Modulation of Spontaneous Transmitter Release during Depression and Posttetanic Potentiation of *Aplysia* Sensory-Motor Neuron Synapses Isolated in Culture

Lise S. Eliot, Eric R. Kandel, and Robert D. Hawkins

Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, New York, New York 10032

An *Aplysia* motor neuron cocultured with a single presynaptic sensory neuron exhibits spontaneous miniature EPSPs or EPSCs (“minis”) that can be used to assay the release process directly, independent of the presynaptic action potential. Sensory-motor synapses in culture undergo homosynaptic depression with low frequency stimulation (< 1 Hz) and posttetanic potentiation (PTP) with high-frequency stimulation (20 Hz) much as they do in intact ganglia, except that PTP does not occur in culture when sensory neurons are impaled. We measured spontaneous release during each of these two forms of homosynaptic plasticity as a way of testing whether they involve depletion or mobilization of synaptic vesicles (Gingrich and Byrne, 1985). We find that PTP is accompanied by an increase in mini frequency that decays with a time course parallel to the decay of evoked EPSP facilitation. In contrast, depression is not paralleled by a reduction of mini frequency, although extensive stimulation reduces mini frequency for a brief period immediately following stimulation. Neither form of plasticity altered miniature EPSP or miniature EPSC amplitude, corroborating previous evidence that both are presynaptically mediated. These findings suggest that PTP is mediated by a presynaptic mechanism independent of the action potential, such as vesicle mobilization. This presumably Ca^{2+} -dependent mechanism does not involve protein kinase C, since we found that the inhibitor H7 does not specifically block PTP. In contrast to PTP, depression appears to involve changes unique to excitation–secretion coupling, such as reduced Ca^{2+} influx during the action potential (Klein et al., 1980).

[Key words: synaptic plasticity, homosynaptic, presynaptic, spontaneous release, evoked release, vesicle depletion]

The simplest and most universal forms of synaptic plasticity are homosynaptic: frequency-dependent depression and facilitation. Individual synapses tend either to decrease or increase

in strength when repeatedly activated. However, both forms of homosynaptic plasticity can be revealed at most synapses using appropriate manipulations, suggesting that their underlying mechanisms are inherent to the machinery of synaptic transmission (Zucker, 1989).

Early work by Del Castillo and Katz (1954) established that both depression and facilitation are due to presynaptic changes in transmitter release. The most enduring form of short-term homosynaptic enhancement, posttetanic potentiation (PTP), has similarly been shown to be presynaptically mediated at both central and peripheral synapses (Liley, 1956; Hirst et al., 1981; Clamann et al., 1989; Griffith, 1990). In principle, presynaptic mechanisms of short-term plasticity can be divided into two classes: those involving changes in the action potential or Ca^{2+} influx, and those independent of changes in the action potential, such as changes in vesicle distribution or direct modulation of the release apparatus. Homosynaptic plasticity is generally thought to involve changes of the second class, based in part on findings at the squid giant synapse that neither depression nor facilitation can be accounted for by changes in action potential shape (Takeuchi and Takeuchi, 1962; Charlton and Bittner, 1978), presynaptic Ca^{2+} current (Charlton et al., 1982), or in the amount of Ca^{2+} that enters the terminal with each action potential (Miledi and Parker, 1981; Charlton et al., 1982).

The synapses made by *Aplysia* mechanosensory neurons onto motor neurons and interneurons exhibit both homosynaptic depression and potentiation, but these sensory neurons are unusual in the dramatic extent to which depression is their dominant form of plasticity. Sensory neuron synapses depress at every interval of stimulation, from tens of milliseconds to tens of minutes (Castellucci et al., 1970; Byrne, 1982). At shorter intervals (<100 msec), synapses may facilitate during the first two or three stimuli, but then depress throughout the remainder of the train (Walters and Byrne, 1984). Following a train of stimuli at 6–25 Hz, the synapses display PTP lasting a few minutes (Clark and Kandel 1984; Walters and Byrne, 1984).

Aplysia sensory neuron synapses are advantageous for assessing the functional significance of short-term homosynaptic plasticity. The sensory neurons are the first afferent relay in both the mono- and polysynaptic components of the gill and siphon withdrawal reflex (Koester and Kandel, 1977; Byrne et al., 1978). With repeated activation, the synaptic potential and the reflex depress comparably in magnitude and time course, suggesting that homosynaptic depression in sensory neurons contributes to behavioral habituation (Castellucci et al., 1970). Although

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Correspondence should be addressed to Lise Eliot, Division of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

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the behavioral relevance of PTP is less well understood, homosynaptic facilitation would be expected to contribute to two forms of learning that involve brief, high-frequency sensory neuron activity: classical conditioning (Hawkins et al., 1983; Walters and Byrne, 1983) and site-specific sensitization (Walters and Byrne, 1985; Walters, 1987).

Of the two forms of homosynaptic plasticity, depression has been more extensively studied at sensory neuron synapses. Quantal analysis of sensory neuron depression under conditions of low release indicated that it is mediated presynaptically (Castellucci and Kandel, 1974). In contrast to depression in the squid giant synapse, Klein et al. (1980) found that depression during repeated voltage-clamp depolarizing steps is paralleled by a progressive decline in sensory neuron Ca^{2+} current, suggesting that it is due to a decrease in Ca^{2+} influx with successive depolarizations. Subsequently, Gingrich and Byrne (1985) modeled the sensory neuron synapse using the published Ca^{2+} current inactivation parameters and concluded that the inactivation is insufficient to account for the magnitude of synaptic depression observed with repeated action potentials. The authors posited a depletion mechanism to account for the bulk of synaptic depression. In their model, vesicles reside in two pools, a releasable pool and a backup or storage pool. The former is depleted by activity and then slowly replenished by a Ca^{2+} -dependent mechanism. This model accounts for various parametric features of depression (Byrne, 1982) and even predicted the occurrence of PTP. However, the model may underestimate the contribution of presynaptic Ca^{2+} current inactivation to depression, because sensory neurons were recently found to have two different types of Ca^{2+} channels, only one of which, a dihydropyridine-insensitive channel, contributes to normal synaptic transmission in sensory neurons (Edmonds et al., 1990). Since Klein et al. (1980) measured inactivation of total Ca^{2+} current, it remains possible that the component contributing to release undergoes greater inactivation than the total current.

