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Presynaptic Induction and Expression of Homosynaptic Depression at *Aplysia* Sensorimotor Neuron Synapses

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The cellular mechanisms underlying the induction and expression of homosynaptic depression at the glutamatergic synapse between *Aplysia* sensory and motor neurons were studied in dissociated cell culture. Intracellular microelectrodes were used to stimulate action potentials in the presynaptic sensory neuron and record the depolarizing EPSP from the motor neuron. Homosynaptic depression (HSD) was induced by repeatedly stimulating the sensory neuron at rates as low as one action potential per minute. Activation of postsynaptic Glu receptors was neither sufficient nor necessary to induce HSD. Thus, repeated applications of exogenous Glu did not depress the synaptically evoked EPSP. Moreover, normal HSD was observed when the sensory neuron was stimulated during a period when the Glu receptors were blocked with the antagonist DNQX. The induction of HSD is thus likely to occur within the presynaptic terminal. We explored the role of presynaptic calcium in the induction of HSD by injecting the sensory neuron with EGTA, a relatively slow calcium chelator that does not alter rapid release but effectively buffers the slow residual calcium transient thought to be important for plasticity. EGTA had little effect on HSD, indicating that residual Ca_i is not involved. HSD does not appear to involve a decrease in presynaptic calcium influx, because there was no change in the presynaptic calcium transient, measured by calcium indicator dyes, during HSD. We conclude that HSD is induced and expressed in the presynaptic terminal, possibly by a mechanism directly coupled to the release process.

Key words: synaptic transmission; synaptic plasticity; Glu receptors; presynaptic calcium; transmitter release; learning and memory

Synaptic plasticity is an important aspect of neuronal function that underlies certain forms of memory and learning in both invertebrates and vertebrates (Hawkins et al., 1993). It occurs by both heterosynaptic mechanisms in which one synaptic input modifies the efficacy of a second and by homosynaptic mechanisms that are intrinsic to a single synapse. Homosynaptic depression (HSD), a progressive decrease in the amplitude of the postsynaptic potential in response to successive presynaptic stimuli, is one of the simplest examples of synaptic plasticity. Compared with other forms of plasticity that require trains of presynaptic action potentials, such as long-term potentiation (Bliss and Lomo, 1973) and long-term depression (Dudek and Bear, 1992), HSD is intriguing because even a single presynaptic action potential is sufficient to produce a long-lasting change in synaptic strength. This may imply that HSD involves a mechanism that is fundamental to the transmitter release process itself.

Although HSD has been characterized in a number of systems, its mechanism of induction and expression are not completely understood. Here, we investigate the properties of HSD at the well characterized synapse between *Aplysia* mechanoreceptor sensory neurons and their target motor neurons, in which this form of plasticity is thought to underlie behavioral habituation (Castellucci et al., 1970). At this synapse, HSD is particularly robust and occurs at presynaptic firing rates as low as once every 5 min (Byrne, 1982).

Studies using quantal analysis at the sensorimotor neuron synapse in the abdominal ganglion have shown that HSD is caused by a decrease in transmitter release, with no postsynaptic change in the quantal amplitude (Castellucci and Kandel, 1974; Eliot et al., 1994). However, the mechanism underlying the decrease in transmitter release has not been identified. Although a decrease in presynaptic calcium current caused by prolonged inactivation has been suggested as a potential mechanism (Klein et al., 1980), a modeling study indicated that depletion of synaptic vesicles may also be required (Gingrich and Byrne, 1985). Indeed, ultrastructural studies indicate that the number of synaptic vesicles docked at the active zone is decreased after multiple stimuli (Bailey and Chen, 1988). However, the extent of the decrease is not large enough to account for the decrease in EPSP amplitude.

Finally, it is not known whether HSD is induced postsynaptically, similar to long-term potentiation and long-term depression (Bear and Malenka, 1994), or whether presynaptic activity alone is sufficient. In the simplest model, the site of induction of HSD would be presynaptic, because the site of expression is presynaptic. However, many forms of plasticity do not conform to such a simple scheme. For example, the induction of posttetanic potentiation (PTP) of transmitter release at the *Aplysia* sensorimotor neuron synapse has recently been shown to require postsynaptic depolarization and calcium influx (Bao et al., 1997).

To address the mechanism of expression and induction of HSD, we studied the monosynaptic connection between a single *Aplysia* pleural sensory neuron and postsynaptic L7 motor neuron in dissociated cell culture. The transmitter at this synapse is most likely Glu (Dale and Kandel, 1993; Trudeau and Castellucci,

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1993), acting on postsynaptic receptors similar to vertebrate NMDA receptors (Dale and Kandel, 1993). Our data indicate that both the induction and the expression of HSD involve presynaptic mechanisms. Furthermore, the depression of transmitter release occurs downstream of calcium influx.

MATERIALS AND METHODS

Identified *Aplysia* pleural sensory neurons and L7 motor neurons were grown together in cell culture to form synapses as described previously (Rayport and Schacher, 1986). Cells were grown in a solution containing 50% Leibovitz's L-15 medium and 50% hemolymph. The L-15 medium (Sigma, St. Louis, MO) was supplemented to yield a solution with the following final salt concentrations (in mM): 397 NaCl, 9.9 KCl, 11.4 CaCl2, 29 MgCl2, and 29.3 $MgSO_4$, adjusted to pH 7.6 with NaOH. During experiments, cells were bathed in a solution containing 50% L-15 and 50% artificial seawater (ASW) (in mM): 460 NaCl, 10 KCl, 11 CaCl2, 55 MgCl2, and 10 HEPES, adjusted to pH 7.6 with NaOH).

A gravity-fed multichamber microperfusion system was used to apply most solutions to the cells, and the reservoirs were enclosed in tin foil to protect the solutions from light. DNQX (Sigma) was used as a 10 μM solution in L-15–ASW and was applied by microperfusion. To ensure a rapid solution exchange necessary for fast and thorough washout of DNQX, the total volume around the cells was maintained at ~25 μl. The L-type calcium channel antagonist nitrendipine (a gift from Miles Pharmaceuticals) was applied at a concentration of 10 μM dissolved in 0.1% EtOH and L-15–ASW and was applied by microperfusion. Serotonin (Sigma) was prepared at a concentration of 10 μM in L-15–ASW and was applied by microperfusion. Glu (Sigma) was dissolved to a concentration of 10 mM in L-15–ASW and adjusted to pH 7.6. This solution was pressure-applied through a pipette with an opening 1–5 μm in diameter, located 10–15 μm from the site of synaptic contact, with a Picospritzer (General Valve, Fairfield, NJ) set to deliver a 100 msec pulse at 15 psi.

Electrical recording was performed using intracellular microelectrodes. When substances were to be injected intracellularly, the electrodes were pulled to have an initial resistance of 50–70 MΩ when filled with a 0.5 M NaCl solution and then beveled to 30–40 MΩ. All other electrodes were pulled to a resistance of 5–15 MΩ and filled with 3.0 M KCl. Recordings were made using an Axoclamp-2A amplifier in bridge mode. Action potentials were generated by passing 2–8 nA depolarizing current for 5 msec into the sensory neurons. Electrical responses were recorded in analog form on tape. For some experiments, data were also recorded in digital form directly to the computer using the pCLAMP (version 5.5 and 6.0.2.; Axon Instruments, Foster City, CA) acquisition program CLAMPEX. The amplitude of the postsynaptic response was determined using CLAMPAN (version 5.5) or CLAMPFIT (version 6.0.2).

