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Additional component in the cellular mechanism of presynaptic facilitation contributes to behavioral dishabituation in *Aplysia*

(sensitization/homosynaptic depression/serotonin/cyclic AMP)

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ABSTRACT **Sensitization of defensive gill and siphon withdrawal reflexes in *Aplysia* results, in part, from presynaptic facilitation of transmitter release from mechanoreceptor sensory neurons that innervate the siphon skin and synapse with interneurons and motor neurons. Presynaptic facilitation also can be elicited by application of serotonin. This facilitation is associated with two phenomena, a prolongation of the presynaptic action potential resulting from a decrease in a specific K^+ current and an enhancement of the Ca^{2+} transients elicited by depolarization. Previous work has shown that prolongation of the action potential enhances synaptic transmission at normal levels of release. Here we report that an additional set of processes also contributes to facilitation. When repeated activation of the sensory neurons induces profound homosynaptic depression, prolonging the duration of action potentials (or of depolarizing commands under voltage clamp) has little effect on transmitter release. Nonetheless, serotonin is still capable of enhancing release. Since homosynaptic depression underlies the behavioral process of habituation, the second set of processes, by counteracting the consequences of the depression, seems to mediate the effects of dishabituation in the sensory neuron. Prolongation of the action potential by closure of the K^+ channel seems to mediate the effects of sensitization.**

Presynaptic facilitation of transmitter release from the terminals of the sensory neurons on their central target cells contributes importantly to sensitization of the gill and siphon withdrawal reflex in *Aplysia*. Presynaptic facilitation is associated with at least two changes at the cellular level: a decrease in the S current, a specific serotonin (5-HT)-sensitive K^+ current (1–5), and an increase in Ca^{2+} transients measured with the indicator Arsenazo III (6). In the two preceding papers of this series (7, 8), we reported that depression of K^+ current by 5-HT and other facilitating transmitters causes (*i*) an increase in the excitability of the sensory neurons, causing them to fire more action potentials (7), and (*ii*) a prolongation of each action potential, increasing Ca^{2+} influx and enhancing transmitter release (8, 9).

In this paper, we have attempted to determine the limits of the contribution to presynaptic facilitation made by modulation of the K^+ current, so as to ascertain what role, if any, the alteration in Ca^{2+} transients and other processes might play. We find that as synaptic transmission becomes depressed with repeated stimulation, changes in duration of the action potential have a progressively weaker influence on transmitter release. Under these circumstances, 5-HT produces facilitation primarily by recruiting a second set of mechanisms that do not involve alterations in spike shape. This component counteracts the synaptic depression and increases transmitter release in some other way, perhaps by increasing the availability of releasable transmitter. Once synaptic strength is restored, the sensitivity of transmitter release to spike duration is also restored.

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METHODS

For experiments on intact ganglia, we used abdominal ganglia dissected from *Aplysia californica* (see ref. 8). Facilitating nerve stimulation was done with suction electrodes using 2-msec shocks at 0.17 Hz for 5–20 sec. Cultured sensory neurons and follower cells were prepared by the method of Schacher and Proshansky (10). (For other details of electrophysiological and culture methods, see refs. 8 and 10.) Arsenazo III experiments were carried out by the methods described by Boyle *et al.* (6). Cyclic AMP was determined by the method of Abrams *et al.* (11). Ganglia were first desheathed and bathed in either control or K^+-channel-blocking solution (see Fig. 1 legend) for 0.5 hr before incubation with 100 μM serotonin for 3 min. Basal levels of cAMP were measured in ganglia that had been bathed in the control or K^+-channel-blocking solution without addition of 5-HT. 5-HT–creatinine sulfate complex, 4-aminopyridine (4-AP), and 3,4-diaminopyridine (3,4-DAP) were from Sigma, and tetraethylammonium chloride (Et_4NCl) was from Kodak. Et_4NCl was purified before use (12).

RESULTS

K^+-Channel Blockers That Reduce Modulation of K^+ Current also Reduce Facilitation by 5-HT. To examine further the contribution to presynaptic facilitation of processes other than S-channel modulation and spike broadening, we first attempted to eliminate the contribution of S-channel closure by pharmacological means. In earlier experiments, Klein and Kandel (2) found that they could block the 5-HT-induced change in outward current in two ways: (*i*) by using a combination of K^+-channel blockers in high concentrations and (*ii*) by substituting Cs^+ for intracellular K^+. We utilized both methods to eliminate the S-current response to 5-HT while examining transmitter release from the sensory neurons to determine whether a residual component of facilitation remained.