To analyze further the mechanisms of short-term homosynaptic plasticity in *Aplysia* sensory neurons, we studied the phenomenon in culture. Both depression and PTP have been shown to persist in isolated cultures containing single *Aplysia* sensory and motor neurons, confirming that each form of plasticity is intrinsic to the synapse, and does not require additional circuit elements (Rayport and Schacher, 1986; Schacher et al., 1990). The ability to reconstitute the synapse between single adult sensory and motor neurons in culture provides a number of other advantages for mechanistic analysis. In particular, we have exploited the ability to measure miniature excitatory postsynaptic responses (mEPSPs or mEPSCs, "minis") that unambiguously reflect transmitter release from a single presynaptic neuron. Measurements of mini amplitude allow a direct assay for changes in quantal amplitude, while measurements of mini frequency provide an assay for modulatory mechanisms that are independent of the action potential, such as vesicle mobilization and depletion.

Materials and Methods

Cell culture. The methods for reconstituting synapses between single *Aplysia* sensory and motor neurons have been previously described (Rayport and Schacher, 1986; Schacher et al., 1990; Eliot et al., 1994). Synapses between abdominal or pleural sensory neurons and motor neurons L7 or LFS were reestablished in polylysine-coated plastic dishes in a solution containing 50% sterile filtered *Aplysia* hemolymph and 50% L15 medium (Flow Laboratories, supplemented to the following final concentrations: 385 mM NaCl, 10 mM KCl, 11 mM CaCl_2 , 28 mM

MgCl_2 , 27 mM MgSO_4 , 2.3 mM NaHCO_3 , 35 mM D-glucose, 50 U/ml streptomycin, 50 $\mu\text{g}/\text{ml}$ penicillin). Synaptic connections appeared as early as 24 hr after plating and growth at room temperature. Dishes were transferred to an 18°C incubator after 24 hr. Experiments were performed 1–4 d after plating.

Electrophysiology. All recording was performed at room temperature in medium containing 50% supplemented L15 and 50% artificial seawater (460 mM NaCl, 10 mM KCl, 55 mM MgCl_2 , 11 mM CaCl_2 , 2 mM NaHCO_3 , 10 mM Na-HEPES, pH 7.6). Sensory neurons were either stimulated intracellularly, using 20–30 M Ω microelectrodes (filled with a solution of 2 M K-acetate, 0.5 M KCl, 10 mM K-HEPES, pH 7.2) or extracellularly, using an unpolished patch pipette (1–3 M Ω) filled with recording medium and coated with silver paint to allow concentric, bipolar stimulation. Extracellular stimulus strength was adjusted by gradually increasing the intensity of a 0.1 msec voltage pulse until a single EPSP was observed in the motor neuron. For most experiments, the synapse was tested once with a single presynaptic action potential and then rested 15 min before beginning the experiment. Motor neurons were held hyperpolarized (to -80 to -90 mV) to measure evoked EPSPs. All recording was performed using an Axoclamp 2A amplifier. Current-clamp measurements were performed in bridge mode, while mEPSCs were recorded in continuous single electrode voltage-clamp mode.

Measurements of spontaneous release. Spontaneous transmitter release was measured exclusively in cultures containing a single sensory neuron in contact with a single motor neuron, and was assessed in two ways. Initially, we recorded mEPSPs in L7 motor neuron synapses (2–3 d postplating), while in later experiments we recorded mEPSCs in synapses onto LFS motor neurons (1–2 d postplating). L7 is quite large (300–400 μm) and thus can be impaled with a low-resistance sharp microelectrode (6–8 M Ω beveled and filled with 1.25 M K-acetate, 1.25 M KCl, 10 mM K-HEPES, pH 7.2). The frequency of detectable minis is higher in the smaller (50–100 μm) LFS cells, probably because their input resistance is 3–10 times higher than L7s. The disadvantage of LFS followers is that they tend to have large oscillations in their membrane potential which are reduced, but not eliminated by holding the cell hyperpolarized. These oscillations made it difficult to accurately detect mEPSPs in most cultures. However, the oscillations disappeared when the cells were voltage clamped and internally dialyzed with Cs^+ -containing solutions. To record mEPSCs, LFS motor neurons were held at -50 mV under whole-cell voltage clamp with fire-polished patch pipettes (3–4 M Ω) filled with 465 mM Cs-gluconate, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM K-EGTA, 5 mM Na_2ATP , 0.1 mM Tris-GTP, 10 mM reduced glutathione, and 50 mM Cs-HEPES, buffered to pH 7.2 with CsOH.

Analyses of mEPSP and mEPSC ("mini") frequencies and amplitudes were performed as previously described (Dale and Kandel, 1990; Eliot et al., 1994). Briefly, high-gain recordings (AC coupled) were filtered (300 Hz for mEPSPs, and 2–4 kHz for mEPSCs), digitized (4 kHz sampling frequency) and measured using a PDP-11 computer and programs written in BASIC-23. The starting point of each mini was detected manually based on rapid rise time and amplitude greater than the peak-to-peak noise level (approximately 40 μV in current-clamp recordings and 6 pA in voltage-clamp recordings; see Figs. 5B, 11B) and then the peak amplitude and the interval between minis were determined automatically. Distributions of mini amplitudes generally yielded multiple peaks, with the majority of events in the lowest amplitude class. Larger-amplitude events tended to be integral multiples of the smallest mean amplitude, particularly in the case of mEPSP distributions. Unitary mini amplitude is taken as the mean of the first peak of amplitude histograms fit with one to three Gaussian distributions using the simplex algorithm and the method of maximum likelihood (Dale and Kandel, 1990). Average unitary mEPSP amplitude was 99.4 ± 12.0 (SEM) μV ($n = 11$). Average unitary mEPSC amplitude was 20.7 ± 3.0 pA ($n = 13$). Experiments were rejected if the noise level increased during the experiment to the point of exceeding the smallest signals detectable at the beginning of the experiment.