For calcium imaging experiments, the presynaptic sensory neuron was loaded with the free acid form of calcium Green-1 (Molecular Probes, Eugene, OR) by iontophoresis from an intracellular microelectrode. The microelectrode was filled with a solution containing 10 mM of the dissolved dye in 0.5 M KCl. Five hundred millisecond hyperpolarizing current pulses (0.1–0.5 nA) applied at 1 Hz for 10–20 min were used to eject the dye. Based on the intensity of staining, we estimate the final dye concentration to be 50–100 μM.

In some experiments, cells were injected with the calcium buffer EGTA from an electrode filled with 100 mM or 1 M K_2EGTA. Rhodamine was included in the electrode to determine when the EGTA had diffused to the region of the presynaptic terminals and to estimate its concentration there. This process took 20–30 min and resulted in an EGTA concentration that was 1–10% of that included in the pipette.

Cells were viewed on a Zeiss (Oberkochen, Germany) IM-35 inverted microscope with an Olympus Optical (Tokyo, Japan) 40×/1.30 NA oil immersion objective. Illumination was provided by a Xenon arc lamp and was filtered through a standard FITC filter set. Images were collected by a Hamamatsu intensified CCD camera and stored in analog form on a Panasonic TQ-2026F optical memory disk recorder. The storage of video images to disk was triggered by an electronic signal synchronized with a current pulse that stimulated action potentials in the presynaptic sensory neuron. In this way, full frame images, temporally correlated with presynaptic action potentials, were collected at video rates.

Image analysis was accomplished using the VIDEOPROBE program (ETM Systems, Irvine, CA). We designated several "areas of interest" within a cell, and then the program calculated the average fluorescence intensity of each area in all frames. Regions were selected based on their location (e.g., presynaptic varicosities that contacted the primary motor neuron axon), their morphology (local swellings that were >50% of the neurite diameter), and/or their calcium responses (uniform increase in intensity that was >5 times the SD of the baseline). For each area of interest, the resting intensity values from the five frames before the onset of the train (F) were averaged, as were the intensity values from the five frames starting at the peak value of the calcium concentration transient (Fpeak). The relative amplitude of the calcium transient was then approximated by the ratio $\Delta F/F$ = (Fpeak - F)/F. We did not correct for background fluorescence before loading with indicator dye, because this was negligible compared with the dye signal (<10%).

For all statistical analyses of significance, a paired Student's t test was used. Error values and error bars reflect SE of the mean.

RESULTS

Effects of postsynaptic activation on HSD

An initial set of experiments was aimed at elucidating the site of induction of HSD. As shown in Figure 1*A*, repeated stimulation of the presynaptic sensory neuron at a rate of one action potential per minute induced a cumulative depression in the EPSP recorded from the motor neuron, which reached a steady-state level after five to seven presynaptic stimuli, which was ~30% of the initial EPSP amplitude. To test whether postsynaptic activity alone is sufficient to induce HSD, we gave repeated pulses (100 msec) of exogenous Glu to induce depolarizing postsynaptic responses of similar magnitude to the synaptically generated EPSPs (Fig. 1*A*). To maximize the likelihood that the same receptors activated in response to sensory neuron stimulation were also activated by the exogenous Glu, we applied the transmitter to the region of the motor neuron that was innervated by the sensory neuron, as determined by imaging of presynaptic terminals. In any given motor neuron, the amplitude of the depolarization in response to exogenous Glu ranged from 0.2 to 3 times the amplitude of the EPSP evoked by presynaptic stimulation. Unlike the evoked responses, the depolarizing responses to repetitive Glu applications at 1 min intervals showed no significant change in average amplitude (Fig. 1*B*). This suggests that either the induction and/or the expression of HSD must have a presynaptic component.

