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Activity-Dependent Potentiation of Synaptic Transmission From L30 Inhibitory Interneurons of *Aplysia* Depends on Residual Presynaptic Ca^{2+} But Not on Postsynaptic Ca^{2+}

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Activity-dependent potentiation of synaptic transmission from L30 inhibitory interneurons of *Aplysia* depends on residual presynaptic Ca^{2+} but not on postsynaptic Ca^{2+} . *J. Neurophysiol.* 78: 2061–2071, 1997. Activity-induced short-term synaptic enhancement (STE) is a common property of neurons, one that can endow neural circuits with the capacity for rapid and flexible information processing. Evidence from a variety of systems indicates that the expression of STE depends largely on the action of residual Ca^{2+} , which enters the presynaptic terminal during activity. We have shown previously that a Ca^{2+} -dependent STE in the inhibitory synapse between interneurons L30 and L29 in the abdominal ganglion of *Aplysia californica* has a functional role in regulating the gain of the siphon withdrawal circuit through facilitated recurrent inhibition onto the L29s. In the present paper, we further explore the role of Ca^{2+} in L30 STE by examining two basic issues: 1) What is the role of residual presynaptic Ca^{2+} in the maintenance of L30 STE? We examine this question by first inducing STE in the L30s then rapidly buffering presynaptic free calcium through the use of the photoactivated Ca^{2+} chelator diazo-4, which was preloaded into the L30 neurons. Three forms of STE in the L30s were examined: frequency facilitation (FF), augmentation (AUG), and posttetanic potentiation (PTP). In each case, the activation-induced enhancement of the L30 to L29 synapse was reduced to preactivation levels at the first test pulse following photolysis of diazo-4. 2) What is the role of postsynaptic Ca^{2+} in the induction of L30 STE? We examine whether there is a postsynaptic requirement of elevated Ca^{2+} for the induction of L30 STE by first injecting the calcium chelator bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) into the postsynaptic cell L29 (at levels sufficient to block transmitter release from the L29s), to prevent any increase in postsynaptic intracellular Ca^{2+} that may occur during L30 (presynaptic) activation. We found that BAPTA injection did not effect either the induction or the time course of FF, AUG, or PTP in the L30s. Taken collectively, our data indicate that all forms of STE in the L30s depend on presynaptic free cytosolic Ca^{2+} for their maintenance but do not require the elevation of postsynaptic Ca^{2+} for their induction.

INTRODUCTION

Synaptic transmission can be regulated both intrinsically and extrinsically on time scales ranging from milliseconds to days. Intrinsic modifications of synaptic efficacy can result solely from activity in a presynaptic neuron, with a time course dependent on both the duration and frequency of presynaptic activation. At least three basic forms of short-term synaptic enhancement (STE) can be distinguished, each defined by its time course of expression: *facilitation*,

with a time course of milliseconds; *augmentation*, which lasts for seconds to tens of seconds; and *posttetanic potentiation* (PTP), with a time course on the scale of minutes (reviewed in Fisher et al. 1997; Magleby 1987; Zucker 1989, 1996). A large body of evidence, beginning with the work of Katz and Miledi (1968), suggests that STE depends on the action of Ca^{2+} , which enters the nerve terminal during activation ($[\text{Ca}^{2+}]_i$). Support for this hypothesis, generally known as the ‘residual calcium hypothesis’, derives largely from three lines of evidence: extracellular calcium is necessary for the induction of synaptic enhancement (i.e., Katz and Miledi 1968); manipulations that directly increase $[\text{Ca}^{2+}]_i$ increase synaptic strength (e.g., Kamiya and Zucker 1994; Zengel et al. 1994); and calcium imaging experiments demonstrate a strong correlation between the accumulation of $[\text{Ca}^{2+}]_i$ during activation and synaptic enhancement (e.g., Atluri and Regehr 1996; Delaney and Tank 1994; Delaney et al. 1989; Regehr et al. 1994).