Data analysis. Group data are presented throughout as mean $\pm$ SEM, normalized to initial or pretetanus level. In experiments measuring spontaneous release, both mini frequencies and evoked EPSP amplitudes were log transformed and then normalized for both statistical analysis and graphical display, where the data are plotted as geometric mean $\pm$ SEM (see Figs. 6, 9, 10A, 12). Because spontaneous release frequency is quite low, it was necessary to sample it over long intervals. To compare evoked and spontaneous release we thus compared average mini frequency during an interval to the average amplitude of two

Figure 1. Homosynaptic depression in culture. *A*, Depression in a sensory-LFS motor neuron synapse stimulated intracellularly at 60 sec intervals. *B*, Shorter stimulus intervals produce greater depression. In this data set, depression at a 60 sec interval ($n = 13$) is significantly different from both the 10 sec interval ($n = 8$) the 1 sec interval ($n = 6$), but the 10 sec and 1 sec intervals are not significantly different from each other ($p < 0.05$, by Student-Newman-Keuls' test). Each experiment was performed on a naive synapse.

evoked EPSPs tested at either end of the interval. This procedure is preferable to comparing a single EPSP measurement to average mini frequency in two bracketing intervals because it allows some averaging to correct for variability in evoked release.

Within-group comparisons to pretest measures were evaluated using one-way ANOVA with repeated measures followed by Dunnett's multiple range test. Between-group comparisons were evaluated using two-way ANOVA with repeated measures (with time and treatment as factors) followed by Student-Newman-Keuls' test for comparing multiple groups, or t tests for comparing two groups at individual time points.

Results

Homosynaptic depression and potentiation occur in culture but are altered by sensory neuron impalement

As was found previously for cultures containing single sensory and L7 motor neurons (Rayport and Schacher, 1986; Schacher et al., 1990), sensory-to-LFS motor neuron synapses show both homosynaptic depression and facilitation in culture. Figure 1*A* illustrates depression of the EPSP to repeated intracellular stimulation of the sensory neuron at 60 sec intervals. Depression occurred at every stimulus interval tested, from 50 msec to 10 min. Average depression resulting from stimulating different synapses at 1, 10, or 60 sec intervals is plotted in Figure 1*B*. Depression kinetics in culture are very similar to the ganglion (Byrne, 1982), with about half of the total decrease occurring

between the first and second stimuli, and shorter stimulus intervals producing a greater amount of depression. This is reflected as a significant overall effect of stimulus interval on the magnitude of depression ($F_{2,24} = 7.40$, $p < 0.05$).

In contrast to the depression exhibited during low-frequency stimulation, sensory neuron synapses in culture undergo PTP following a high-frequency (20 Hz, 2 sec) tetanus (Fig. 2*B₁*). Considerable depression occurs during the train, but 1 min later, the synapse is substantially facilitated, and remains enhanced for 5–10 min following the tetanus. Group data from 17 such experiments are shown in Figure 3*A*. Facilitation was significant overall ($F_{1,18} = 8.11$, $p < 0.02$) and at the 1 min ($p < 0.05$) and 5 min ($p < 0.02$) time points, when compared to test-alone controls ($n = 3$). Potentiation also occurs in synapses that are substantially depressed. Thus, the same tetanus largely reverses the depression induced by 10 stimuli delivered at low frequency (Fig. 3*B*). This is reflected as a significant overall difference between tetanized and control depressed groups ($F_{1,9} = 6.44$, $p < 0.05$) and significant differences at all individual posttests at early time points ($p < 0.05$, one-tailed t test), but not at 7, 8, and 10 min posttetanus.

The most dramatic difference between PTP in culture and in the ganglion is that PTP does not occur when the sensory neuron is impaled and stimulated intracellularly (Fig. 2*B₂*). Although extracellular stimulation may elicit action potentials in a somewhat different manner from intracellular stimulation, it did not produce PTP in cells that were concurrently impaled by a microelectrode (not shown). It was possible, in some cases, to induce PTP with extracellular stimulation 30 min after removal of an intracellular electrode. A likely explanation for these observations is that impalement elevates intracellular Ca^{2+} , effectively occluding PTP. Two additional observations are consistent with this interpretation. First, synaptic depression was significantly greater when elicited with extracellular stimulation than in sensory neurons that were impaled and stimulated intracellularly ($F_{1,20} = 8.86$, $p < 0.01$; Fig. 4). Second, the interval between consecutive mEPSCs was significantly shorter in LFS synapses in which the presynaptic sensory neuron was impaled (6.8 ± 2.6 sec, $n = 5$) compared to synapses in which the sensory neuron was not impaled (14.7 ± 2.5 sec, $n = 8$; $p < 0.05$ by one-tailed t test). Both of these observations support the interpretation that impalement elevates intracellular Ca^{2+} , producing a modest level of facilitation that counteracts depression, elevates resting mini frequency, and interferes with PTP.

These results demonstrate that both depression and PTP can be induced in culture, where they display many of the same properties as in the ganglion. We next took advantage of the ability to measure spontaneous transmitter release in culture to gain insight into the mechanisms underlying these two forms of homosynaptic plasticity.

PTP is accompanied by an increase in frequency, but not amplitude, of spontaneous mEPSPs

Figure 5 illustrates spontaneous and evoked EPSPs in an L7 synapse before and after tetanization. Resting mEPSP frequency is quite low, especially since the sensory neuron was not impaled in these experiments (mean rate = 1 mEPSP every 20.9 ± 1.6 sec, $n = 5$). Immediately following tetanization, mEPSP frequency increases dramatically, by about 15-fold on average. We omitted the 1 min posttest of evoked release in these experiments to provide an uninterrupted interval for measuring spontaneous release after the tetanus. Although the initial increase

Figure 2. PTP can be elicited in culture, but not when the sensory neuron is impaired. *A*, Diagram of experimental design. Two sensory neurons (SNs) synapsing on a common motor neuron were stimulated at 5 min intervals (offset from each other by 2.5 min). SN-2 was stimulated with an intracellular microelectrode while SN-1 was stimulated extracellularly as described in Materials and Methods. *B₁*, The EPSP from SN-1 shows PTP up to 10 min after extracellular tetanization (20 Hz for 2 sec). *B₂*, In contrast, the EPSP from SN-2 shows depression, rather than PTP following the same tetanus delivered intracellularly.

in mini frequency appears greater than the facilitation of evoked release at 1 min seen in the previous set of experiments (Fig. 3*A*), this initial increase decays rapidly, leaving a smaller, though still substantial enhancement of spontaneous release whose decay roughly parallels the decay of PTP of the evoked EPSP (Fig. 6). Both the increases in mini frequency and evoked EPSP amplitude were significant overall ($F_{5,20} = 58.48$ and $F_{3,12} = 11.30$ for spontaneous and evoked release, respectively; $p < 0.001$) and at individual time points through 10 min posttetanus ($p < 0.01$ for both effects, Dunnett's test), but not at later time points. Changes in mEPSP frequency are larger than changes in evoked release, such that a twofold increase in mEPSP frequency in the interval between 5 and 10 min posttetanus corresponds to about 50% facilitation of the EPSP.