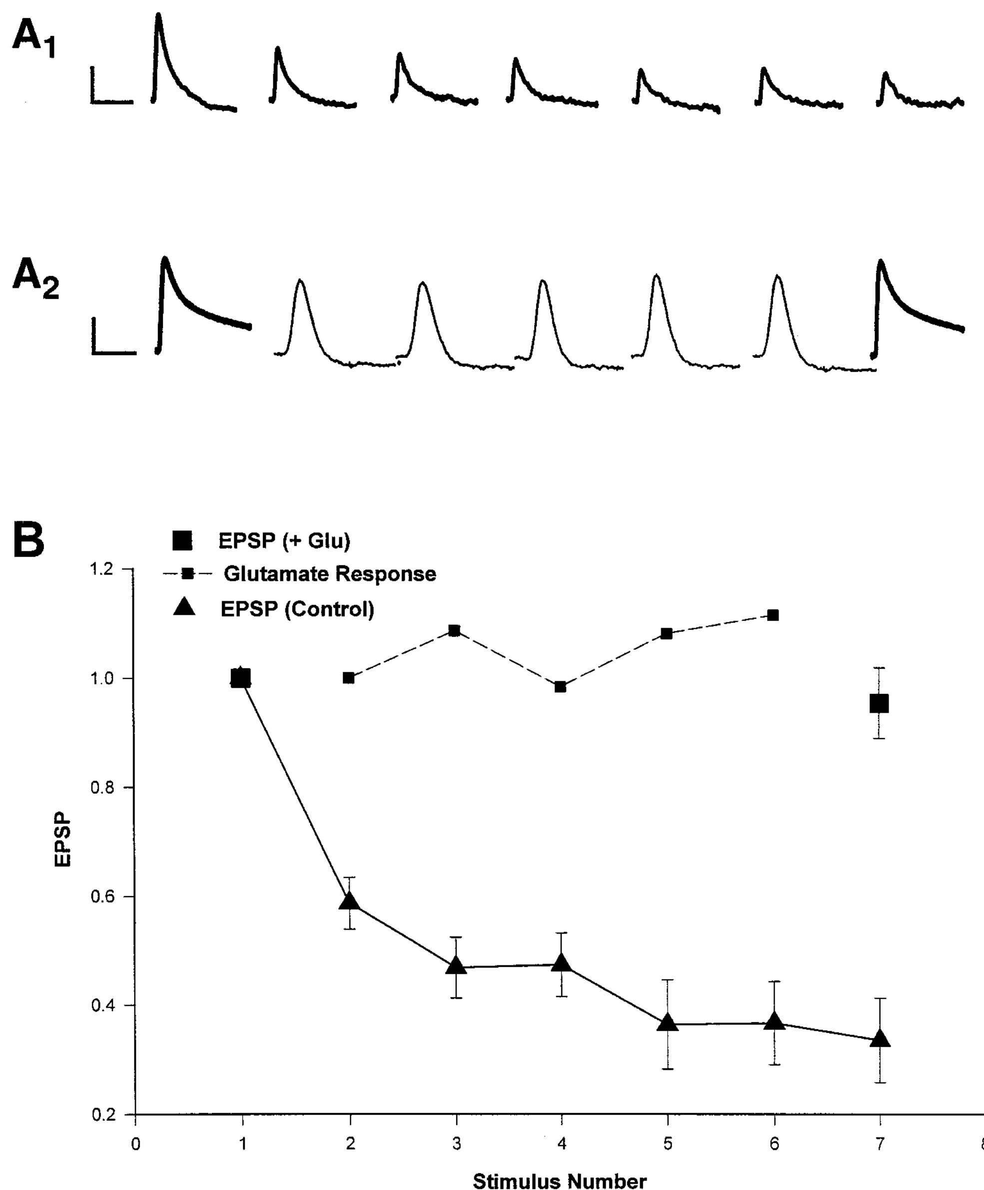

Figure 1. Glu receptor activation is not sufficient to induce HSD. *A*, Effect of Glu at a representative sensory to motor neuron synapse. *A1*, HSD in response to low-frequency presynaptic stimulation. EPSPs were recorded from a postsynaptic motor neuron in response to intracellular stimulation of presynaptic action potentials in a sensory neuron. Responses were elicited at 1 min intervals. *A2*, The effect of exogenous Glu application on synaptically evoked EPSP. The *first trace* (*bold*) shows the first EPSP recorded from a motor neuron in response to firing an action potential in the presynaptic sensory neuron. Five brief Glu pulses (100 msec) were then applied from a puffer pipette to elicit postsynaptic depolarizations (*thin traces*) of approximately equal amplitude to the synaptically evoked EPSP. The *final trace* (*bold*) is a second synaptically evoked EPSP. All responses were elicited at 1 min intervals. Calibration: *A1*, 10 mV, 100 msec; *A2*, 10 mV, 250 msec. *B*, Summary of mean data for Glu applications. Protocol is identical to that shown in *A*. For each synapse analyzed using presynaptic stimulation, the amplitudes of the EPSPs evoked during successive presynaptic stimuli were normalized to that of the first EPSP (*triangles*, *Control*). The amplitudes of depolarizing responses to exogenous Glu were normalized to the first response to Glu (*small squares*, *+Glu*). In the same experiments, the EPSP evoked by presynaptic stimulation after the Glu pulses was normalized to the first evoked EPSP before the Glu pulses (*large squares*). The normalized responses were then averaged among the different cells. *Triangles*, $n = 4$; *squares*, $n = 5$. *Error bars* indicate SEM.

Although postsynaptic receptor activation does not depress the response to exogenous Glu application, it is possible that it may depress the EPSP evoked by presynaptic stimulation. To investigate this possibility, we measured the postsynaptic response to a presynaptic action potential before and after five repeated applications of exogenous Glu. The amplitude of the evoked response 1 min after the last of five Glu applications was not significantly different from the amplitude of the first evoked response (95 ± 6 vs 100%; $n = 5$; $p > 0.25$) (Fig. 1*B*). This is in sharp contrast to the result when five presynaptic action potentials were substituted for the five exogenous applications of Glu. In this case, a robust depression occurred in which the amplitude of the last EPSP was significantly different from that of the initial event (34 ± 8 vs 100%; $n = 4$; $p < 0.01$) (Fig. 1*B*).

Effects of presynaptic activation on HSD

The above results show that postsynaptic Glu receptor activation is not sufficient to induce HSD. The next question we addressed was whether activation of the postsynaptic receptors that underlie the fast EPSP is necessary for the induction of HSD or whether presynaptic activity alone is sufficient. We evoked presynaptic action potentials while the postsynaptic Glu receptors were blocked with DNQX (Fig. 2), an effective antagonist of the Glu receptors at this synapse (Dale and Kandel, 1993). After the stimulation of a presynaptic action potential to assay the initial synaptic strength, 10 μM DNQX was applied to the cells for 10 min. Then, in the continued presence of DNQX, the presynaptic neuron was triggered to fire an action potential five times at 1 min intervals. No postsynaptic responses were observed in response to these stimuli because of the blockade of Glu receptors. To test whether the presynaptic stimuli were still able to elicit depression, even with the postsynaptic receptors inhibited, DNQX was washed out of the bath for 10 min. The synaptic strength was retested with a second series of five presynaptic stimuli at 1 min intervals ($n = 5$). The evoked EPSP recorded after washout of DNQX was substantially depressed compared with the first EPSP (Fig. 2). EPSPs generated in response to subsequent presynaptic action potentials did not show any additional depression, suggesting that the eight presynaptic stimuli in the presence of DNQX

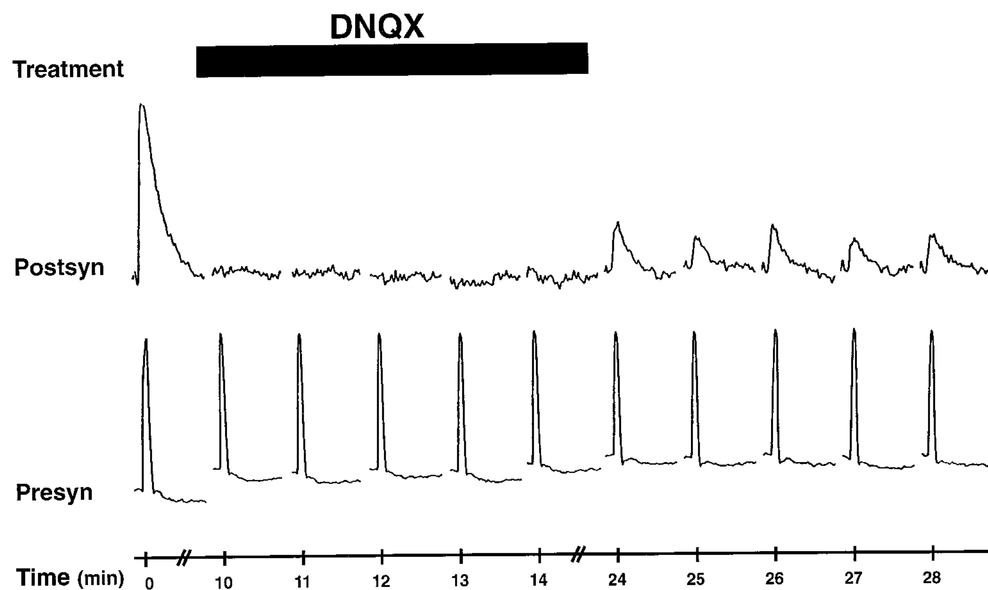

Figure 2. Effect of blockade of postsynaptic Glu receptors on induction of HSD. EPSPs were recorded from a postsynaptic motor neuron (*top traces*) in response to intracellular stimulation of action potentials in a presynaptic sensory neuron once per minute (*bottom traces*). After the first stimulus, 10 μM DNQX (*filled bar*) was applied by microperfusion. After the fifth stimulus, presynaptic stimulation was halted, and DNQX was washed out for a period of 10 min. Presynaptic stimulation was then resumed.