However, until the development of photolabile Ca^{2+} buffers capable of producing rapid and controlled manipulations of $[\text{Ca}^{2+}]_i$ (Adams and Tsien 1993; Adams et al. 1989), it was difficult to demonstrate directly that the maintenance of synaptic enhancement depends on the continued action of residual $[\text{Ca}^{2+}]_i$ in the synaptic terminal. Using the diazo series of Ca^{2+} buffers, which undergo a rapid rise in Ca^{2+} affinity with binding kinetics similar to bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) on photolysis by ultraviolet (UV) light, Kamiya and Zucker (1994) demonstrated that facilitation, augmentation, and PTP at the crayfish neuromuscular junction are all eliminated on reduction of $[\text{Ca}^{2+}]_i$ by diazo photolysis. In the present work, we have employed a similar methodology to examine the calcium dependence of STE at a central synapse formed from the L30 inhibitory interneurons onto the L29 excitatory interneurons in the abdominal ganglion of *Aplysia californica* (Fig. 1). The L30 to L29 inhibitory synapse exhibits forms of STE similar to those that have been described in other systems. Synaptic enhancement at these synapses can be induced either by tactile stimulation of the skin, which is a potent stimulus for L30 activation, or by direct intracellular activation of L30 at firing frequencies similar to that induced by skin stimulation. We previously have provided evidence that STE in the L30s provides dynamic gain regulation of the siphon withdrawal reflex in *Aplysia*, in part through facilitated recurrent inhibition of the L29s (Fischer and Ca-

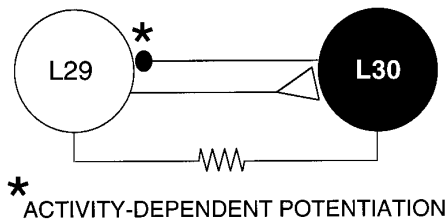


FIG. 1. Simplified schematic of the recurrent synaptic circuit formed by the L29 excitatory interneurons and the L30 inhibitory interneurons. These neurons are connected via both chemical and electrical synapses (Hawkins et al. 1981a). The experiments in the present paper examine short-term synaptic enhancement (STE) at the L30 to L29 synapse (*).

rew 1993, 1995; reviewed in Fischer and Carew 1997). We also have shown that tail shock [or serotonin (5-HT) application] attenuates augmentation, but not facilitation, in the L30s. Reducing extracellular calcium ($[Ca^{2+}]_o$) produced similar effects, suggesting that tail shock-induced modulation may be due in part to a 5-HT induced reduction in Ca^{2+} influx into the L30s during activation (also see Frost et al. 1988). These experiments also demonstrated that the induction of STE in L30 neurons is dependent on the presence of $[Ca^{2+}]_o$ (Fischer and Carew 1994).

In this paper, we further explore the role of Ca^{2+} in L30 STE by examining two basic issues. 1) What is the role of residual presynaptic Ca^{2+} in the maintenance of L30 STE? We examine this question by first inducing STE in the L30s then rapidly buffering free Ca^{2+} through the use of the photoactivated Ca^{2+} chelator diazo-4. 2) What is the role of postsynaptic Ca^{2+} in the induction of L30 STE? Recent experiments examining potentiation of synaptic transmission at sensory to motor neuron synapses in *Aplysia* have revealed a novel postsynaptic component for the induction of short-term synaptic enhancement; in these studies, enhancement is abolished by postsynaptic hyperpolarization or BAPTA injection (Bao et al. 1997; Cui and Walters 1994; Lin and Glanzman 1994). In the present paper, we investigate whether there is a similar postsynaptic requirement for the induction of synaptic enhancement in L30 by first injecting the Ca^{2+} chelator BAPTA into the postsynaptic cell (L29) to block any increase in intracellular Ca^{2+} . Collectively, we find that three specific forms of synaptic enhancement in the L30s (frequency facilitation, augmentation, and PTP) depend on presynaptic free cytosolic Ca^{2+} for their maintenance; however, no form of STE examined required the elevation of postsynaptic Ca^{2+} for its induction.

Some of these results have been presented previously in abstract form (Fischer et al. 1996).

METHODS

Animals

Adult *A. californica* (150–250 g) were obtained commercially (Marinus, Long Beach, CA; Marine Specimens, Long Beach, CA; *Aplysia* Resource Center, Coral Gables, FL) and maintained at 15°C in a 600-l aquarium with continuously circulating artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH). Animals were housed in individual containers and fed dried seaweed weekly.