We first examined membrane currents and transmitter release from sensory neurons in a medium containing 10 mM 4-AP in which 410 mM Et_4NCl was substituted for an equimolar amount of NaCl. The control solution contained 410 mM Tris and 10 mM Et_4NCl (the Et_4NCl was added to maintain action potential generation in the low-Na^+ medi-

Abbreviations: (E)PSP, (excitatory) postsynaptic potential; 5-HT, serotonin; Et_4NCl, tetraethylammonium chloride; 4-AP, 4-aminopyridine; 3,4-DAP, 3,4-diaminopyridine.

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um). We voltage-clamped the cell body and stepped from −40 mV to +10 mV for 50 msec. The K^+-channel blockers reduced the outward current at the end of the step from 15.46 nA ± 1.06 (mean ± SEM, $n = 16$) to 4.25 nA ± 0.82 ($n = 25$). Confirming Klein and Kandel (2), we found that 5-HT produced much smaller changes in membrane current in the presence of the K^+-channel blockers [2.99 nA ± 0.43 ($n = 25$) in the presence of the blockers as opposed to 7.63 nA ± 0.55 ($n = 16$) in control solution]. Not only was modulation of the outward current reduced by these K^+-channel-blocking agents, but the peak synaptic facilitation produced by 5-HT was also dramatically reduced to 8% of control (13% ± 7 compared to 155% ± 35; Fig. 1 *A* and *B*).

In a second series of experiments, we blocked K^+ currents by iontophoretically injecting Cs^+ ions into sensory neurons. In these experiments also, the reduction of outward current produced by 5-HT was greatly depressed [to 1.14 nA ± 0.47 compared to a control of 5.6 nA ± 1.24 ($n = 6$)], and the facilitation was totally blocked ($n = 7$).

Since the effect of 5-HT on outward current and transmitter release is mediated by a rise in intracellular cAMP, we examined the possibility that the K^+-channel blockers might interfere with the action of 5-HT in inducing a rise in cAMP. We found this not to be the case; 5-HT doubled the level of cAMP in both sensory neurons bathed in K^+-channel blockers and those bathed in control solution (Fig. 1*C*).

K^+-Channel Blockers also Reduce Modulation of Ca^{2+} Transients by 5-HT. The finding of Boyle *et al.* (6) raised the possibility that altered handling of Ca^{2+} might play a role in facilitation. Therefore, we examined the effect of the K^+-channel blockers on the 5-HT-induced increase in Ca^{2+} transients. In contrast to its lack of effect on cAMP synthesis, we found (Fig. 1*D*) that the K^+-channel-blocking solution reduced the 5-HT-induced increase in Ca^{2+} transients measured with Arsenazo III from 61.25% ± 22.39 ($n = 4$) to 21.57% ± 6.30 ($n = 13$; $P < 0.05$). In another set of experiments, in which we injected Cs^+, we found that Cs^+ completely blocked the change in Arsenazo III signals produced by 5-HT ($n = 6$). Since the experiments of Boyle *et al.* (6) showed that 5-HT does not act on the Ca^{2+} transients by improving voltage-clamp control through a reduction of K^+ conductance, it is not clear why K^+-channel blockers should affect modulation of the Arsenazo signal. But irrespective of their mechanism of action, the fact that both of the treatments that we used to block facilitation blocked not only K^+-current modulation by 5-HT but also the modulation of the Ca^{2+} transients suggests that both of these processes might contribute to facilitation. It was therefore necessary to use other means to try to dissociate facilitation from spike broadening.

