Long-Term Sensitization in *Aplysia*: Biophysical Correlates in Tail Sensory Neurons

KENNETH P. SCHOLZ AND JOHN H. BYRNE

A fundamental problem in the cellular analysis of learning and memory is the identification of the neuronal substrates of long-term information storage and their relation to short-term cellular alterations. In this report, biophysical correlates of long-term sensitization of a simple withdrawal reflex in the mollusc *Aplysia* were examined. A voltage-clamp analysis of the sensory neurons that control the reflex, 24 hours after sensitization training, revealed a significant reduction in net outward current. The results indicate that one mechanism for the storage of long-term sensitization is the regulation of membrane currents that influence the characteristics of the action potential and the excitability of individual neurons. The results also provide insights into the relation between short- and long-term sensitization in that the biophysical loci involved in the storage of long-term sensitization appear similar to those involved in short-term sensitization.

THE MARINE MOLLUSC *Aplysia* HAS proved to be a useful animal model for the study of cellular and molecular mechanisms underlying short- and long-term behavioral modifications. For example, the mechanisms for short-term sensitization of the defensive withdrawal reflex of the tail (*1*) and of the gill and siphon (*2*) have been examined extensively. Sensitizing stimuli trigger the release of neuromodulatory substances that act on sensory neurons to stimulate the production of adenosine 3′,5′-monophosphate (cAMP). At least one function of increased cAMP is to promote the closure of specific K^+ channels, thereby enhancing the excitability of the sensory neurons and reducing the amount of outward current contributing to repolarization of the action potential. Consequently, there is an increased probability that a single brief stimulus will elicit multiple spikes and that individual spikes will have a greater duration. These modifications lead to increased excitation of the follower neurons (motor neurons and interneurons) and to more intense and prolonged reflex withdrawal.

Long-term sensitization of reflex withdrawal has also been demonstrated and is associated with enhancement of the monosynaptic excitatory postsynaptic potential (EPSP) from sensory neurons to motor neurons (*3*) [see also (*14*)]. Little is known, however, about the biophysical mechanisms underlying the long-term sensitization. We have begun to address this question by examining membrane currents in tail sensory neurons 1 day after sensitization training. We confirmed and extended earlier studies and found that, as with short-term sensitization, the sensory neurons constitute a cellular locus for long-term sensitization. Furthermore, our results demonstrate that the membrane currents of the sensory neurons are subject to long-term regulation, thus providing a cellular basis for long-term information storage.

For behavioral testing, *Aplysia* (*4*) were placed in a 10-liter chamber filled with artificial seawater (ASW) that was aerated continuously. Before sensitization training was begun, both sides of each animal were tested by the delivery of a-c shock (10 mA) for 500 msec through a hand-held electrode applied to a site on the posterior part of the body wall. Each side was tested between two and five times (*5*). The test stimuli produced a coordinated set of defensive responses, including reflex withdrawal of the tail and siphon. The siphon is a midbody structure that is withdrawn when stimuli are applied to either side of the animal. We used the siphon component of this reflex as a measure of the sensitivity of the animal to the test stimuli, and changes in the siphon response relative to the pretest responses as a measure of sensitization (*6*).

Immediately after the initial testing, one randomly chosen side of the animal was subjected to sensitization training (*7*). The other side of the animal served as the untrained control. Twenty-four hours after sensitization training, the animal was again tested as described above. All behavioral and electrophysiological experiments were done by separate individuals who did not know which side of the animal received the sensitization training.

For each animal, the averages of the test scores before and after training were calculated, and the percent change in the average test score after training relative to the average test score before training was determined for each side. Thus there were two separate scores for each animal. Seventeen animals were used in the behavioral study. The mean duration of siphon withdrawal in response to tail stimulation was increased 24 hours after sensitization training. For the control side the posttest scores were 93 ± 12% (mean ± SEM) of the pretest, while for the sensitized side the posttest scores were 169 ± 30% of the pretest. This effect was statistically significant ($t_{16} = 2.41$; $P < 0.025$) (*8*). There was no significant difference in the pretest scores for the control (23.4 ± 5.0 seconds) and sensitized (16.0 ± 2.1 seconds) sides ($t_{16} = 1.52$; t test for paired values). This lateralized effect allowed us to examine biophysical correlates of long-term sensitization, with each animal as its own control. Specifically, we compared the membrane properties of neurons mediating the response induced by test stimuli to one side, with those neurons mediating the response to test stimuli delivered to the other side.