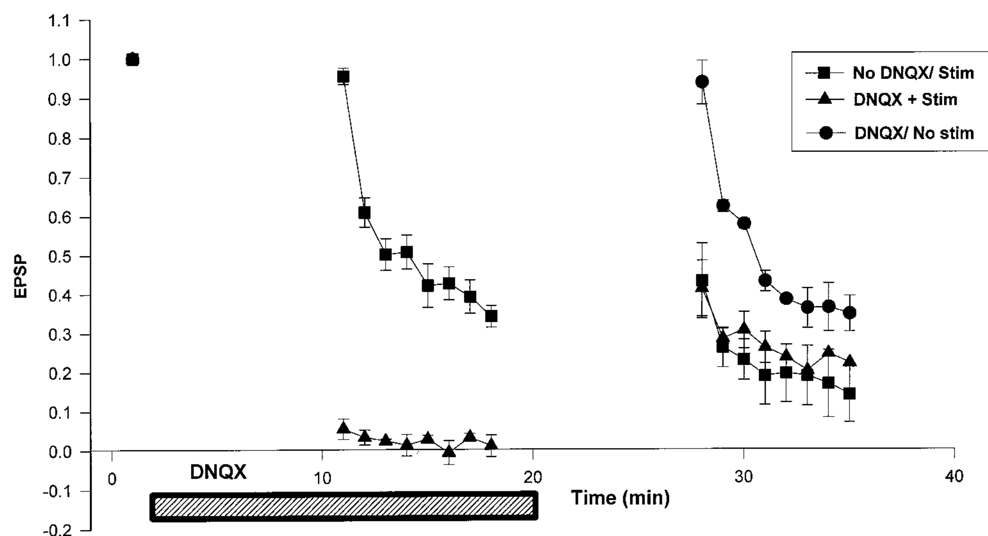

Figure 3. Activation of ionotropic Glu receptors is not required for induction of HSD. Mean data shown for induction of HSD during three experimental protocols to test the effects of DNQX. The first protocol is similar to that shown in Figure 2 (*triangles*). The presynaptic cell was first stimulated to evoke an EPSP (*left point*, 0 min). Cells were then exposed to 10 μM DNQX for 10 min (*bottom bar*). In the continued presence of DNQX, eight additional presynaptic stimuli were applied at a rate of one per minute (*middle points*, 11–18 min). The DNQX was then washed from the bath for 10 min, and eight more presynaptic stimuli were applied (*right points*, 28–35 min). The second protocol was identical to the first, except that the cells were not exposed to DNQX (*squares*). In the third protocol, cells were exposed to DNQX as in the first protocol, except that the first group of eight presynaptic stimuli during DNQX were omitted (*circles*). For each cell, EPSP amplitudes were normalized to the first EPSP, and then the normalized values in each group were averaged. $n = 5$ for each protocol.

were sufficient to elicit a steady-state level of depression. Preliminary experiments with control cells indicated that the five action potentials in the first stimulation series were not always sufficient to achieve a steady state of depression. Therefore, in most subsequent experiments, trains of eight action potentials were used (Fig. 3).

To compare the extent of depression when the presynaptic cell alone was activated to that observed when activity occurred in both cells, a group of control cells received an identical presynaptic stimulation protocol but without DNQX application (Fig. 3). The amplitudes of all EPSPs in a given cell were normalized to the initial response to allow comparisons among cells. There was virtually no difference in the extent of HSD between the DNQX-treated cells and the control cells, as determined by the average normalized amplitude of the EPSPs in response to the second series of presynaptic stimuli ($n = 5$).

A trivial explanation for the decreased EPSP amplitude after the presynaptic stimuli in the presence of DNQX is that the postsynaptic receptors had not fully recovered from blockade, so that the postsynaptic response remained partially inhibited, independent of any HSD. To rule out this possibility, a second control experiment was performed in which a group of cells was treated identically to the DNQX-treated cells (as above) but without the first series of presynaptic action potentials ($n = 5$). For these cells, the response to the first stimulus of the second series of action potentials showed no significant decrease as a result of the DNQX treatment when compared with the initial EPSP, demonstrating that 10 min was sufficient for complete washout of DNQX (Fig. 3). From these experiments, we conclude that presynaptic stimulation alone is sufficient to induce robust HSD independent of postsynaptic receptor activation.

Induction of HSD is not mediated by residual presynaptic Ca_i

Because HSD appears to be both induced and expressed presynaptically, we next investigated the potential role that presynaptic

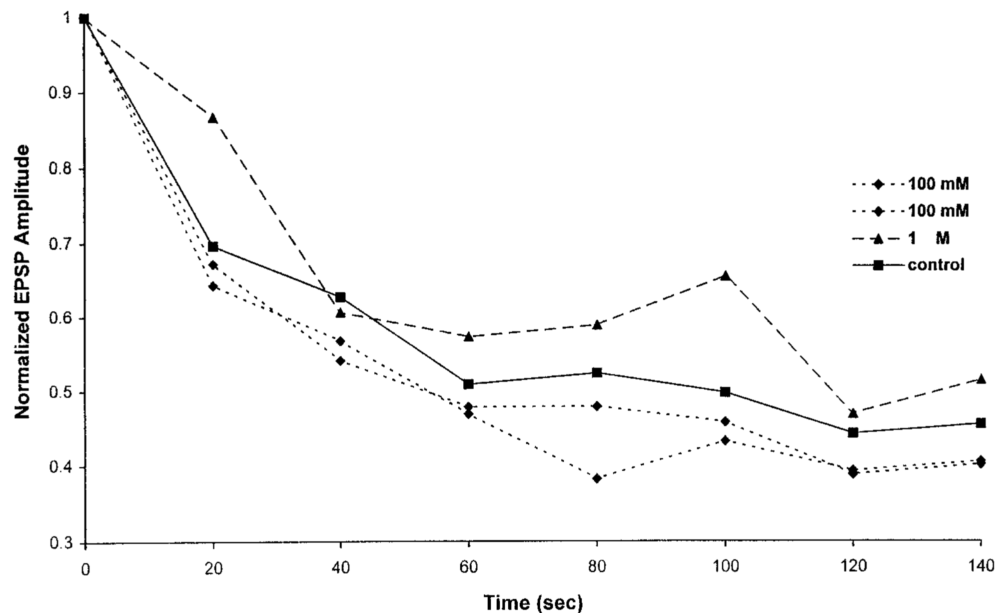

Figure 4. Presynaptic injection of EGTA does not inhibit induction of HSD. Peak EPSP amplitudes from four motor neurons are plotted in response to successive stimulation of the sensory neurons. EPSP amplitudes are normalized to that of the first EPSP. In two cells, the presynaptic microelectrode contained 100 mM EGTA (*diamonds*), and in a third cell, the microelectrode contained 1 M EGTA (*triangles*). In a control cell, the presynaptic electrode did not contain EGTA (*squares*). The presynaptic cell was stimulated at 20 sec intervals. Before these measurements were obtained, solution was ejected from the presynaptic electrode using 500 msec pressure pulses.

Ca influx plays in the induction of HSD, given the important role of Ca_i in other forms of synaptic plasticity. Because a rise in Ca_i triggers transmitter release, it was necessary to dissociate any slow modulatory effect of increased Ca_i that may underlie HSD from the rapid transient Ca_i increase that mediates transmitter release. To accomplish this, we injected the slow calcium buffer EGTA into the presynaptic cell. At the squid giant synapse, EGTA has been shown to be relatively ineffective in altering fast transmitter release, presumably because the kinetics of Ca binding to the buffer are too slow to affect the large rapid Ca_i transient near the membrane (Smith et al., 1984; Adler et al., 1991). In contrast, EGTA does serve as an effective buffer for slow changes in Ca_i that occur on the time scale of milliseconds to minutes, blocking some forms of synaptic plasticity without altering release per se (Swandulla et al., 1991; Regehr et al., 1994).