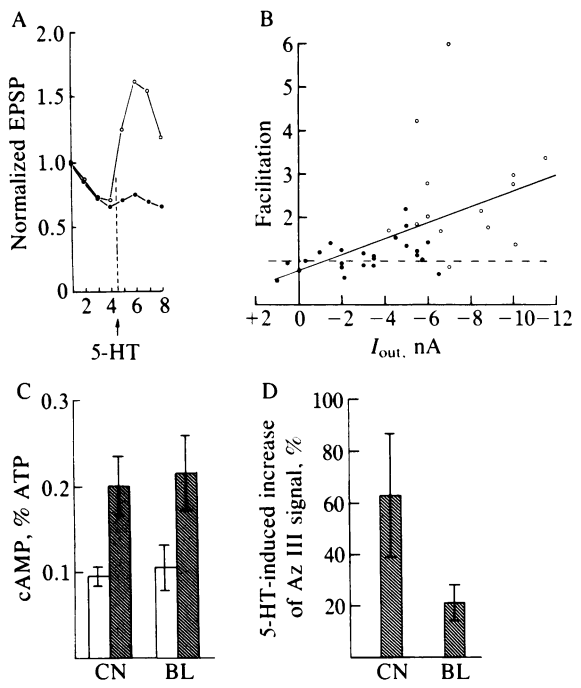

FIG. 1. K^+-channel blockers reduce responses to 5-HT without affecting cAMP. (*A*) Comparison of facilitation of EPSPs by 100 μM 5-HT in the presence of K^+-channel blockers (●; $n = 19$) and in control medium (○; $n = 17$). (*B*) Magnitude of the facilitation as a function of the reduction in outward current (I_{out}) produced by 5-HT in the presence (●) and absence (○) of K^+ blockers. The correlation coefficient is 0.529 ($n = 38$; $P < 0.01$). (*C*) Increase in cAMP caused by 5-HT in sensory neuron clusters is not affected by K^+-blocking solution. For controls (bars CN), $n = 5$ for basal levels (□) and $n = 6$ for 5-HT application (▨); for K^+-blocking solution (bars BL), $n = 6$ for basal (□) and $n = 5$ for 5-HT application (▨). (*D*) 5-HT induced an increase in the Ca^{2+} transients measured with Arsenazo (Az) III in control solution (410 mM Tris/10 mM Et_4N^+) (bar CN) but caused much less of an increase in blocking solution (410 mM Et_4N^+/10 mM 4-AP) (bar BL).

Voltage Clamp of Sensory Neurons in Cell Culture Reveals That When Transmitter Release Is Depressed, Spike Broadening Becomes Less Effective in Enhancing Transmitter Release. In the preceding study (8), we found that when sensory neurons are plated very close to follower cells in dissociated cell culture, it is possible to control transmitter release from the terminals by voltage-clamping the cell body. Under these circumstances, prolonging the presynaptic depolarizing step increases transmitter release in a steep but graded manner at durations below about 5 msec. Since the normal action potential in the sensory neuron falls in this range, the broadening of the action potential that accompanies facilitation can contribute significantly to the enhancement of release. In further support of the importance of spike broadening in facilitation, we found that when 5-HT was applied with the cell voltage clamped, so that the duration of the depolarization was kept constant, much less facilitation occurred than when a presynaptic spike was permitted to broaden. Block of facilitation by voltage clamping was more effective at short durations (<5 msec) than at longer durations, suggesting that a process other than broadening might play a role in facilitation with steps of long duration that demand the continued availability of releasable transmitter.

To define this process better, we returned to voltage-clamping and attempted to determine the circumstances under which facilitation could be dissociated from spike broadening. In the course of these experiments we found that when transmitter release became significantly depressed, as occurs with repeated stimulation that accompanies habituation, spike broadening became progressively less effective in enhancing release (Fig. 2). Fig. 2*A* shows the steep relation between step duration and the normal (nondepressed) level of transmitter release at the beginning of an experiment. Repeated stimulation caused the input–output curve to flatten (Fig. 2*B*); the dependence on transmitter release was still steep, but the rate-of-rise of the synaptic potential and the maximal level of release was reduced. Finally, when the synaptic potential was further reduced (Fig. 2*C*), changes in duration caused only small increases in release. Nonetheless, 5-HT (10 μM) could still produce large facilitation (Fig. 2*D*). These changes in the input–output curves are unlikely to be a result of a decreasing voltage-clamp control during depression and its restoration by 5-HT. Outward current normally inactivates during repeated stimulation, which would improve control rather than decrease it. In addition, K^+-channel blockers do not mimic the effect of 5-HT in counteracting the depression. Therefore, the facilitation induced by 5-HT under these circumstances must involve a set of