Experimental preparation

Experiments were performed using isolated abdominal ganglia. Ganglia were removed under $MgCl_2$ anesthesia and pinned ventral side up on a microscope slide coated with a thin layer of silicone elastomer (Sylgard; Dow-Corning). To facilitate dark field imaging, the left ventral ganglion was pinned over a small hole cut into the Sylgard. A small plastic wall was built around the perimeter of the slide to allow for perfusion of artificial sea water (ASW) throughout the experimental sessions [ASW contained (in mM) 460 NaCl, 55 $MgCl_2$, 11 $CaCl_2$, 10 KCl, and 10 Tris, pH 7.4]. ASW was at room temperature (20°C) and was perfused at a rate ≈ 1 ml/min.

Postsynaptic responses were measured in L29 interneurons, which were identified on the basis of their ability to recruit recurrent inhibitory input on intracellular activation. L30 interneurons were identified solely on the basis of their recurrent synaptic relationship with L29 (Fig. 1): L30 interneurons are excited by L29 activation and in turn produce IPSPs back onto L29 (Fischer and Carew 1993; Hawkins et al. 1981a). Standard intracellular recording techniques were used. Recordings were made on a Zeiss compound microscope fitted with a fixed recording stage. Neurons were impaled with glass microelectrodes (resistance 5–10 M Ω) containing 3 M KCl. Postsynaptic responses were recorded as inhibitory postsynaptic currents (IPSCs) in L29 neurons, which were placed under two-electrode voltage clamp at holding potentials of -85 mV. This is below the reversal potential for this synapse (approximately -55 mV), so postsynaptic currents appear inward.

Diazo-4 injection and photolysis

Diazo-4 (50 mM in 250 mM KMOPS, pH 7.2) was injected iontophoretically into L30 neurons using negative current pulses (-4.0 nA, 500-ms duration at 1 Hz, for 3 min). Based on our estimate of cell volume and amount of diazo-4 injected iontophoretically, the estimated final diazo-4 concentration was in the low millimolar range (estimated at 5 mM). The diazo-4 containing electrode usually was replaced with one containing 3 M KCl immediately after injection. Preliminary experiments showed that a diffusion time of 10 min after injection was sufficient for diazo-4 to be effective on photolysis. This time course is consistent for a molecule of $<1,000$ molecular weight with expected diffusion constant of 2×10^{-7} cm 2 /s to reach processes <100 μ m from the cell body. Before photolysis by UV light, diazo-4 has a low resting affinity for Ca^{2+} , with a dissociation constant (K_d) of 89 μ M (Adams and Tsien 1993; Adams et al. 1989). Consistent with a low initial affinity for Ca^{2+} , we observed no change in the L30 to L29 IPSC 10 min after injection (Fig. 2), indicating that at the concentrations we inject, the unphotolyzed buffer has no noticeable effect on synaptic transmission.

Photolysis was accomplished using a 100-W mercury lamp reflected through a 400 nm dichroic mirror (which reflects UV light) and focused onto the ganglia through a $\times 10$ objective. To calibrate our system, we measured flash-induced Ca^{2+} changes in microcurvettes filled with known mixtures of diazo-4, Ca^{2+} , and the Ca^{2+} indicator dye fluo-3. Changes in fluo-3 fluorescence were measured using a Hamamatsu video imaging system and a photodiode connected to a custom-built photoamplifier. Using these measurements in conjunction with a model of diazo-4 photolysis (Fryer and Zucker 1993), we estimated that a 3-s flash should photolyze diazo-4 by $\sim 60\%$. Assuming a prephotolysis residual $[Ca^{2+}]_i$ of 2 μ M immediately after L30 activation (Blumenfeld et al. 1992) and allowing for tissue absorbance, our model estimates that photolysis using a 3-s flash should reduce residual $[Ca^{2+}]_i$ to ~ 25 nM, less than the assumed resting $[Ca^{2+}]_i$ of 150 nM (Blumenfeld et al. 1992).