We next analyzed mEPSP amplitudes to look for changes in quantal size during PTP. Figure 7*A* shows the distribution of mEPSP sizes before and after tetanus in the experiment illustrated in Figure 5. Although the sample sizes were fairly small before tetanus, in four of five experiments it was possible to fit

amplitude histograms with gaussian distributions to determine the size of the unitary potential. Unitary mEPSP amplitude did not change after tetanization ($+0.6 \pm 3.3\%$ of pretetanus amplitude; Fig. 7*B*), indicating that changes in quantal size cannot account for facilitation of the evoked EPSP. The fact that PTP of the evoked EPSP is accompanied by an increase in frequency, with no change in amplitude of spontaneous mEPSPs, suggests that it is mediated by a presynaptic mechanism. That the same mechanism underlies the enhancement of both evoked and spontaneous release is further suggested by the fact that intracellular tetanization, which fails to induce PTP, did not produce the dramatic enhancement of spontaneous release seen following extracellular tetanization (not shown).

PTP is not blocked by H7

The enhancement of spontaneous release following tetanization is reminiscent of a similar increase in the presence of 5-HT (Dale and Kandel, 1990). The effect of 5-HT on spontaneous release appears to be mediated, at least in part, by protein kinase

Figure 3. Time course of PTP: group data. *A*, PTP following a tetanus (20 Hz $\times$ 2 sec) in mildly depressed synapses ($n = 17$) compared to test alone controls ($n = 3$). *B*, PTP following homosynaptic depression ($n = 7$) compared to test alone controls ($n = 4$). The same tetanus reverses the depression produced by 10 stimuli. All experiments were performed using extracellular stimulation of sensory neurons.

C, because it is both mimicked by phorbol esters and blocked by the kinase inhibitor H7 (Ghirardi et al., 1992). Furthermore, 5-HT has been shown to translocate protein kinase C activity from cytosol to membrane (Sacktor and Schwartz, 1990) and to activate specifically the Ca^{2+} -dependent form of the enzyme in sensory neurons (Sossin and Schwartz, 1992). If the large Ca^{2+} influx that occurs during a tetanus is by itself capable of activating protein kinase C, this activation could contribute to PTP and the enhancement of spontaneous release that accompanies it. Such a mechanism would be consistent with evidence from Ca^{2+} imaging studies suggesting that posttetanic forms of facilitation are mediated by a Ca^{2+} -dependent process distinct from the release mechanism itself (Delaney et al., 1989; Swandulla et al., 1991). Protein kinase C is a good candidate for playing a role in PTP based on its Ca^{2+} dependence and the fact that it enhances both spontaneous and evoked release from

Figure 4. Homosynaptic depression is greater when the sensory neuron is not impaired. Depression was evoked either by extracellular stimulation ($n = 19$) or intracellular stimulation ($n = 13$, data replotted from Fig. 2*B*). Average EPSP amplitude at trial 1 was 39.5 ± 4.4 mV in the extracellular group and 43.8 ± 3.5 mV in the intracellular group.

many different synapses (Eusebi et al., 1986; Malenka et al., 1986; Yamamoto et al., 1987).

To test the role of protein kinase C in PTP, we used the blocker H7 (Seikagaku, Japan). This inhibitor, which is only modestly selective for protein kinase C *in vitro* (Hidaka et al., 1984), has nonetheless been demonstrated in intact sensory neurons to block phorbol ester-stimulated processes without altering cAMP-mediated effects (Braha et al., 1993). When we applied 200 μM H7 (the same concentration used by Braha et al.) to depressed synapses shortly before sensory neuron tetanization, PTP was not affected. One minute after tetanus, synapses were facilitated by 2.46 ± 0.47 -fold ($n = 5$) in the presence of H7 as compared to 2.60 ± 0.34 -fold facilitation ($n = 6$) in its absence (Fig. 8*A*). In contrast, briefly applying the same concentration of H7 2 min after tetanization dramatically reduced the EPSP to its pretetanus level. This result might suggest that protein kinase C participates in the maintenance, but not the initial induction of PTP. However, H7 had a similar effect on synaptic transmission from sensory neurons that had not been tetanized (Fig. 8*B*).

These results indicate that protein kinase C activation is not necessary for PTP. The general depressive effect of H7 on synaptic transmission may be nonspecific, or it might also reflect a role of protein kinase C in basal transmitter release or vesicle mobilization. This seems plausible in light of a number of reports that activation of protein kinase C facilitates both evoked and spontaneous release (Eusebi et al., 1986; Shapira et al., 1987; Ghirardi et al., 1992; Sugita et al., 1992).

Homosynaptic depression affects neither the amplitude nor the frequency of spontaneous mEPSPs or mEPSCs

Homosynaptic depression has been demonstrated in the ganglion to be mediated by a decrease in quantal content, with no change in quantal size (Castellucci and Kandel, 1974). Analysis of spontaneous release in culture allows a second method for quantal analysis of synaptic depression. Another and perhaps more novel use of spontaneous release is to address the issue of vesicle depletion during synaptic depression.

Spontaneous release was analyzed during synaptic depression

Figure 5. PTP is accompanied by an enhancement of spontaneous release. Recordings of evoked (*A*) and spontaneous (*B*) release in an L7 synapse during PTP. mEPSP frequency is substantially enhanced immediately after tetanus (20 Hz), and remains somewhat elevated for the duration of evoked facilitation. mEPSPs are indicated by dots.

using two different stimulation protocols. In the first, depression was produced by stimulating extracellularly with single action potentials at 5 min intervals, and mEPSP or mEPSC frequency was compared in the intervening intervals. As can be seen from the test-only controls in Figure 3*A*, sensory neuron synapses depress substantially, even at 5 min stimulation intervals. In 20 experiments, the EPSP was reduced to an average of $71 \pm 1\%$ of its starting value by the third stimulus, whereas the frequency of spontaneous release was unaltered (Fig. 9). The relationship between depression and spontaneous release frequency in individual experiments is plotted in Figure 10*A*. There is no correlation between changes in spontaneous and evoked release.