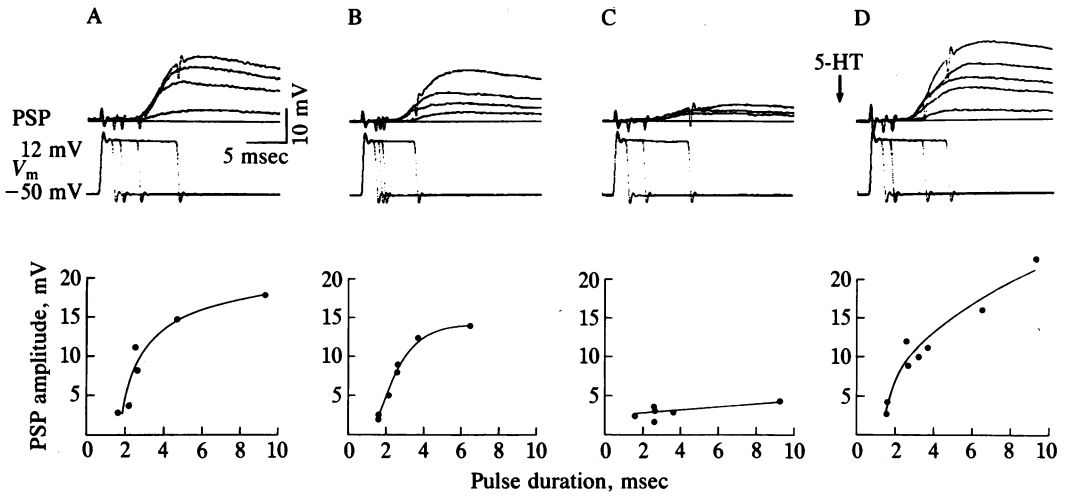

FIG. 2. Enhancement of EPSPs by 5-HT in a depressed synapse under voltage clamp. A sensory neuron and follower were grown in dissociated cell culture, the sensory neuron was voltage-clamped and stepped to a depolarized potential for different durations, and the EPSP was recorded. (*A*) Initial dependence of EPSP amplitude on duration is seen in sample records (*Upper*) and input-output curve (*Lower*). (*B*) Repeated activation of the synapse caused the EPSP to decline, making the input–output curve less steep and its maximum lower. (*C*) With further depression, a point was reached where increasing the duration of the voltage-clamp step had little effect on the amplitude of the EPSP. (*D*) Application of 5-HT to this depressed synapse still caused a large facilitation.

processes independent of the spike broadening produced by modulation of the S current (see also ref. 13).

Once this second process restored the transmission capability of the synapse, the duration of the depolarizing command again became a powerful agent in controlling release (Fig. 2*D*). Moreover, after exposure to 5-HT, increases in duration had a greater effect at longer durations (>10 msec) than in the nondepressed synapse, as if availability of transmitter was less limiting than it was in the absence of 5-HT. In their model of this synapse, Gingrich and Byrne (9) have also suggested that, in the range of action-potential duration where broadening is no longer effective (>10–15 msec), 5-HT must produce facilitation by some additional mechanism. A possible morphological basis for these results is suggested by the studies on short-term habituation by Bailey and Chen (14). They found that habituation leads to depletion of synaptic vesicles from the active zone and a failure to mobilize vesicles from the storage pool to the active zone. The second process we have encountered here seems to overcome depletion, perhaps by mobilizing transmitter vesicles into the active zone. Consistent with this idea is the finding that the second process did not increase the synaptic potential primarily by increasing the time-to-peak, as occurs with spike broadening, but rather acted mainly by increasing the rate-of-rise (Fig. 2*D*).

The Second Component of Presynaptic Facilitation Is Present in the Intact Ganglion As Well As in Cell Culture. To determine whether this component is also present in the intact ganglion, we carried out experiments in the ganglion using low concentrations of K^+-channel-blocking agents to broaden the action potential (Fig. 3). Increased release was accompanied by a characteristic increase in the time-to-peak of the excitatory postsynaptic potential (EPSP). Procedures that alter release without changing spike duration do not produce changes in time-to-peak and affect only the rate-of-rise of the EPSP (8). Fig. 3 compares the response to various procedures that broaden the action potential in synapses that have been depressed to about 20% of control. The figure shows both histograms (*A*, *B*, and *C*) and averaged records (*A'*, *B'*, and *C'*), illustrating EPSP amplitude, its rate-of-rise, and its time-to-peak.