We used two different concentrations of EGTA in the microelectrodes, 100 and 1 M, which should result in 1–10 or 10–100 mM concentrations of EGTA, respectively, at the axon terminals. This estimate was based on a comparison of rhodamine fluorescence in the electrodes and terminals. Rhodamine has a similar size (577 MW) to EGTA (380 MW), distributes uniformly throughout the cytoplasm, and has a fluorescence intensity sufficient to allow rapid measurement at low light levels, thereby minimizing photodamage.

Experimental support that EGTA actually reached the synaptic terminals at sufficient concentrations to buffer calcium effectively was provided by the observation that EGTA injection inhibited synaptic transmission. Thus, when the synapse was tested once before loading with EGTA and then stimulated 30 min later, the EPSP was decreased to 80 and 36% of its initial amplitude with electrodes containing 100 and 1 M EGTA, respectively. The 30 min interval between preloading and postloading stimuli is too long to produce any significant HSD. Thus, the decrease in synaptic strength most likely reflects altered Ca_i buffering by EGTA. Apparently, fast release at the *Aplysia* sensorimotor neuron synapse is more sensitive to EGTA than the squid giant synapse.

Despite the decrease in synaptic transmission, marked HSD persisted in response to a series of presynaptic action potentials in experiments using electrodes containing either 100 or 1 M EGTA. The magnitude and time course of the HSD was not substantially different from the HSD under control conditions. After eight presynaptic stimuli, the EPSP was decreased on average to 43% of its initial amplitude in control cells (n = 3), to 40% of its initial amplitude in cells injected with 100 mM EGTA (n = 2), and to 51% of its initial amplitude in a cell injected with 1 M EGTA (n = 1) (Fig. 4). Thus, HSD does not appear to depend on a residual increase in Ca_i (although we cannot rule out the possibility that there was some residual Ca_i transient that was not altered by the EGTA).

HSD is not associated with altered calcium transients in presynaptic terminals

We next investigated the expression of HSD by testing the hypothesis that the decrease in transmitter release is a result of inhibition of voltage-dependent calcium influx (Klein et al., 1980). The presynaptic neuron was filled with the fluorescent calcium indicator dye calcium Green-1 and imaged during a series of action potentials used to evoke release and elicit HSD. These experiments were performed in the presence of 10 μM nitrendipine to block the dihydropyridine-sensitive Ca channels present in the presynaptic terminals. This was necessary to ensure that we selectively measured Ca influx through the dihydropyridine-insensitive Ca channels, the type that mediate transmitter release at this synapse (Edmonds et al., 1990).

Two possible mechanisms for HSD that depend on changes in Ca influx were investigated. The first involves a progressive decrease in calcium influx into presynaptic terminals in response to successive stimuli. To address this possibility, we evaluated the Ca_i signals in presynaptic varicosities, local swellings of the thin axonal branches of the sensory neuron that have been shown to contain active zones (Glanzman et al., 1989), during the induction of HSD. Figure 5 shows that the Ca_i transient recorded in a single varicosity elicited by six successive action potentials delivered at 20 sec intervals did not decrease, despite a marked HSD.

We obtained images of Ca_i using the $\Delta F/F$ ratio at the peak of the Ca_i transient during each of the six action potentials. Although the Ca_i image obtained during a single action potential

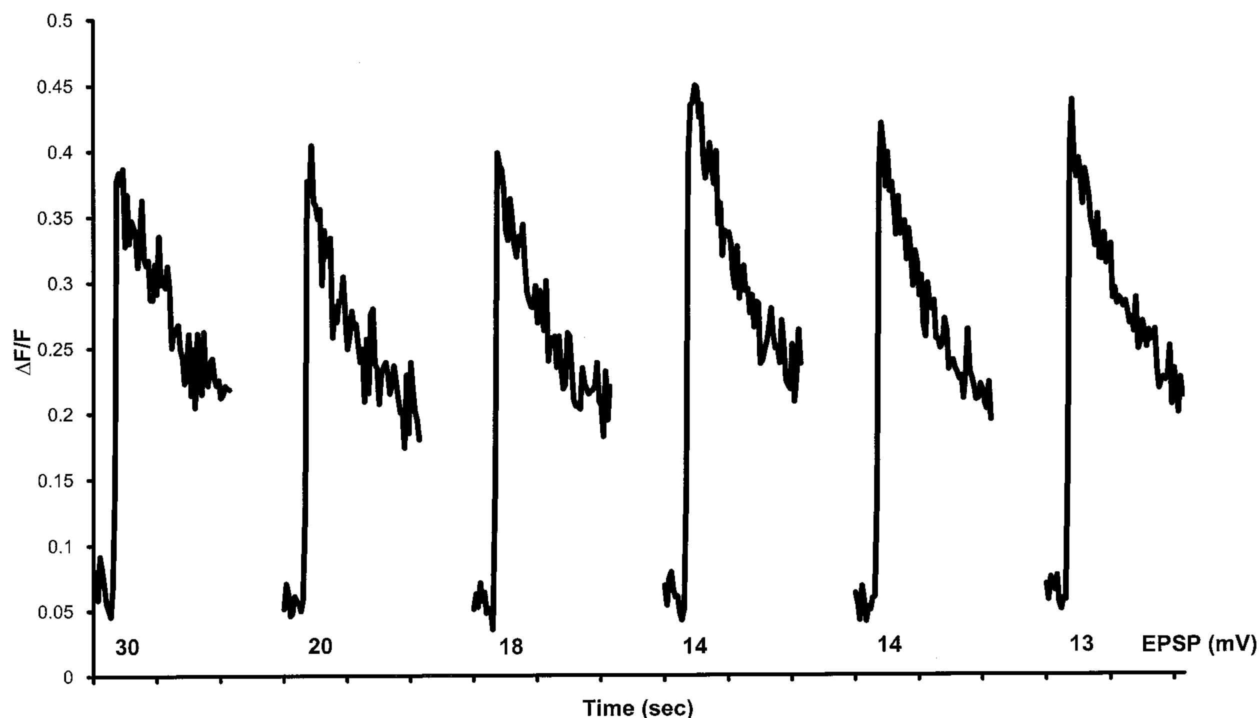

Figure 5. Ca influx in a single presynaptic varicosity does not change during induction of HSD. Ca_i transients, plotted as $\Delta F/F$, were imaged from a single presynaptic varicosity in response to stimulation of six presynaptic action potentials at 20 sec intervals. The peak EPSP amplitude recorded from the postsynaptic motor neuron in response to the same presynaptic stimulus is given *below* each calcium transient. Time is shown in 1 sec intervals.

was somewhat noisy, we could clearly resolve localized increases in Ca_i. Comparison of Ca_i images during the first and sixth action potential showed no marked differences (Fig. 6).

The presynaptic Ca_i transient was measured in a total of 38 varicosities from nine sensory cells while the EPSP was simultaneously recorded from the postsynaptic motor neuron. Whereas the EPSP produced in the motor neuron elicited by successive action potentials during the train showed a progressive depression, there was virtually no change in the mean size of the Ca_i transient elicited by successive action potentials (Fig. 7). The average amplitude of the calcium transient in response to the sixth stimulus was 107 ± 6% (n = 38) of the Ca_i transient amplitude in response to the first stimulus (100%; $p > 0.1$). In contrast, the average EPSP in these same cells in response to the sixth stimulus decreased to 37 ± 4% (n = 9) of its size relative to the first stimulus (100%; $p < 0.0005$). Therefore, despite the substantial decrease in transmitter release during HSD, there is no change in calcium influx in the presynaptic boutons.