In separate control experiments, we substituted diazo-3 for di-

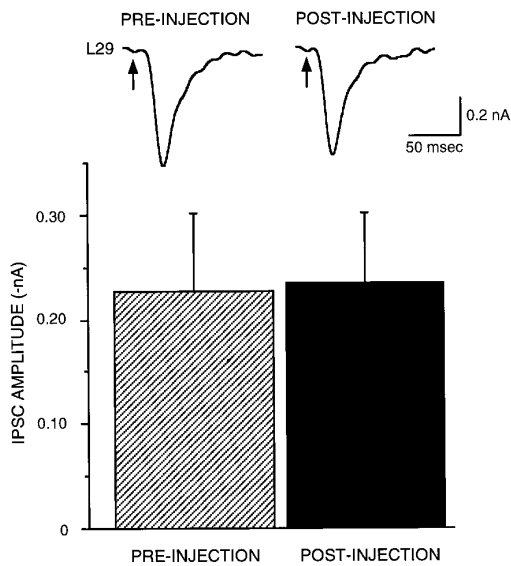


FIG. 2. Diazo-4 injection does not affect basal synaptic transmission. After measurement of the baseline L30 to L29 inhibitory postsynaptic current (IPSC; preinjection), diazo-4 was injected iontophoretically. Ten minutes later, a second baseline measure was obtained (postinjection). *Top*: voltage-clamp recording from a L29 interneuron. \rightarrow , times of L30 presynaptic spikes. *Bottom*: summary data from neurons examined both preinjection and postinjection ($n = 7$). There was no significant difference between the 2 time points.

azo-4, which on photolysis produces similar photoproducts and changes in pH without a change in Ca^{2+} affinity (Adams and Tsien 1993; Adams et al. 1989). At least three of these control experiments were performed for each of our experimental protocols examining diazo-4 photolysis. No effects of diazo-3 were found, therefore data are not reported.

Statistics

A repeated measures analysis of variance (ANOVA) was used when comparing data obtained by repeated sampling across different time points; for example, when comparing preflash time points with postflash measures or postflash measures between different experimental groups (i.e., Fig. 4). For other data, a factorial ANOVA was used for comparison of single measures across groups. Normalized data are calculated so that no change = 0%. Data are reported as means \pm SE.

RESULTS

Diazo-4 injection and photolysis do not affect basal synaptic transmission

Before investigating the effects of Ca^{2+} buffering by photolysis of diazo-4 on STE in L30 neurons, we performed an initial series of control experiments to examine whether diazo-4 injection or diazo-4 photolysis would affect basal (nonpotentiated) synaptic transmission. Experiments examining possible effects of diazo-4 injection alone are summarized in Fig. 2. In these experiments, we measured the amplitude of the L30 to L29 IPSC before injection, then measured the same synaptic connection 10 min after iontophoretic injection of diazo-4 (these data are taken from experiments examining augmentation, which are summarized in Fig. 6). We found no significant difference between pre- and postinjection measures ($n = 7$, $P = 0.78$), indicating that the

unphotolyzed chelator has no discernible effect on synaptic transmission.

In a separate series of experiments, we determined whether flash photolysis of diazo-4 would affect basal synaptic transmission. Two groups were examined: diazo-4-loaded neurons, and a noninjected control group exposed to light flash (flash alone). This latter group controls for the photoresponse commonly observed in L29 neurons during the light flash (see Fig. 4). In these experiments, single spikes were elicited in L30 neurons at a 30 s interstimulus interval (ISI), which is both nondecrementing and nonpotentiating. After four baseline measures, the UV light was turned on for 3 s; the postflash test was given 5 s after the light was turned off. Data from these experiments are summarized in Fig. 3. First, no significant differences were found between the four baseline measures within either group, indicating that baseline measures of synaptic strength were stable. Because of this, the four baseline trials were averaged for subsequent comparison to postflash measures. No significant difference was found comparing the average baseline scores and the postflash test within both experimental groups (diazo-4: $n = 6$, $P = 0.66$; flash alone: $n = 5$, $P = 0.74$). These data indicate that the level of chelator induced after photolysis is not sufficient to affect baseline synaptic transmission.

