and PKA-dependent facilitation of EPSPs at the SN–motor neuron synapses and also broadening of action potentials and an increase in membrane resistance of the SNs, all of which are larger during associative plasticity (Hawkins et al. 1983; Walters and Byrne 1983; Clark et al. 1994; Eliot et al. 1994; Bao et al. 1998; Antonov et al. 2001, 2003). Subsequent studies of associative and nonassociative plasticity have found that they also both involve sensorin synthesis and secretion (Hu et al. 2007), neurotrophin signaling, RNA synthesis, and DNA methylation (Yang et al. 2018), and PKM Apl II in the SN (Hu et al. 2017a). However, other studies have found mechanisms that are unique to associative plasticity including Hebbian potentiation initiated by Ca^{2+} influx through postsynaptic NMDA receptor channels (Lin and Glanzman 1994a,b; Murphy and Glanzman 1996, 1997, 1999; Schacher et al. 1997; Bao et al. 1998; Antonov et al. 2003), which are modulated by NO and Ih (Antonov et al. 2007; Yang et al. 2015). Furthermore, these pre- and postsynaptic mechanisms interact through retrograde signaling (Bao et al. 1998; Antonov et al. 2003). In addition, associative and nonassociative plasticity involve different kinases in the SN (Hu et al. 2007) and different calpains and PKM isoforms in the SN and motor neuron (Hu et al. 2017a,b).

These results suggest that associative and nonassociative plasticity have both shared and unique mechanisms, raising questions about the relationship between the two types of plasticity. Furthermore, one of the mechanisms that is thought to be shared, spike broadening in the SNs, could actually involve modulation of different ionic currents during the different forms of plasticity and therefore be unique to one of them. We have investigated that possibility, and have found that activity-dependent facilitation appears to involve modulation of the same serotonin-sensitive current, I_S, as normal presynaptic facilitation. That result is consistent with the finding that serotonin can serve as the US for activity-dependent facilitation (Clark et al. 1994; Eliot et al. 1994; Schacher et al. 1997; Bao et al. 1998), and supports the idea that associative and nonassociative plasticity involve at least some shared mechanisms.

Classical conditioning requires that an US such as shock be temporally paired with and contingent on a CS during training, whereas sensitization does not involve a CS during training. What properties of a stimulus are necessary for it to be able to act as a CS for conditioning? All previous studies of activity-dependent facilitation have used a train of action potentials in the SN (Hawkins et al. 1983; Walters and Byrne 1983; Clark et al. 1994; Eliot et al. 1994; Schacher et al. 1997; Bao et al. 1998). We found that a 20 or 40 mV depolarizing voltage clamp pulse could substitute for the train of action potentials as the CS. If the 20 mV pulse caused relatively little transmitter release these results would imply that postsynaptic receptor activation may not be required for the presynaptic changes in I_S, although it is thought to contribute (Bao et al. 1998; Antonov et al. 2003). Similarly, Hebbian potentiation is traditionally said to require coincident pre- and postsynaptic action potentials, but postsynaptic depolarization can substitute for the postsynaptic action potentials (Wigström et al. 1986; Kelso et al. 1986).