Although we observed no change in the average Ca_i transient in these experiments, we considered a second possible mechanism for HSD that involves a decrease in Ca influx that could be obscured by this averaging. Thus, if HSD were caused by a failure of the action potential to invade a subset of presynaptic boutons, the Ca_i transient averaged among all boutons might not change dramatically (for example, if the Ca_i transient were to increase in boutons that remained excitable). To investigate this possibility, we followed the behavior of a large number of individual varicosities in response to the six action potentials triggered at 20 sec intervals (Fig. 8). Among varicosities that showed a Ca_i transient in response to the first action potential, there was never an instance in which a subsequent action potential failed to elicit a measurable change in calcium concentration. In only 3 of 38 varicosities did the transient fall below 50% of its initial value (Fig. 8). In addition, in sites in which there was a decrease in calcium transient amplitude, there were only two instances in which this decrease was maintained throughout the train of action potentials (subsequent responses being equal to or less than previous responses). In all the other cases, the amplitude of the Ca_i transient fluctuated up and down during the action potential train. Therefore, the number of presynaptic boutons responding to an action potential remained relatively constant and cannot explain the accompanying decrease in transmitter release caused by HSD.

DISCUSSION

The induction of HSD is presynaptic

The above results suggest that the induction of HSD is presynaptic. Thus, when the postsynaptic cell was stimulated by exogenous Glu, no depression resulted. A trivial explanation for this failure to induce HSD is that Glu is not the endogenous transmitter. Although definitive proof is lacking, two lines of evidence support Glu as the sensory neuron transmitter (Dale and Kandel, 1993; Trudeau and Castellucci, 1993). First, the biophysical properties of the postsynaptic response to the native transmitter closely resemble the response to exogenous Glu (Dale and Kandel, 1993). Both show a reversal potential close to 0 mV, and both display a flattened *I–V* curve at negative voltages in the presence of external Mg. After Mg removal, the *I–V* curve becomes linear. Second, some of the known Glu receptor antagonists, including CNQX and DNQX, block the responses to endogenous transmitter and to exogenously applied Glu to similar extents. Therefore, it is likely that even if Glu is not the endogenous transmitter, it induces a postsynaptic response that very closely mimics the endogenous response. A second explanation for the failure of exogenous Glu to induce HSD is that the agonist activated extrasynaptic receptors but failed to activate synaptic receptors,

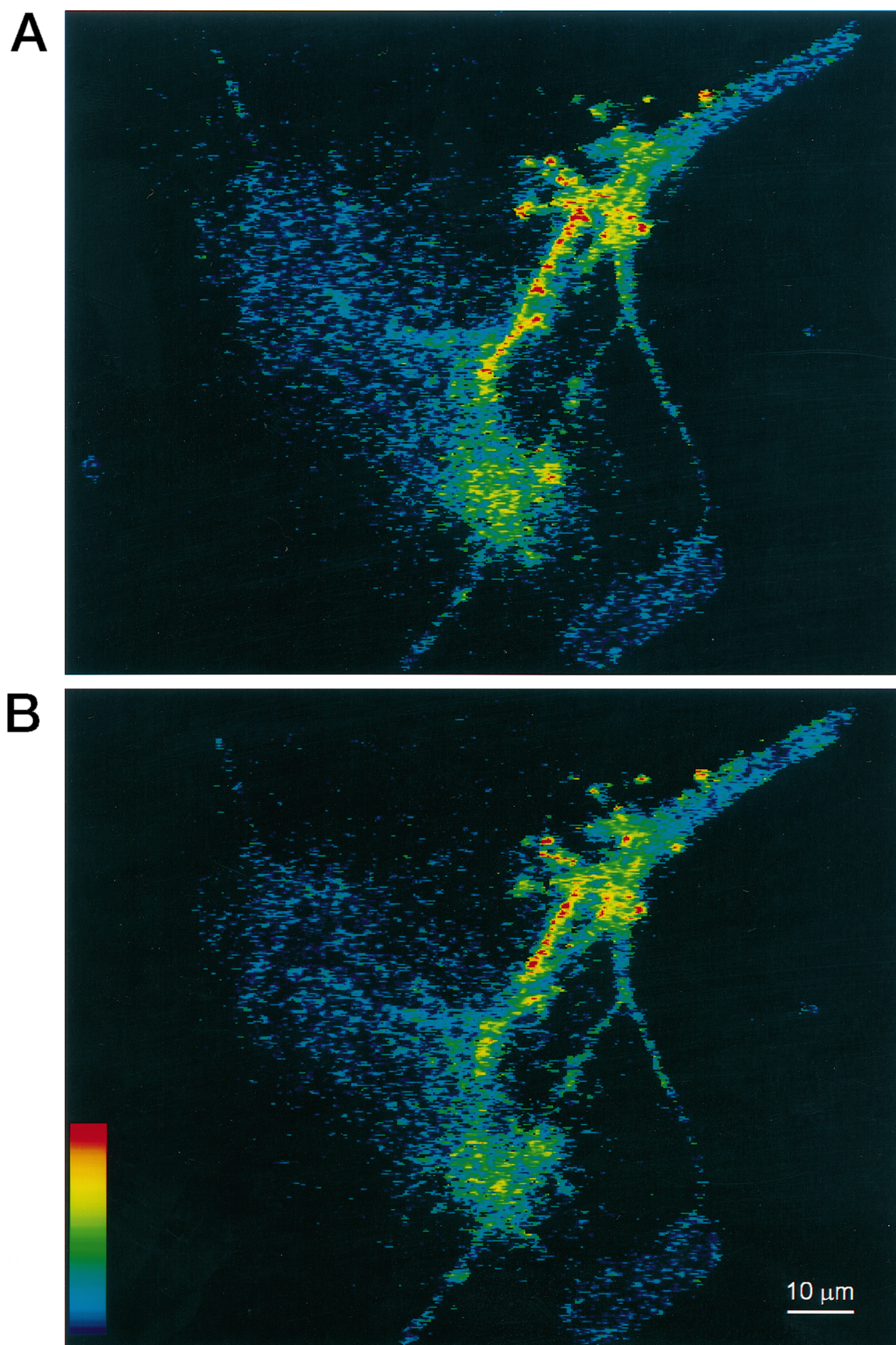

Figure 6. Spatial distribution of Ca_i influx does not change during HSD. Pseudocolor images of the peak calcium response ($\Delta F/F$) in a presynaptic sensory neuron elicited by a single action potential. *Red* indicates high calcium concentration; *blue* indicates low calcium concentration. *A* shows the Ca_i response to the first stimulus, that in *B* to the sixth stimulus, during a series of six action potentials, elicited at 20 sec intervals.

which mediate HSD. However, cross-desensitization experiments show that exogenous Glu can primarily block the EPSP elicited by presynaptic stimulation, indicative of an efficient activation of synaptic receptors by the exogenous Glu (S. Schacher, personal communication).