These experiments also provide an opportunity to test for changes in quantal size with depression. Mini amplitude does not decrease during depression of the evoked EPSP at 5 min intervals (Fig. 10*B*). Average mini size instead slightly increased in these experiments, but the effect was not significant ($+4.9 \pm 4.7\%$ change, $p > 0.10$). These results confirm the finding of Castellucci and Kandel (1974) that sensory neuron depression is presynaptic, a reduction in evoked release without a change in postsynaptic responsiveness or vesicle loading.

The second protocol used to test for depletion during homosynaptic depression employed extensive stimulation at 1 Hz. While stimulation at 5 min intervals reliably depressed the synapse, it is possible that depletion only plays a role in more extensive depression, after a number of action potentials have been evoked. mEPSCs were recorded in LFS cells under whole-cell voltage clamp before and after 80–120 sec of sensory neuron stimulation at 1 Hz. Such extensive stimulation profoundly depresses the evoked EPSP, but has relatively little effect on spontaneous release (Fig. 11). Group data from five similar experiments are plotted in Figure 12. Stimulation reduces the EPSP to an average of $11 \pm 3\%$ of its starting amplitude. In contrast, the frequency of spontaneous release is unaffected, except im-

mediately after the end of stimulation. While stimulation has an overall effect on the rate of spontaneous release ($F_{3,20} = 4.14$, $p < 0.01$), mini frequency is only significantly reduced (to $51.8 \pm 9.8\%$ of resting frequency) in the first 30 sec interval after stimulation ($p < 0.05$, Dunnett's test). It recovers almost completely by 1 min after stimulation and does not significantly differ from resting frequency in this or any later interval. In contrast, even 4 min after the end of stimulation, the evoked EPSP remains substantially depressed, to $42.7 \pm 9.8\%$ of its starting amplitude ($t = 3.89$, $p < 0.02$). As in the experiments using stimulation at 5 min intervals, the size of unitary mEPSCs was not reduced by extensive depression of the evoked EPSP.

Figure 6. The decay of mEPSP frequency parallels the decay of PTP. Group data are from five experiments like the one illustrated in Figure 6. Horizontal bars indicate the interval over which mEPSP frequency was sampled for each data point. The tetanus was delivered at the arrow. Although the increase in mEPSP frequency is many times larger than the increase in EPSP amplitude, both are significant through 10 min posttetanus, but not later. In this and subsequent figures, evoked EPSP amplitude is normalized to the first EPSP of the tetanus, and mEPSP frequency is normalized to the average of both intervals before the tetanus. Note the log scales for both y-axes.

Figure 7. Tetanus does not change the amplitude of spontaneous mEPSPs. *A*, Distribution of mEPSP amplitudes in all intervals before and after tetanus for the experiment shown in Figure 5. The mean unitary size was $91.5 \pm 23.7 \mu\text{V}$ before tetanus and $90.2 \pm 22.9 \mu\text{V}$ after tetanus, determined by fitting two Gaussian distributions to each set. *B*, Group data illustrating that mean amplitude of the unitary mEPSP does not change following tetanus whereas the evoked EPSP is significantly elevated ($p < 0.005$). The change in evoked amplitude is the average of EPSP amplitudes at 5 and 10 min posttetanus ($n = 4$, because in one of the five experiments, there were too few mEPSPs before the tetanus to fit a Gaussian distribution). Average unitary mEPSP amplitude was $70.8 \pm 7.2 \mu\text{V}$ before tetanus, and $71.0 \pm 7.0 \mu\text{V}$ after tetanus.

Average unitary mEPSC amplitude was $14.1 \pm 2.6 \text{ pA}$ in all intervals before depression compared to $15.5 \pm 2.8 \text{ pA}$ in all intervals after stimulation (average change was $+11.5 \pm 5.6\%$, $n = 5$, NS).

These findings are consistent with our results on depression at 5 min intervals. Depression cannot be accounted for either by changes in mini amplitude or frequency. Extensive stimulation does briefly reduce the frequency of spontaneous mEPSCs, indicating that such changes can be detected, but the duration of the decrease does not parallel the depression of evoked release.

Discussion

Previous studies have established that homosynaptic depression and PTP are preserved when *Aplysia* sensory-motor synapses are reconstituted in culture (Rayport and Schacher 1986, Schacher et al., 1990). Here we further characterized these two

Figure 8. H7 blocks neither induction nor maintenance of PTP. *A*, Average results of five experiments in which PTP was induced in the presence of H7 and six control PTP experiments. In both groups, EPSPs were depressed to 25–30% of initial amplitude before receiving a 2 sec, 20 Hz extracellular tetanus (TET). Amplitudes were normalized to the average of tests at -2 , -1 , and 0 (first EPSP of the tetanus) min. H7 does not block PTP induction. *B₁*, Diagram of experimental protocol used to test the effect of H7 on PTP maintenance. Cultures containing two sensory neurons synapsing on a common LFS motor neuron were used. After pretesting, one cell (PTP) received the same tetanus used in *A*. Stimulation of the second sensory cell (TEST ALONE) began only after the first cell was tetanized. *B₂*, H7 reduces synaptic transmission of both facilitated and control EPSPs. Results are average of five experiments as depicted in *B₁*. H7 (200 μM) was applied for 1 min, beginning 2.5 min after the tetanus, by microperfusion. The effect is not a perfusion artifact because cells were similarly perfused with normal saline both before and after H7 application.

forms of plasticity and exploited the ability to measure spontaneous transmitter release from a single presynaptic sensory neuron in culture to analyze further the mechanisms underlying depression and PTP.

Figure 9. Modest depression does not alter spontaneous release frequency. Results are average of 20 experiments in which the EPSP was elicited once every 5 min using extracellular stimulation, and spontaneous release frequency was measured in the intervening intervals (five of these experiments are from Fig. 6).

We found that changes in mini amplitude cannot account for either form of plasticity (Figs. 7, 10*B*), suggesting that both depression and PTP are presynaptically mediated. One class of presynaptic mechanism proposed to underlie short-term homosynaptic plasticity involves changes in the availability of vesicles for release: vesicle "mobilization" in the case of PTP and depletion in the case of depression (Gingrich and Byrne, 1985). We therefore looked for changes in spontaneous release frequency that might provide evidence for such changes in vesicle availability.

PTP and depression in culture

Homosynaptic depression exhibits very similar properties in culture compared to those in the ganglion (Byrne, 1982). The largest increment of depression occurs between the first and second EPSPs. Shorter stimulus intervals produce more profound depression (Fig. 1). PTP also exhibits similar magnitude and kinetics in culture and in the ganglion (Clark and Kandel, 1984; Walters and Byrne, 1984). These findings indicate that the mechanisms underlying both forms of homosynaptic plasticity are intrinsic to the synapse and do not importantly involve additional neural circuitry.