Fig. 3 *A* and *A'* shows the increase in transmitter release produced by 0.1 mM 3,4-DAP, a K^+-channel blocker. This increase could be totally accounted for by an increase in the time-to-peak of the EPSP, reflecting an increase in spike

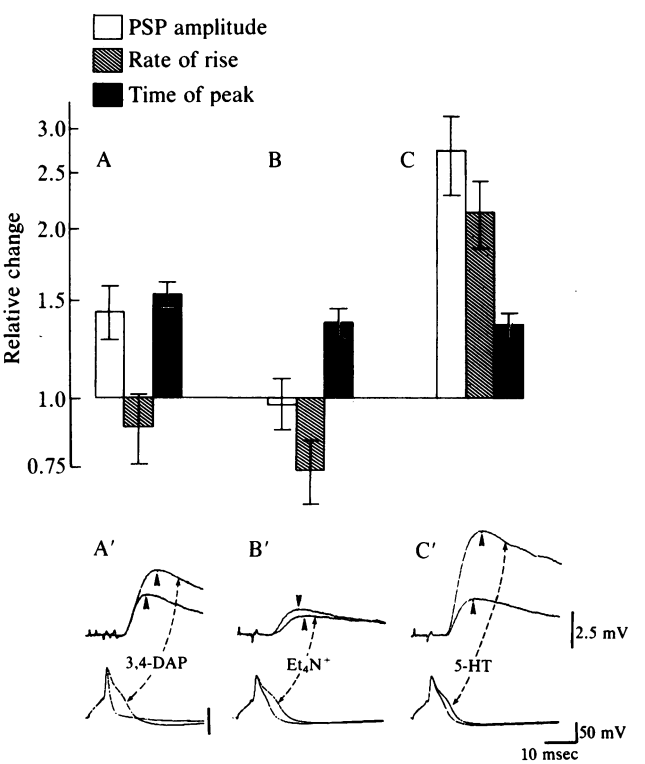

FIG. 3. Effects of 5-HT on the amplitude of the EPSP, its rate-of-rise, and its time-to-peak in the presence of low concentrations of K^+-channel blockers. (*A*) The K^+ blocker 3,4-DAP (0.1 mM) prolongs the presynaptic action potential, enhances the amplitude of the EPSP, and increases the time-to-peak without significantly affecting the rate-of-rise. (*A'*) The EPSP in the presence of 3,4-DAP has been shifted to the left to superimpose it on the control EPSP. (*B*) Further prolongation of the action potential in *A* by adding Et_4N^+ (50 mM) in the presence of 3,4-DAP causes no further increase in EPSP amplitude. It causes a reduction in the rate-of-rise, and an increase in time-to-peak. (*C*) Addition of 5-HT in the presence of 3,4-DAP facilitates the EPSP and increases both rate-of-rise and time-to-peak. These results suggest that part of the facilitation of the depressed EPSP that is induced by 5-HT cannot be accounted for by prolongation of the presynaptic action potential. (*A'*, *B'*, *C'*) Sample records are averages of four to six traces. Arrowheads indicate peaks of EPSPs.

duration. However, further broadening of the spike by a second K^+-channel blocker (Et_4NCl at 50 mM) did not cause a further increase in the synaptic potential (Fig. 3 *B* and *B'*). By contrast, adding 5-HT in the presence of 3,4-DAP (or in the presence of both 3,4-DAP and Et_4NCl) immediately increased synaptic strength (Fig. 3 *C* and *C'*). This facilitation was mostly accounted for by an increase in rate-of-rise, reflecting the recruitment by 5-HT of the second process. But it also was accompanied by an increased time-to-peak, presumably reflecting the broadening of the spike produced by 5-HT, a broadening that was now effective in contributing to facilitation.

To test the idea that the second process initiated by 5-HT actually involves increased availability of transmitter, we carried out the previous experiment in reverse (Fig. 4). We first depressed the EPSP and then gave 5-HT to restore transmission capability. Now with transmission restored, we broadened the presynaptic spike to about 40 msec by adding Et_4NCl. This further broadening, although considerably beyond the normal plateau of the input–output curve (Fig. 2*A*), nonetheless caused a substantial increase in transmitter release. This result suggests that the relative inefficiency of action-potential prolongation in increasing transmitter release at depressed synapses reflects a limit in the amount of releasable transmitter, a limit that can be overcome by 5-HT.