Long-term biophysical correlates of the sensitization training were examined in the first central relay of the response pathway—the sensory neurons in the pleural ganglion, which innervate the tail and body wall (*9*). There are two symmetric clusters of sensory neurons located in the left and right pleural

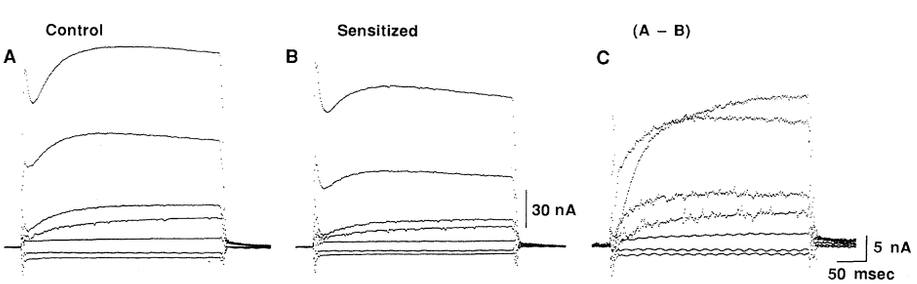

Fig. 1. Membrane currents in response to a series of different voltage pulses were recorded and digitized. Thus, for each cell, a response family was generated. The response families of the sensory neurons in each cluster were then averaged by computer. The overall average of control (**A**) and sensitized (**B**) sides was then obtained by averaging the characteristic response families of the respective clusters. The data in (**C**) were obtained by subtracting the response family of the sensitized side of each animal from its corresponding control family. The results for all of the animals were then averaged. Thus, the family of traces in (C) represent the net outward currents that are reduced 24 hours after sensitization training.

Department of Physiology and Cell Biology, University of Texas Medical School, Houston, TX 77225.

ganglia, each innervating one side of the animal (*10*). The separated left and right pleural-pedal ganglia were removed and pinned to the floor of a 10-ml chamber and bathed in ASW. The connective tissue sheath covering the pleural ganglia was removed surgically to expose the sensory neuron clusters.

Sensory neurons in each cluster were impaled with two microelectrodes that were filled with 3*M* potassium acetate. In all experiments cells were voltage-clamped at a holding potential of −50 mV, and the current-voltage (*I-V*) relation was examined (*11*). Input conductance was measured with an initial 15-mV hyperpolarizing pulse. Only cells with resting potentials more negative than −35 mV and input resistances greater than 7 megohms were used in the analysis. An average of 4.4 cells were successfully clamped in each cluster (*12*).

In order to examine possible correlates of the sensitization training, we averaged the families of current response curves from all of the cells in each cluster (the response family for each cell contains current responses to seven pulse potentials). This yielded two characteristic response families from each animal—one for the control side and one for the sensitized side. We obtained an overall average for all of the experiments by averaging the response families on the control side of all the animals (Fig. 1A) and also on the sensitized side of all the animals (Fig. 1B). Figure 1C illustrates the net outward currents that are reduced as a consequence of sensitization training. It is evident that, in the sensory neurons innervating the sensitized side, there is a considerable reduction in net outward current for most of the duration of the 300-msec depolarizing pulse 1 day after sensitization training.

The *I-V* relation of sensory neurons innervating the control side and the side receiving the sensitization training were averaged (Fig. 2A). For a range of potentials (−35 to +25 mV), the net outward current at the end of the pulse in sensory neurons innervating the sensitized side was significantly less (*8*) than that of the cells innervating the control side. Subtraction of the *I-V* relation of the sensitized group from the *I-V* relation of the control group (Fig. 2B) indicates that the changes in conductance are relatively voltage-independent up to potentials that approach +10 mV. In this depolarized region voltage dependence can be seen. The cells from the sensitized side seemed to display a reduction of input conductance (as measured by stepping to more negative potentials from a holding potential of −50 mV), but this effect was not significant. Similarly, the resting membrane potentials of sensitized cells (−40.4 ± 4.0 mV) were more positive than those of control cells (−41.5 ± 5.1 mV), but this was also not significant (*8*).