The only discrepancy we observed is that PTP does not occur in culture when the sensory neuron is impaled with an intracellular microelectrode. Impalement has been observed to increase levels of somatic Ca^{2+} in cultured cerebellar Purkinje cells, an effect that takes a number of minutes to recover (Connor and Tseng, 1988). Four findings suggest that a similar elevation of intracellular Ca^{2+} occurs when cultured sensory neurons are impaled: (1) the frequency of spontaneous release is higher in impaled cells; (2) evoked EPSP amplitude increased after impalement in a few cases (not shown); (3) depression is less profound with intracellular as compared to extracellular stimulation (Fig. 4), suggesting that impalement promotes some Ca^{2+} -dependent mobilization process; and (4) the loss of PTP can be reversed after removing the intracellular electrode if adequate time is allowed for recovery. Presumably, elevated intracellular Ca^{2+} interferes with PTP by dampening the increase in Ca^{2+} produced by tetanus. Sensory neurons in the ganglion appear

Figure 10. Modest depression is not associated with changes in mini frequency or amplitude. *A*, Relationship between mini frequency and evoked depression in individual experiments from Figure 9 ($R = -0.22$). Mini frequency in the interval from 5 to 10 min is normalized to the frequency from 0 to 5 min. Average EPSP amplitude at 5 and 10 min is normalized to the average at 0 and 5 min. *B*, A change in unitary size does not account for evoked depression. Average amplitude of spontaneous mEPSPs or mEPSPs in the interval from 5 to 10 min is normalized to the interval from 0 to 5 min. The EPSP is significantly depressed ($p < 0.001$) whereas the change in mini amplitude is not significant. The number of minis in each interval was too small to fit amplitude distributions, so average amplitudes were used to calculate changes. Thus, the small increase in mini amplitude could be due to an increased incidence of multiple quantal events. For L7 synapses, average mEPSP amplitude was $107.5 \pm 14.0 \mu\text{V}$ in the first interval and $107.3 \pm 11.8 \mu\text{V}$ in the second interval, $n = 12$; for LFS synapses, mEPSC amplitude averaged $24.8 \pm 4.2 \text{ pA}$ in the first interval and $25.5 \pm 4.2 \text{ pA}$ in the second interval ($n = 8$).

not to suffer this impalement effect, because they exhibit robust PTP following intracellular tetanization (Clark and Kandel, 1984; Walters and Byrne, 1984). The difference may be due to the greater distance between the soma and presynaptic terminals in sensory neurons in the ganglion as compared to in culture.

Figure 11. Extensive stimulation reduces both EPSP amplitude and mEPSC frequency, but spontaneous release recovers quickly, while evoked release does not. *A₁*, mEPSCs measured in an LFS cell (MN) under voltage clamp before and after 1 Hz stimulation of the sensory neuron. Note that mEPSC frequency is reduced only for the first 30–40 sec poststimulation. *A₂*, Voltage recording in the LFS cell during intracellular stimulation of the sensory neuron at 1 Hz. The first EPSP produced a spike in the motor neuron that was off scale at this gain. The gap in the record indicates the 4 min interval between the end of stimulation and the first test for recovery of the EPSP. *B*, Examples of mEPSCs recorded during the prestimulation interval in *A₁*. Sweeps containing mEPSCs (indicated by dots) were selected, because at this expanded time scale, many of the sweeps would be empty. This figure illustrates the waveform of mEPSCs in an LFS synapse and is not meant to depict actual frequency, which is considerably lower. *C*, Expanded recordings of three synaptic responses from *A₂*. Note that the EPSP undergoes depression despite presynaptic spike broadening during the train, consistent with depletion playing a role in depression during the train.

Mechanisms underlying PTP in cultured synapses

Spontaneous mEPSPs measured before and after PTP induction showed no change in unitary amplitude (Fig. 7). This result agrees with quantal analyses of PTP at a number of other synapses (Liley, 1956; Hirst et al., 1981; Clamann et al., 1989; Griffith, 1990). Sensory neuron PTP is evidently mediated by an increase in release, rather than a change in postsynaptic responsiveness to transmitter.

In contrast to its lack of effect on quantal size, PTP produced a large enhancement in the frequency of spontaneous release. mEPSP frequency was elevated 15-fold in the 1 min interval following tetanization (Fig. 6). This enhancement appears to decay with both fast and slow components, perhaps reflecting the same augmentation and potentiation phases of evoked and mEPSP facilitation seen at the neuromuscular junction (Zengel and Magleby, 1981). Although we did not test evoked release shortly after tetanus in these experiments, comparison to Figure

3A suggests that the initially large enhancement in mini frequency is only partially reflected in facilitation of evoked release at 1 min posttetanus. However, the slower phase of mEPSP frequency decay appears to parallel closely the decay of evoked EPSP facilitation. Both mEPSP frequency and EPSP amplitude remained significantly increased over pretetanus levels for 10 min, but not at later time points. This parallel between the posttetanus frequency of spontaneous release and facilitation of evoked release has been observed at neuromuscular synapses (Liley, 1956; Lev-Tov and Rahamimoff, 1980; Zengel and Magleby, 1981; Zucker and Lara-Estrella, 1983), and suggests that PTP is mediated by a mechanism common to both forms of release—that is, some process independent of excitation–secretion coupling. This finding is consistent with the model that high-frequency stimulation “mobilizes” vesicles, in some way making them more available for release (Gingrich and Byrne, 1985).

PTP at sensory-motor neuron synapses thus appears to be

quite similar to PTP at the neuromuscular junction and other synapses. One notable difference is the longer duration of sensory neuron PTP (up to 10 min) produced by fewer spikes than at other well-studied synapses. For example, the same 20 Hz, 2 sec tetanus produces facilitation with 47 and 130 msec time constants at the crayfish neuromuscular junction (Zucker and Lara-Estrella, 1983), and a 100 times larger tetanus (200 Hz for 20 sec) produces PTP that lasts only 80 sec at spinal cord 1A afferent synapses (Hirst et al., 1981). This difference, together with the fact that sensory neurons show profound depression to very low frequency stimuli, indicates that *Aplysia* sensory neurons are exquisitely sensitive to their recent history of activity.