As a second approach to investigate the site of induction of HSD, we stimulated the presynaptic cell in the presence of DNQX to block the postsynaptic response. Under these conditions, the synapse became depressed to the same extent to that which occurred with stimulation in the absence of DNQX. Although the simplest interpretation of the above two results is that the induction of HSD is presynaptic, there are two schemes consistent with a role for the postsynaptic cell. In the first scheme, induction of HSD would result from the co-release of a substance with Glu from presynaptic terminals. This would explain why Glu application did not induce HSD and why depression occurred in the presence of Glu receptor antagonists. One candidate cotransmitter is the sensory neuron peptide sensorin (Brunet et al., 1991), which may be present in small clear synaptic vesicles, as indicated by staining of varicosities with indirect immunocyto-

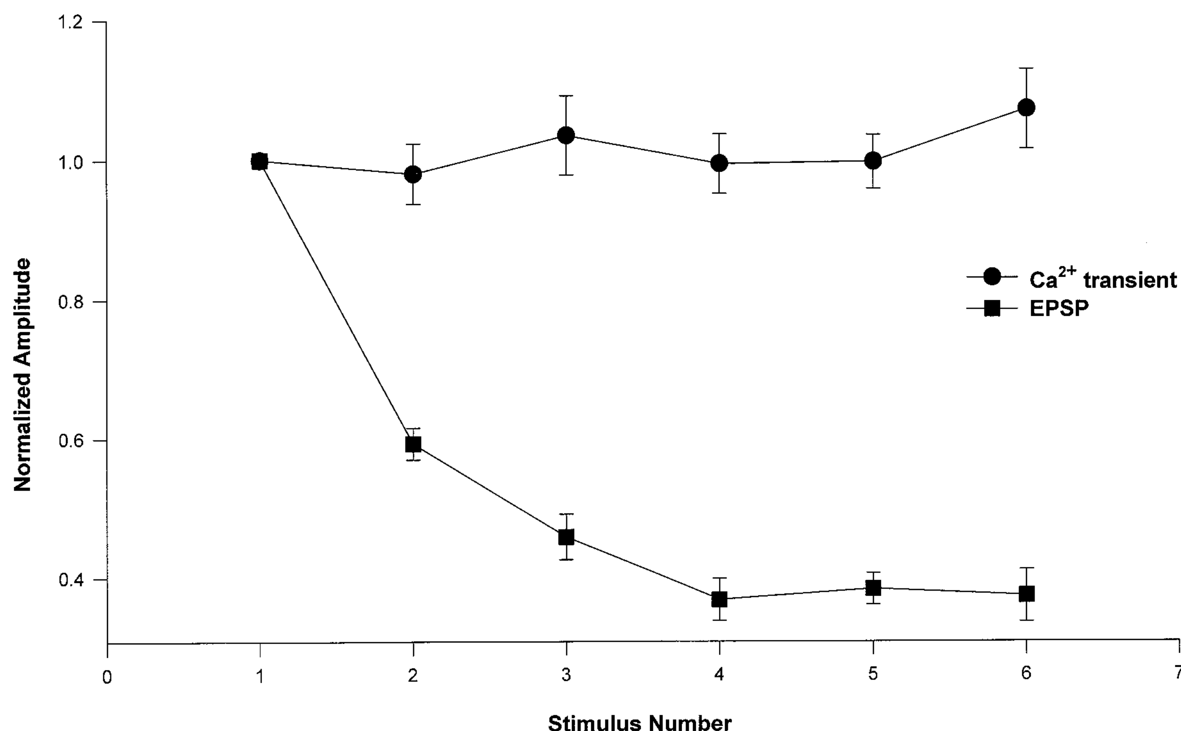

Figure 7. Mean data for Ca_i transient amplitude during HSD. The mean peak amplitude of the presynaptic Ca_i transient ($\Delta F/F$; *circles*) and the associated mean peak EPSP amplitude (*squares*) are plotted during successive presynaptic stimuli. A presynaptic action potential was elicited once every 20 sec. The average calcium transient in response to a given stimulus was calculated by first measuring the calcium transient in four to five individual varicosities per experiment. For each varicosity, the amplitude of the transient was then normalized to that of the first stimulus. These normalized values were then averaged for all the varicosities ($n = 38$) from all cells ($n = 9$). For each postsynaptic cell, the EPSP amplitudes were normalized to that of the first EPSP, and then the normalized values from all cells ($n = 9$) were averaged. *Error bars* indicate SEM.

chemistry (Santarelli et al., 1996). Application of exogenous sensorin elicits a hyperpolarizing response in certain postsynaptic cells (Brunet et al., 1991), although the physiological role of this peptide is currently unknown. Because DNQX completely inhibits the EPSP (Fig. 2), such substances probably do not directly gate postsynaptic ion channels. However, they could act through a modulatory second messenger-dependent postsynaptic action. In the second scheme, activation of DNQX-insensitive postsynaptic metabotropic Glu receptors would be necessary, but not sufficient, to induce HSD. For example, combined presynaptic activity and postsynaptic metabotropic Glu receptor stimulation may be required. Although we cannot rule out the above two hypotheses, we have not found any evidence for a slow modulatory component to the postsynaptic response in the presence of DNQX, as might be expected. Given the presynaptic site of expression of HSD, we favor the simple view that its induction is also presynaptic.

Residual calcium is not the induction event

There are three principal components of presynaptic activity that could induce HSD: (1) calcium influx, (2) transmitter release acting on presynaptic receptors, and (3) vesicle fusion.

The calcium influx during an action potential could trigger a second messenger pathway that modulates transmitter release. When calcium first enters the cell, it exists momentarily within domains of high concentration localized just under the membrane, which are required to trigger exocytosis of synaptic vesicles. The calcium then rapidly diffuses down its steep concentration gradient to the surrounding cytoplasm, producing a relatively small longer-lasting elevation in Ca_i concentration. This residual Ca_i has been implicated in other forms of synaptic plasticity, most notably PTP (Swandulla et al., 1991; Regehr et al., 1994; Fischer et al., 1997). In imaging experiments of the sensory neurons, the decay of the residual Ca_i transient had a time constant of ~2 sec (Fig. 5). To test the role of this residual Ca_i transient, the calcium buffer EGTA was injected into the presynaptic cell. The failure of EGTA to inhibit HSD is probably not attributable to a lack of effect on calcium buffering, because the EGTA injections diminished evoked release, which is relatively resistant to EGTA, in a dose-dependent manner. Thus, HSD is not likely to depend on the slow residual Ca_i transient, although HSD might be mediated by the more rapid Ca_i transient.

A second possible mechanism for HSD depends on a presynaptic feedback mechanism in which one or more transmitters act on presynaptic receptors to induce HSD. Activation of presynaptic Glu receptors alone is not sufficient to induce HSD, because application of exogenous Glu to the synaptic region did not change the synaptic strength. However, induction may require simultaneous receptor binding and presynaptic activity and/or binding of a cotransmitter (e.g., sensorin) to its presynaptic receptor.

The third possibility is that induction of HSD could result from the fusion event itself and not directly depend on an effect of any substances that are released. It is now known that there are numerous proteins on the vesicle surface, on the plasma membrane, and in the cytoplasm, which interact to regulate vesicle fusion (Sudhof, 1995). It is possible that the fusion event itself alters one or more of these proteins such that the probability of fusion occurring in response to the next stimulus is decreased.