The Contribution to Presynaptic Facilitation of Rate-of-Rise and Time-to-Peak of the Synaptic Potential Is Correlated with the Level of Depression. To compare the relative contributions to facilitation of changes in rate-of-rise and time-to-peak under conditions that best stimulate normal functioning of the synapses, we performed experiments in intact ganglia in normal medium, using action potentials to evoke EPSPs and connective nerve stimulation to induce facilitation. In synapses that had not been depressed, increases in time-to-peak accounted for roughly 70% of the facilitation of the EPSP. The remainder was accounted for by an increased rate-of-rise (see also ref. 8). On the other hand, in depressed synapses, the relative contributions of these two parameters were reversed (Fig. 5). If, as our earlier analysis suggested (8), these two parameters of the EPSP shape represent two sets of facilitatory processes, then the two processes seem to carry different weights in facilitation of depressed as opposed to undepressed EPSPs.

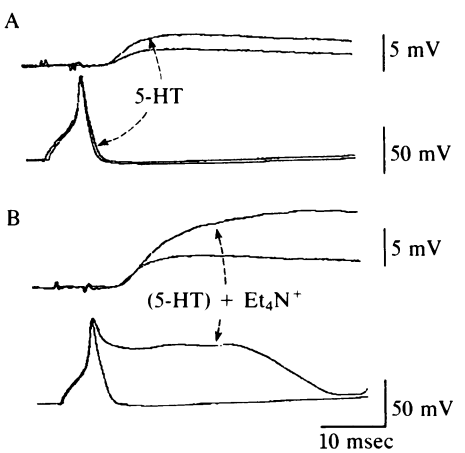

FIG. 4. 5-HT (10 μM) enhances the effectiveness of spike broadening in releasing transmitter. (*A*) Application of 5-HT causes a small prolongation of a sensory neuron action potential and enhancement of transmitter release. (*B*) Addition of Et_4N^+ (50 mM) in the presence of 5-HT causes marked spike broadening and marked enhancement of release.

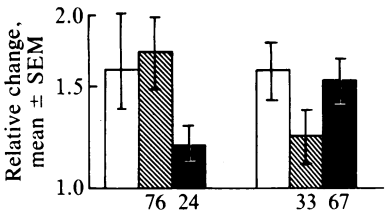

FIG. 5. Differential effects of facilitating nerve stimulation on the shape of the EPSP in depressed (*Left*; $n = 6$) and undepressed (*Right*; $n = 9$) synapses. To induce homosynaptic depression, action potentials were initiated every 10 sec, generating 10–15 EPSPs before nerve stimulation. For undepressed synapses, action potentials were initiated only two or three times at 1-min intervals prior to nerve stimulation. The numbers under the bars indicate the relative contributions (in percent) of the increases in rate-of-rise (▨) and time-to-peak (■) of the EPSP to the facilitation (calculated by the method of ref. 8). □, PSP amplitude. Facilitation in depressed synapses is accompanied by a larger change in rate-of-rise, while facilitation in undepressed synapses is accompanied by a larger change in time-to-peak. These results suggest that two processes may contribute differentially to facilitation, depending on the state of the synapse.

DISCUSSION

A Second Process in Presynaptic Facilitation Becomes Evident When the Synaptic Potential Is Depressed. Whereas spike prolongation is a powerful contributor to presynaptic facilitation at normal levels of release, as release becomes depressed, broadening becomes progressively less effective. Nonetheless, exposing the ganglion to the facilitating transmitter 5-HT or stimulating a facilitating pathway overcomes the depression. Thus, a process other than spike broadening must come into play when, as a result of habituation, transmitter release is limited by some factor other than spike configuration. That this process might be related to mobilization of available transmitter is consistent with the morphological findings of Bailey and Chen (14) that short-term habituation at this synapse involves depletion of vesicles in the active-zone regions. Therefore, to overcome habituation, a dishabituating stimulus must first mobilize vesicles into the active zones.