PTP is well known to involve Ca^{2+} influx during the tetanus (Rosenthal, 1969; Weinreich, 1971). Various measures indicate that Ca^{2+} accumulates during trains of action potentials, and that the decay of $[\text{Ca}]_i$ is closely related to the decay of potentiation (Kretz et al., 1982; Connor et al., 1986; Delaney et al., 1989; Regehr and Tank, 1991; Swandulla et al., 1991). Moreover, preventing Ca^{2+} buildup with EGTA and other chelators blocks PTP (Kretz et al., 1982; Swandulla et al., 1991). Such findings support the early proposal that PTP is due to residual Ca^{2+} in the nerve terminal (Gage and Hubbard, 1966; Rosenthal, 1969; Miledi and Thies, 1971; Weinreich, 1971; Magleby and Zengel, 1975). In these early formulations, residual Ca^{2+} was thought to act directly on the Ca^{2+} -activated release machinery. More recent assessments suggest a different possibility. Transmitter release is now thought to be mediated by low-affinity Ca^{2+} binding sites requiring very high ($>100 \mu\text{M}$) local $[\text{Ca}]_i$ (Simon and Llinas, 1985; Adler et al., 1991; Roberts et al., 1991; Yamada and Zucker, 1992), whereas PTP is considerably more sensitive to $[\text{Ca}]_i$, varying approximately linearly with $[\text{Ca}]_i$ in the submicromolar range (Delaney et al., 1989; Regehr and Tank, 1991; Swandulla et al., 1991). These findings suggest that residual Ca^{2+} does not act directly on the exocytotic machinery but at another site, for example, a Ca^{2+} -dependent enzymatic reaction that modulates release by another mechanism.

The long duration of sensory neuron PTP seems especially to favor a mechanism whereby Ca^{2+} influx triggers a biochemical cascade producing facilitation, as opposed to residual Ca^{2+} acting directly on release. Various Ca^{2+} -dependent enzymes have been hypothesized to play such a role in PTP. Protein kinase C is one candidate, given the Ca^{2+} dependence of some of its isoforms, and the fact that it enhances both spontaneous and evoked release from many different synapses. However, our evidence indicates that protein kinase C activation is not necessary for the induction of PTP (Fig. 8A). Similar results have been reported for PTP in hippocampal slices using two other inhibitors of protein kinase C, polymyxin B and K-252b (Reymann et al., 1988a,b). H7 did reverse facilitation 2 min post-tetanus in our experiments, but it had a comparable effect on basal synaptic transmission, indicating that the effect is not specific to PTP (Fig. 8B). Other studies have also reported some reduction of basal synaptic transmission by H7 (Malinow et al., 1988; Muller et al., 1988). These effects could be specific, reflecting a role of basal protein kinase C activity in synaptic transmission, or they could be nonspecific, for example, due to H7 blocking postsynaptic transmitter receptors (Hirsh et al., 1990; Leidenheimer et al., 1990; Amador and Dani, 1991; Reuhl et al., 1992). Since Ghirardi et al. (1992) found no effect of H7 on sensory neuron transmission onto L7 motor neurons, its

Figure 12. Depression of evoked release is more profound and lasts longer than the decrease in mEPSC frequency following 1 Hz stimulation. *A*, Group data from five experiments like the one shown in Figure 11. Evoked release is profoundly depressed by stimulating at 1 Hz for 120 sec (80 sec in one experiment). By 4 min after the end of stimulation, the evoked EPSP has partially recovered, but remains significantly depressed compared to its initial amplitude. In contrast, mEPSC frequency is reduced for only the first 30 sec following stimulation, and fully recovers after that. Horizontal bars indicate the interval over which mEPSC frequency was sampled for each data point. For clarity, only the first and average of the last 10 evoked EPSPs of the train are depicted in *A*. The full course of evoked EPSP depression during 1 Hz stimulation is plotted in *B*, which shows the first five trials, followed by the average of groups of 10 trials.

inhibition of transmission onto LFS synapses observed here may be due to such a postsynaptic effect.

Two other candidate enzymes for a role in PTP are cAMP-dependent kinase and Ca^{2+} /calmodulin-dependent kinase (CaM kinase). Although the cAMP-dependent kinase itself is not Ca^{2+} dependent, Ca^{2+} /calmodulin stimulates adenylate cyclase, especially cyclase in the nervous system (Eliot et al., 1989), and cAMP increases evoked release in sensory neurons (Brunelli et al., 1976; Braha et al., 1990). However, we recently demonstrated that the same 20 Hz, 2 sec tetanus has no effect on either

sensory neuron excitability or action potential width, both of which are sensitive to cAMP levels in sensory neurons (Eliot et al., 1994), suggesting that activity alone does not activate adenylyl cyclase.

CaM kinase may thus be a better candidate for a role in PTP. At the squid giant synapse, CaM kinase increases transmitter release in a Ca^{2+} -dependent manner, apparently by mobilizing vesicles for release (Llinas et al., 1985; Lin et al., 1990). A recent study showing reversible blockade of PTP by a specific peptide inhibitor directly implicates CaM kinase at the squid synapse (M. Hans, D. Swandulla, and G. J. Augustine, unpublished observations). If CaM kinase mediates PTP in *Aplysia*, however, it must be somewhat different from the CaM kinase in squid and hippocampal preparations, which are blocked by H7 (Malinow et al., 1988; Hans, Swandulla, and Augustine, unpublished observations).

Depletion may not be the primary mechanism of homosynaptic depression

Like depression in the ganglion, homosynaptic depression in culture is evidently presynaptically mediated. mEPSC or mEPSP size did not decrease when the EPSP was significantly depressed (Fig. 10*B*). This was true both for modest depression induced by stimulation at 5 min intervals and for profound depression induced by stimulation at 1 sec intervals. These results agree with previous studies on short-term synaptic depression (del Castillo and Katz, 1954; Castellucci and Kandel, 1974) demonstrating that depression is due to a decrease in quantal content, but not in quantal size.