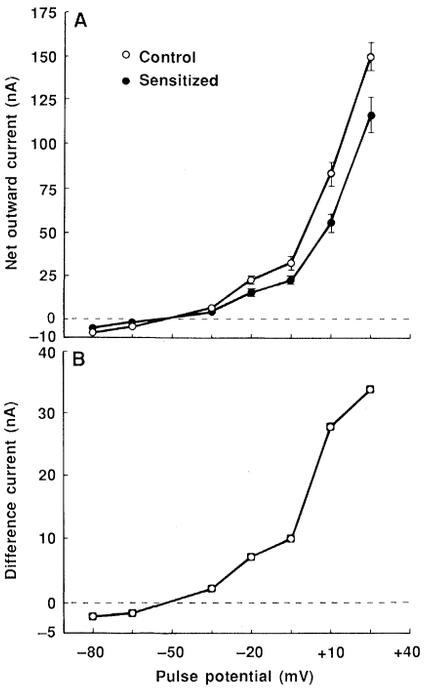

Fig. 2. Current-voltage (*I-V*) relations of response families illustrated in Fig. 1. The values plotted are mean currents at the end of 300-msec voltage pulses. (**A**) *I-V* relations for control (○) and sensitized (●) cells. The *I-V* relation of the sensitized cells was significantly reduced from that of the control cells at potentials from −35 to +25 mV; for example, at −35 mV $t_{10} = 2.11$, $P < 0.05$. (**B**) *I-V* relation of net values for response families from Fig. 1C. Error bars in (A) represent means ± SEM.

The functional consequences of this reduction in net outward current are clear. First, the reduction would lead to a larger or broader spike and enhanced transmitter release. Second, reduction of net outward currents would increase the excitability of the sensory neurons and thus enhance the probability of initiating subsequent action potentials after a single stimulus [see (*13*)].

The results indicate that long-term sensitization is correlated with a considerable reduction in net outward current. The precise nature of this current or currents, however, will require further study. The kinetics and voltage sensitivity of the *I-V* relation (Figs. 1C and 2B) are similar to those of the previously described *S*-current, which is modulated during short-term sensitization (*1, 2*). Thus, one of the membrane channels modified by phosphorylation in these same sensory neurons during short-term sensitization might be regulated in a persistent manner to contribute to the storage and readout of long-term sensitization. This regulation could take the form of persistent covalent modification or alterations in protein synthesis. Indeed, recent results from siphon sensory neurons in tissue culture indicate that protein synthesis is required for long-term enhancement of the monosynaptic EPSP (*14*).

It would be premature to say that the modulation of membrane currents in the tail sensory neurons fully accounts for the enhancement of the reflex withdrawal. Numerous interneurons and motor neurons participate in siphon withdrawal mediated by tail stimulation (*15*). Given that the reflex has many polysynaptic components, it is possible that the properties of some interneurons are altered as well. In addition, it is unlikely that modulation of membrane currents is the sole mechanism of long-term storage of information within the sensory neurons. Indeed, morphological changes in the terminals of the sensory neurons that mediate the gill and siphon withdrawal reflex have been correlated with long-term sensitization (*16*). Thus, it seems likely that long-term behavioral modifications are produced by a coordinated set of cellular modifications that act in concert to provide storage of information over a long period of time.

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4. *Aplysia californica* (150 to 300 g) were purchased from Marine Specimens (Pacific Palisades, California) and Marinus Biomarine (Westchester, California) and maintained in isolated containers in holding tanks containing artificial seawater (Instant Ocean) at 15°C. The behavioral and electrophysiological experiments were done at room temperature (20° to 22°C).
5. Test stimuli were applied at 5-minute intervals to alternating sides of the animal. If inking occurred during the pretest phase (an indication of prior sensitization), the animal was not used for further testing or training. This occurred with one animal.
6. Siphon withdrawal was quantified by measuring the time between the initiation of withdrawal and the reversal of motion of the siphon (*3*).
7. Training consisted of four separate trains of stimuli, each consisting of ten (500-msec duration) 60-mA shocks applied diffusely to the tail and body wall of one randomly chosen side of the animal. Such stimuli reliably produced inking and opaline secretion. Trains were separated by 30 minutes. No

externally visible signs of the sensitization training were apparent 24 hours after training.
8. Statistical significance was assessed by a t test for paired values. One-tailed analysis was used in statistical tests based on pilot studies on five animals.
9. Each of the first 11 animals studied behaviorally were tested electrophysiologically; no selection procedures were used. After the last posttest, each animal was anesthetized by injection of isotonic $MgCl_2$ in an amount equal to approximately one-half the animal's volume, and the ganglia were removed.
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11. *I-V* relations were determined by delivering 300-msec pulses to potentials between −80 mV and +25 mV in increments of 15 mV. Pulses were separated by 40 seconds.
12. No fewer than two and no more than six cells were successfully clamped in any single cluster. A total of 46 cells from the control sides and 51 cells from sensitized sides were successfully clamped.
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Diadema antillarum Was Not a Keystone Predator in Cryptic Reef Environments

JEREMY B. C. JACKSON AND KARL W. KAUFMANN

The ecological impact of the disappearance of a major predator depends on the responsiveness of the prey. Mass mortality of the most abundant grazer in Caribbean cryptic reef environments, the sea urchin *Diadema antillarum*, selectively decreased rates of mortality of encrusting organisms by half, yet community composition hardly changed because alternative species failed to become established.