Thus, the ability in cell culture to control transmitter release from the presynaptic terminals by voltage-clamping the cell body of sensory neurons has allowed us to identify a new component in presynaptic facilitation. The existence of this second process is also supported by experiments in the intact ganglia that examine shape changes in the synaptic potential. These experiments show that when transmitter release is not depressed, facilitation, acting through broadening, produces substantial changes in the duration of the rising phase of the EPSP and smaller changes in its rate-of-rise. However, once transmitter release is depressed, facilitation is primarily produced by changes in the rate-of-rise of the synaptic potential, and the contribution of changes in time-to-peak is diminished.

The Existence of a Second Process Suggests a Fundamental Difference Between Behavioral Sensitization and Dishabituation. Sensitization is a simple form of learning (15) in which a nonhabituated reflex response is increased following a noxious stimulus. Sensitization was initially distinguished from dishabituation, the process whereby a noxious stimulus removes the reflex depression produced by habituation. Subsequent studies (16, 17) suggested, however, that dishabituation may be only a special case of sensitization (see also refs. 18 and 19).

The hypothesis that dishabituation and sensitization involve common mechanisms was based on the behavioral finding that noxious stimuli that produced dishabituation of a

habituated reflex could at the same time sensitize a nonhabituated reflex to above its initial value. However, these experiments could not exclude the possibility that dishabituation may not also use an additional mechanism, a mechanism not shared with sensitization. The experiments that we have described here suggest that, even though dishabituation and sensitization do indeed share a common mechanism (spike broadening), dishabituation in addition utilizes another set of mechanisms that comes importantly into play when the pathway has first been depressed. Thus, our data suggest that it is possible to distinguish between one process that contributes predominantly to the effects of reflex sensitization and a second process that is primarily concerned with restoring synaptic function from a depressed to a nondepressed level.

That dishabituation and sensitization might be different was also suggested in behavioral studies by Whitlow (20). The distinction we suggest emerges even more clearly in studies by Rankin and Carew (21) of the development of learning in the gill and siphon withdrawal reflexes of *Aplysia* that indicate that during development dishabituation of these reflexes emerges before sensitization.

Comparison of the Facilitation Produced by Spike Broadening and by the Second Process. Stimulation of a facilitating pathway or application of 5-HT increases the level of cAMP in *Aplysia* neurons (22, 23). Elevation of cAMP in the sensory neurons, in turn, enhances transmitter release (24, 25). Subsequent work showed that 5-HT, stimulation of the facilitating pathway, or injection into the sensory neurons of cAMP (1, 2) or of the catalytic subunit of the cAMP-dependent protein kinase (26), leads to a broadening of the action potential because of a depression of a specific K^+ current (3). This current was soon identified at the single-channel level (4, 5). Because of the steep input–output curve relating depolarizing step duration to transmitter release (8), it was clear that broadening of the action potential could produce significant facilitation by increasing Ca^{2+} influx. In the preceding paper we provided direct support for this mechanism (8).

Not apparent in the earlier studies in intact ganglia, however, is the present finding in cell culture that, when the synapse is depressed, a process unrelated to S-channel modulation becomes prominent. Although this new component seems to share with spike broadening the feature that it is also driven by cAMP, it does not appear to involve changes in the shape of the action potential. The first suggestion of a second process in presynaptic facilitation emerged from the study of Boyle *et al.* (6), which revealed that, independent of the changes in K^+ channels, Ca^{2+} accumulation in the sensory neurons was increased by 5-HT. Subsequently, Gingrich and Byrne (9) concluded that, when the action potential is already greatly prolonged (as with Et_4NCl), facilitation must involve a process in addition to spike broadening.

Although we do not know, as yet, whether the alteration in the handling of Ca^{2+} is responsible for the second, dishabituating, process that we have described here, our results show that combined pharmacological block of both the enhancement of Ca^{2+} transients by 5-HT and of S-current modulation results in block of facilitation. Evidence from a variety of systems suggests that Ca^{2+} can function at several points in the transmitter release process. For example, the work of Pollard *et al.* (27) and Llinas *et al.* (28) suggests that Ca^{2+} is critical for transmitter availability and mobilization (with which the second process seems to be concerned) as well for the events that bind the vesicle to the release site prior to exocytosis (29). The morphological studies of Bailey and Chen (14) and the theoretical work of Gingrich and Byrne (9) suggest that short-term habituation might involve insufficient mobilization. Our results suggest that this apparent block in mobilization can be overcome by 5-HT.

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