The dominant hypothesis for the presynaptic mechanism of depression at the vertebrate neuromuscular junctions is vesicle depletion (reviewed in Zucker, 1989). Depletion has also been proposed as one of the mechanisms contributing to depression of sensory neuron synapses (Gingrich and Byrne, 1985). Since PTP in sensory neurons is accompanied by an increased frequency of spontaneous release, suggesting it is mediated by vesicle mobilization, it seemed likely that depression would be associated with a decreased frequency of spontaneous release as a consequence of vesicle depletion. We therefore looked for changes in mini frequency during depression as an assay for vesicle depletion. This approach assumes that evoked and spontaneous release are mediated by a common pool of vesicles, which is supported by their parallel modulation during PTP as well as during four other instances of plasticity in sensory neurons: both are (1) increased by 5-HT, (2) decreased by the tetrapeptide FMRF (Dale and Kandel, 1990), (3) increased by phorbol esters (Braha et al., 1990; Ghirardi et al., 1992), and (4) increased by tonic depolarization due to Ca^{2+} influx through L-type channels (Edmonds et al., 1990). Such parallel modulation suggests that the pool of vesicles released spontaneously is the same as the pool of vesicles released by action potentials. If depression of evoked release is due to vesicle depletion, it may then be detectable as a decrease in mini frequency.

We did detect a decrease in spontaneous release in one instance of depression—immediately following fairly extensive stimulation (80–120 spikes at 1 Hz; Fig. 12). This 50% decrease occurs in spite of a 20–70 nM elevation of resting presynaptic Ca^{2+} measured in imaging experiments during the same 30 sec time period immediately following the same stimulation protocol (Eliot, 1991). Since elevated $[\text{Ca}]_i$ generally increases the probability of release, the fact that mEPSC frequency is reduced at a time when resting Ca^{2+} is actually enhanced suggests that

the number of available vesicles is truly limiting, or depleted, for the first 30 sec after stimulation. This conclusion is consistent with morphological evidence that the number of vesicles directly apposed to presynaptic active zones is reduced immediately after extensive sensory neuron stimulation (Bailey and Chen, 1988).

In contrast to this apparent depletion that occurs immediately after extensive stimulation, mini frequency recovers rapidly, and well in advance of evoked release (Fig. 12). This finding is similar to two other situations in which depression of evoked release was not paralleled by a change in mini frequency. First, stimulation at 5 min intervals reduces evoked release without altering spontaneous release frequency (Fig. 9). Second, in PTP experiments, spontaneous release frequency was maximal immediately after tetanization, even though evoked release was substantially depressed at the end of the tetanus (Fig. 5).

Depletion thus appears to contribute significantly to depression only during and immediately after extensive stimulation. Although Bailey and Chen (1988) saw a reduced number of vesicles adjacent to the active zone immediately after stimulation, they did not look at later time points. Indeed, the rapid recovery of spontaneous release we observed may be explained by their finding that the total number of vesicles in presynaptic terminals is not altered immediately after extensive stimulation, despite the decreased fraction at active zones (Bailey and Chen, 1988).

Taken together, our spontaneous release data suggest that depletion is not the primary mechanism of synaptic depression in sensory neurons. However, this conclusion depends on the assumption that evoked and spontaneous release are mediated by the same pool of vesicles. While the above-cited instances of parallel modulation support this assumption, it is possible that evoked and spontaneously released pools of vesicles overlap without being identical. For example, if evoked release utilizes just a small subset of a common pool of releasable vesicles, this subset could be depleted during depression without detectably depleting the total pool. This could occur, for example, if evoked release uses only a small fraction of the total number of presynaptic terminals, whereas spontaneous release occurs from most or all terminals. Alternatively, the evokable subset could be the vesicles immediately apposed to the active zone in each terminal, as implicitly proposed by Bailey and Chen (1988), while spontaneous release might utilize the entire population of vesicles in each bouton. This possibility seems to require the additional assumption that refilling or redocking of the subset involved in evoked release is slow or in some way inactivated, since both our spontaneous release data and electron micrographs of depressed terminals suggest a large population of vesicles is available for this purpose.

Other possible mechanisms contributing to presynaptic depression

Sensory neuron depression differs in a fundamental way from depression in other synapses thought to be subject to depletion in that it does not vary with the starting level of transmitter release. At both the squid synapse (Kusano and Landau, 1975; Swandulla et al., 1991) and neuromuscular junction (del Castillo and Katz, 1954; Thies, 1965), depression is more profound when quantal content is increased by elevating extracellular Ca^{2+} , while it is reduced or even, overridden by facilitation when release is reduced using low extracellular Ca^{2+} solutions. Sensory neuron depression, in contrast, occurs at all levels of release,

whether due to changes in extracellular Ca^{2+} (Kandel, 1976) or natural variation between synapses (Eliot, Kandel, and Hawkins, unpublished observations), and is thus less likely due to depletion. Zucker and Bruner (1977) reached a similar conclusion regarding depression at the crayfish fast flexor neuromuscular junction, which is similarly insensitive to starting level of release, shows kinetics of depression comparable to sensory neurons, and was found to be inadequately described by a depletion model.

The dissociation between evoked and spontaneous release in depression is similar to the case of pairing-specific, activity-dependent facilitation at sensory neuron synapses. In the latter form of plasticity, evoked release was enhanced in an associative manner by pairing tetanus with 5-HT application, but spontaneous release exhibited no such associative enhancement (Eliot et al., 1994). This finding suggested that the associative mechanism involves processes unique to evoked release and, indeed, paired training was found to enhance broadening of the sensory neuron action potential, presumably increasing Ca^{2+} influx and, thereby, evoked release. The fact that mini frequency is not reduced during depression suggests that depression may also be mediated by a mechanism unique to evoked release or excitation-secretion coupling.

One mechanism that has already been implicated in sensory neurons is slow Ca^{2+} current inactivation (Klein et al., 1980). Reduction in Ca^{2+} current has also been correlated with synaptic depression in cultured dorsal root ganglion cells (Jia and Nelson, 1986). However, Pieroni and Byrne (1992) demonstrated depression in the absence of changes in tetraethylammonium-broadened action potentials in sensory neurons, which should be a sensitive measure of total Ca^{2+} current. This issue thus needs to be reassessed by isolating and studying the inactivation properties of the dihydropyridine-insensitive, high-threshold Ca^{2+} current that underlies synaptic transmission in sensory neurons (Edmonds et al., 1990). Preliminary Ca^{2+} imaging studies support a reduction in Ca^{2+} influx with repeated activation, particularly when influx through these dihydropyridine-insensitive channels is isolated using the dihydropyridine antagonist, nitrendipine (Eliot, 1991). Alternatively, depression may be mediated by inactivation of excitation-secretion coupling downstream from Ca^{2+} influx, such as a change in the Ca^{2+} dependence of vesicle docking or fusion. A better understanding of homosynaptic modulation may thus await a more complete description of the mechanism of exocytosis.

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