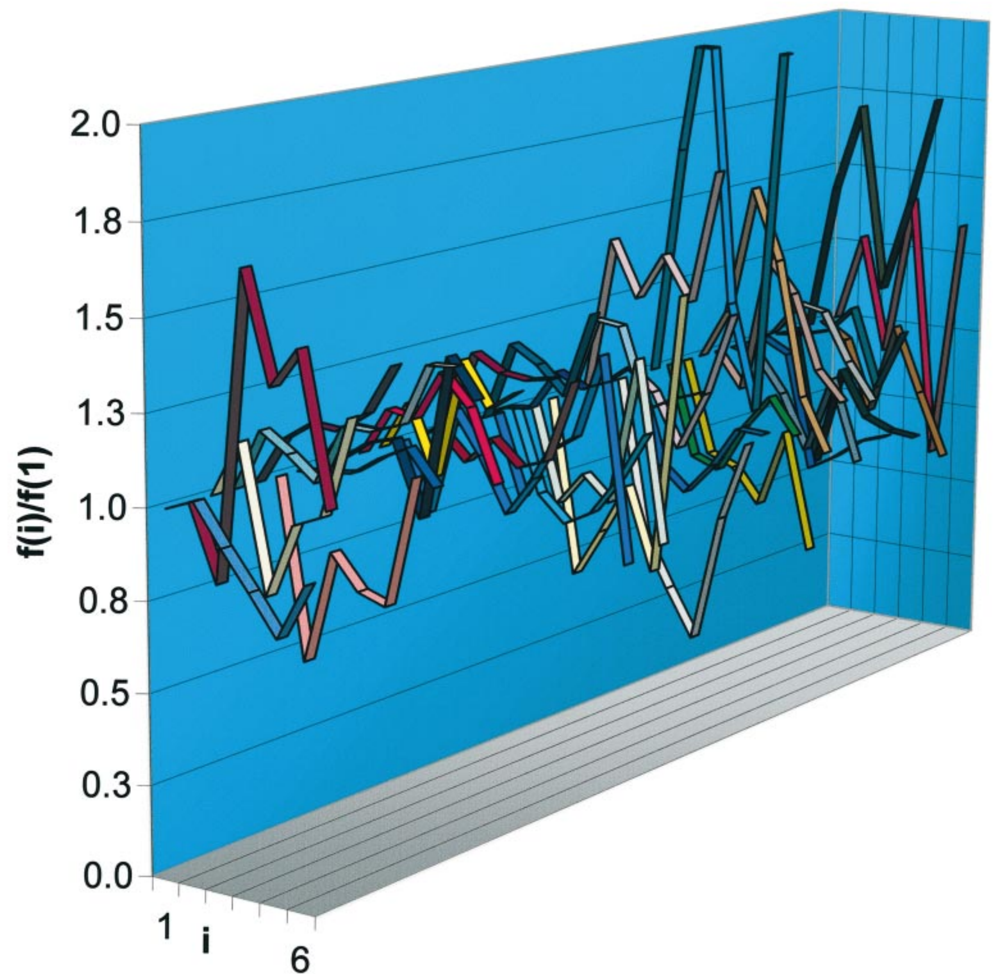

Figure 8. Multiple stimuli activate a constant number of varicosities. Calcium responses in individual varicosities from presynaptic sensory neurons were measured in response to successive action potentials used to induce HSD (n = 38). For each varicosity, the amplitude of the calcium transient in response to each successive action potential, $\Delta F(i)/F$, was normalized to that of the first action potential, $\Delta F(1)/F$, yielding the ratio $f(i)/f(1)$. Normalized Ca_i transients during six successive action potentials are plotted as a function of action potential number (i). Each varicosity is represented by a separate *line*. Action potentials were stimulated once every 20 sec.

Such an alteration could involve a conformational change that occurs as a result of calcium binding or protein–protein interactions, or it could be a posttranslational modification, such as phosphorylation. Investigation of such hypotheses will await a more complete understanding of the process of fusion itself.

The expression of HSD is not caused by decreased calcium influx

Quantal analysis demonstrated that the expression of HSD is presynaptic (Castellucci and Kandel, 1974), involving either a decrease in the number of vesicles available for release, a decrease in the probability of release, and/or a decrease in the number of functional release sites. Two previous studies addressed the depletion hypothesis. In the first, EM images of synaptic terminals were obtained after a long series of stimuli (Bailey and Chen, 1988). It was found that although the total number of vesicles was unchanged, there was a decrease in those vesicles directly apposed to the membrane. However, this decrease was not large enough to account for the decrease in postsynaptic response measured in the same synapses. The second study used spontaneous release as an indication of available vesicles (Eliot, 1991; Eliot et al., 1994). Although a high-frequency train was capable of transiently decreasing the frequency of spontaneous miniature EPSPs, (indicating that depletion may have occurred), there was no prolonged decrease in spontaneous release rate associated with HSD. Therefore, although depletion of the pool of available vesicles may account, in part, for a decrease in transmitter release under certain circumstances, HSD is likely to involve also a decrease in the probability of release and/or a change in the number of functional release sites. The fact that presynaptic EGTA injections did not alter the time course or extent of HSD despite a decrease in the absolute magnitude of the EPSP (see Fig. 4) also argues against a primary role of vesicle depletion.

One potential mechanism for the depression of Ca-evoked transmitter release is via a decrease of Ca influx associated with a presynaptic action potential. It has been shown that the voltage-gated calcium current measured in the sensory cell body undergoes a use-dependent decrease caused by cumulative inactivation during trains of action potentials that induce HSD (Klein et al., 1980). Although this decrease in Ca current is a prime candidate for contributing to HSD, it has been argued that the magnitude of the expected decrease in Ca influx is too small to account quantitatively for the change in EPSP amplitude (Gingrich and Byrne, 1985). One problem with such conclusions is that they are based on calcium current measurements in the cell body, which may differ from the behavior of the calcium current in the presynaptic terminal that triggers release.

Using fluorescence microscopy, we directly measured the calcium transient in response to single presynaptic action potentials in regions of the presynaptic cell that are likely to correspond to the presynaptic terminals (Glanzman et al., 1989). These studies were performed in the presence of nitrendipine, which allowed us

to selectively measure Ca influx via the dihydropyridine-insensitive Ca channels: the type that mediates release from the sensory neurons (Edmonds et al., 1990; Eliot et al., 1993). During stimulation protocols that produced marked HSD, we found no change in either the average peak amplitude of the Ca_i transient or in the number of terminals that were activated by successive stimuli, as judged by the presence of a Ca_i transient. The criteria used for identifying a presynaptic terminal, presynaptic axonal varicosities in contact with the postsynaptic cell (Glanzman et al., 1989), were not definitive and therefore some release sites may have been missed, whereas other regions may have been incorrectly identified as release sites. However, it is unlikely that this bias led to the selective exclusion of those sites that were altered in response to repetitive stimulation. Thus, we conclude that the decrease in release occurs at some step downstream of Ca influx.

Our results thus indicate that both the induction and expression of HSD may be an integral part of the release process itself. With an expanding knowledge of the fusion process and of the molecular components of the release apparatus, it may soon be possible to test the role of specific presynaptic proteins. In particular, future studies of genetically modified animals offer the promise of identifying specific proteins involved in homosynaptic depression.

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