During activity-dependent facilitation the CS is thought to allow an influx of Ca^{2+} that primes an adenylyl cyclase coupled to the serotonin receptor, so that it produces more cAMP if serotonin arrives in the next second or so (Kandel et al. 1983; Ocorr et al. 1985; Abrams et al. 1991, 1998; Yovell et al. 1992). What voltage-dependent Ca^{2+} current might be involved? Two voltage-dependent Ca^{2+} currents were initially characterized in *Aplysia*: a rapidly inactivating, dihydropyridine-insensitive, high voltage activated current and a slowly inactivating, dihydropyridine-sensitive, L-type current (Edmonds et al. 1990). The dihydropyridine-insensitive current is activated by action potentials and is important for normal synaptic transmission and several types of plasticity including presynaptic inhibition, homosynaptic depression, and normal presynaptic facilitation (Edmonds et al. 1990; Eliot et al. 1993). However, that current would be poorly activated by the depolarizing voltage clamp pulse that we found can also act as the CS. In contrast, the dihydropyridine-sensitive current is poorly activated during action potentials and is not important for normal synaptic transmission or for several types of plasticity including activity-dependent facilitation with a train of action potentials as the CS (Edmonds et al. 1990; Eliot et al. 1993, 1994). However, that current is activated by a 20 mV depolarizing pulse from a holding potential of −50 mV (Edmonds et al. 1990) and it or some other low voltage activated current (Lacinová 2005) could allow Ca^{2+} influx when the voltage clamp pulse is the CS (Fig. 4). Thus, different Ca^{2+} currents may be activated when the CS is a train of action potentials or a depolarizing pulse, but in both cases, the resultant Ca^{2+} influx could act to prime the adenylyl cyclase during activity-dependent facilitation.

Materials and Methods

The methods were similar to those of previous experiments on activity-dependent presynaptic facilitation (Hawkins et al. 1983).

Hermaphroditic *Aplysia californica* weighing 100–300 g (supplied by Pacific Biomarine) were anesthetized with isotonic $MgCl_2$, and the central nervous system was dissected out (with or without the tail) and pinned to the Sylgard (Dow Chemical) floor of a dish filled with circulating artificial seawater (ASW, Instant Ocean) at room temperature. The abdominal ganglion was partially desheathed, and two LE siphon SNs were impaled with single-barreled glass microelectrodes filled with 2.5 M KCl (~10 MΩ, shielded with parafilm and aluminum foil) connected to two Dagan 8100 single electrode voltage clamps run on the same clock. The switching frequency was 3 kHz, and the capacity compensation, phase, and gain were adjusted at near maximum to produce the best square wave as seen at the output of the headstage without ringing (Halliwell and Adams 1982). The efficacy of the voltage clamp was confirmed by impaling the same neuron with two microelectrodes, one under voltage clamp and one under the current clamp, and checking that the voltage and current readings of the two electrodes agreed.

During training, there were five trials with a 5 min intertrial interval, during which the amplifier was switched to current-clamp mode. On each trial, a train of six action potentials at 10 Hz (the conditioned stimulus or CS) was produced in each SN by brief depolarizing pulses. The CS in one neuron (the paired neuron) started 0.5 sec before a 1.5 sec train of 20 Hz (Figs. 1–3) or 10 Hz (Fig. 4) shocks to the posterior pedal nerves (the unconditioned stimulus or US), whereas the CS in the other neuron (the unpaired neuron) started 2.5 min after the US. Each neuron was tested under voltage clamp ~5 min before and 5 min after training. Then 10^{-4} M serotonin (5HT) was added to the bath and the neurons were tested again 5 min later. During testing the current (leakage corrected) in each neuron was measured during 500 msec voltage pulses of −20, 20, and 40 mV from a holding potential of −50 mV, first in ascending and then in descending order, and the two measurements for each voltage pulse were averaged. The −20 mV pulse was used to estimate leakage current. The two neurons were tested once every 20 sec I sec apart, counter balanced for the order. In some experiments (Fig. 4) the amplifier was kept in voltage-clamp mode during training and the CS was a 500 msec voltage pulse of either 20 or 40 mV (in interdigitated experiments) from a holding potential of −50 mV. In those experiments, there was no 40 mV pulse during testing and no 5HT.

Experiments comparing paired and unpaired SNs in the same preparation (Figs. 1, 4) were analyzed with paired *t*-tests using Statistica software. Experiments with paired, unpaired, and serotonin training and two levels of voltage step during testing had a full factorial within subjects design, and were analyzed with two-way repeated measures ANOVAs with training (either P, UP, and the average 5HT, Fig. 3A or P-UP and the average 5HT, Fig. 3B) and voltage step (20 and 40 mV) as the factors. The ANOVAs were then repeated after scaling the training groups to the same overall mean to test for differences in the shapes of the curves (the training × voltage step interaction).

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