Activity-dependent forms of short-term synaptic enhancement are abolished by diazo-4 photolysis

Having established that both unphotolyzed and photolyzed diazo-4 do not affect nonpotentiated (basal) synaptic transmission, we next investigated the dependency of L30 STE maintenance on residual presynaptic Ca^{2+} . We did this by

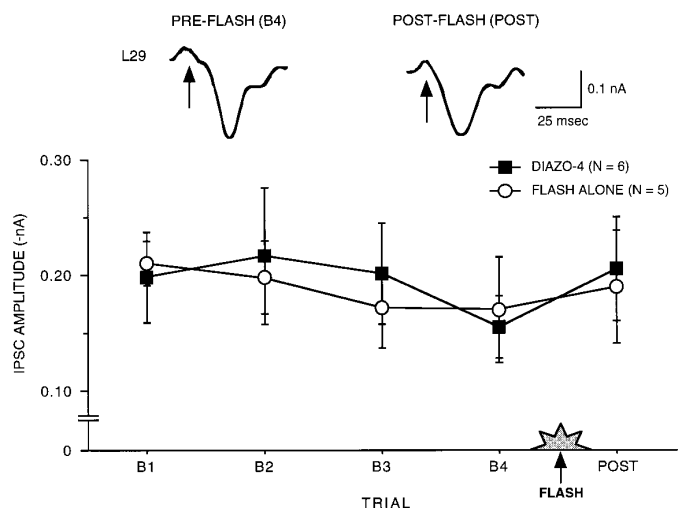


FIG. 3. Diazo-4 photolysis does not affect basal synaptic transmission. L30 to L29 IPSC was measured at a 30 s interstimulus interval (ISI). After 4 baseline measures (B1–B4), the ultraviolet (UV) light was flashed for 3 s. Postflash (POST) measures were obtained 5 s after the flash. *Top*: voltage-clamp recording from a L29 interneuron. \rightarrow , times of L30 presynaptic spikes; the L30 neuron was loaded with diazo-4. No change in IPSC amplitude was observed after photolysis. Apparent broadening of the IPSC is due to spontaneous synaptic noise; a similar broadening is not observed in other cases (see for example Fig. 6). *Bottom*: summary data comparing diazo-4-loaded neurons and a separate flash alone group. There were no significant differences between the 2 groups.