KEYSTONE PREDATORS CAN RADICALLY alter the composition of ecological communities by consuming potentially dominant competitors (*1*). Before 1983, the sea urchin *Diadema antillarum* was the most abundant large grazing invertebrate on most Caribbean coral reefs (*2, 3*). Because of its enormous densities (*4*) and voracious appetite (*5*), *Diadema* has been considered a keystone predator (*3, 4, 6*), a view supported by severalfold increases in algal abundance on many reefs after mass mortality of *Diadema* in 1983 (*7, 8*). *Diadema* was also an important grazer on encrusting organisms in cryptic reef environments such as crevices in the reef framework and under corals (*9*). Thus one would predict that *Diadema*'s disappearance would result in large changes in the composition of the encrusting community, but it did not.

Population dynamics of encrusting organisms and *Diadema* were studied for 27 months at Rio Bueno, Jamaica. Undersurfaces of 38 foliaceous corals situated along 200 m of reef between 8 to 14 m in depth were photographed at approximately weekly intervals (*10*). We determined abundances by projecting photographs onto a digitizer tablet overlain by randomly placed points and then by recording what organisms coincided with the points at each census (*11*). Causes of changes in abundance were determined by observing the fates of organisms at randomly chosen points during five different 6-week periods (*12*). Overgrowth was readily observed in sequences of photographs as one organism advanced over another, and predation by large grazers was noted by their characteristic feeding scars. Together these two processes caused 82% of all changes observed.

More than 98% of the *Diadema* died between 26 July and 4 August 1983, and the population did not recover for the duration of the study (*7, 13*). Before the sea urchins died, predators (*14*) cleared 6.9% of the total surface in 6 weeks, whereas afterward this amount dropped as low as 1.1% and was still only 3.3% after 27 months (Fig. 1A; $\chi^2 = 372$, $P < 0.0001$, 4 df). In contrast, space overgrown by encrusting organisms stayed around 5% ($\chi^2 = 4.89$, $P > 0.10$, 4 df), so that the relative importance of mortality due to predation and competition was reversed.

Surprisingly, mean abundances of major encrusting taxa changed little during the 27 months after the disappearance of *Diadema* (Fig. 1B). However, abundances under individual corals varied considerably during the same period (Fig. 2), demonstrating that the overall stability of the encrusting community is not due to slow growth or lack of disturbance by other organisms. In contrast, animals with erect growth increased more than threefold but were never abundant.

Why was the composition of the cryptic community so stable in view of such a marked ecological change? One possibility is that larval recruitment of species able to dominate space under corals was extremely slow (*15*), so that new or previously uncommon species under a given coral may have simply failed to appear. To test this, we searched the photographs for larval recruits of sponges, which are the best overgrowth competitors under corals (*9, 16*), during two different 6-week intervals after the *Diadema* died (*17*). None appeared. Moreover, only seven bryozoan colonies settled, and there was no obvious increase in recruitment of any other groups under corals, including erect animals, which appear to have increased entirely by clonal propagation. Throughout this period, however, substrata suitable for larval settlement, such as crustose algae, were abundant. In contrast, erect animals recruited heavily after removal of another diadematoid sea urchin from a California rock reef (*18*).

Another possible explanation for the stability of the cryptic community may be associated with the diet of *Diadema* under corals. Before the urchins died, 57% of the predation was upon crustose algae and another 38% upon the bryozoan *Steginoporella* sp. Crustose algae are among the poorest overgrowth competitors under corals (*9*), so that their reprieve from grazing would not be expected to result in much community change. In contrast, *Steginoporella* sp. is among the best overgrowth competitors (*15*), but this ability is restricted to the growing margins of colonies, whereas older regions of the same colonies become senescent and are easily overgrown (*19*). For this reason we examined the condition of grazed versus ungrazed *Steginoporella*. Zooids were classified as young, middle-aged, and old, and their fates were determined after 6 weeks in June and July 1983 (*20*). The oldest zooids constituted 20% of the colonies, yet they sustained 75% of the predation. Thus *Diadema* fed almost exclusively and preferentially (*21*) on organisms or parts of organisms that were not actively affecting the abundance of other organisms.

Keystone predators, by definition (*1*), feed on potentially dominant competitors for space. *Diadema* behaves this way on upper reef surfaces by feeding preferentially on algae that might otherwise overgrow and

J. B. C. Jackson, Smithsonian Tropical Research Institute, Box 2072, Balboa, Republic of Panama.
K. W. Kaufmann, Department of Invertebrates, American Museum of Natural History, New York, NY 10024.