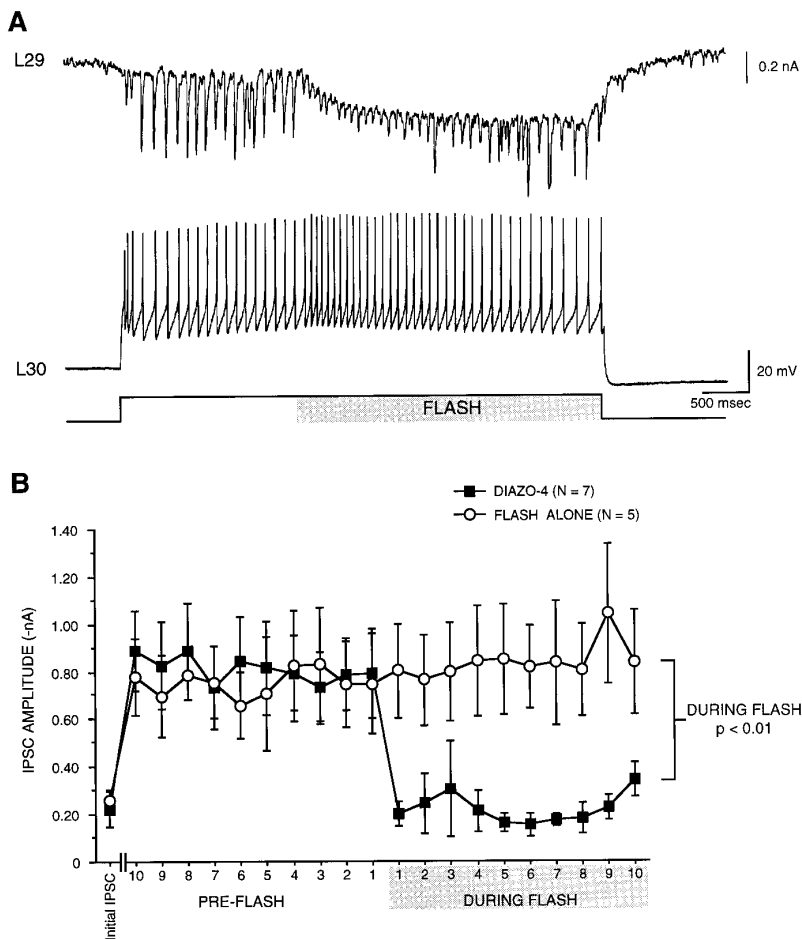


FIG. 4. Diazo-4 photolysis eliminates frequency facilitation. After a preactivation measurement of the L30 to L29 IPSC (not shown), L30 neurons were activated at rates of 6–12 Hz for 5 s. UV light was turned on 2 s into L30 activation and remained on for the duration of the activation (3 s total exposure). *A*: recordings from a voltage-clamped L29 neuron and a diazo-4-loaded L30 neuron. L30 firing rate was 10 Hz preceding the flash. Diazo-4 photolysis had 2 main effects: an immediate reduction in the amplitude of the L30 IPSC and an immediate increase in L30 firing frequency. A photoresponse, which temporally coincides with the UV flash, also can be observed in the L29 neuron. *B*: a comparison of the effects of UV flash on diazo-4-loaded neurons and in noninjected controls (flash alone). Amplitudes of the initial IPSC (nonfacilitated), the 10 evoked IPSCs immediately preceding the flash, and the first 10 IPSCs during the flash are plotted. Significant differences were observed between the 2 groups only for measures obtained during UV flash.

first inducing synaptic enhancement by activating L30 then examining whether a rapid increase in Ca^{2+} buffering through diazo-4 photolysis would reduce the amplitude of the enhanced synapse. In previous work, we have described two forms of STE in L30 neurons, frequency facilitation (FF) and augmentation (AUG) (Fischer and Carew 1994). In addition to examining the calcium dependency for maintenance of these two forms of STE, we also describe and examine a third form of STE in L30 neurons, PTP. Each of these experiments are discussed separately below.

FREQUENCY FACILITATION. We define FF as the synaptic enhancement obtained within the first few seconds during L30 activation (Bittner and Baxter 1991; Schlapfer et al. 1974). In this time range, FF is likely composed of both F1 and F2 forms of facilitation, which have been described in other systems based on decay rates (Fisher et al. 1997; Magleby 1987; Zucker 1989). After a preactivation measurement of the L30 to L29 IPSC, FF was induced by activating L30 neurons, using DC injection, at rates ranging from 6 to 12 Hz for 5 s (mean = 9.3 ± 0.6 Hz). The UV light was turned on 2 s into L30 activation and remained on for the duration of the activation (3 s total exposure). Two experimental groups were examined initially: diazo-4-loaded neurons and noninjected flash alone controls. Results from these experiments are summarized in Fig. 4. The physiological record in Fig. 4*A* depicts the response of a diazo-4-loaded neuron and illustrates the two basic effects of diazo-4 photolysis: a

rapid decrease in the amplitude of the L30 IPSC with the onset of the flash and an increase in L30 firing frequency. Also notable is a photoresponse in the L29 neuron (an inward current) that temporally coincides with the UV flash. This photoresponse also occurs in noninjected controls (not shown) and commonly is observed in L29 neurons.

To illustrate the dynamics of FF and its reduction by diazo-4 photolysis, three aspects of the experimental data are plotted in Fig. 4*B*: the amplitude of the initial IPSC, the amplitudes of the 10 evoked IPSCs immediately preceding the flash, and the amplitude of the first 10 evoked IPSCs during the flash. Summary data from seven experiments with diazo-4-loaded neurons and five experiments examining flash alone controls are illustrated. Preflash measures for both the diazo-4 and flash alone groups are significantly greater than initial IPSC measures, indicating the induction of significant FF ($P < 0.001$, both groups). Further, the amplitudes of the facilitated IPSCs are not different between the flash alone and diazo-4 groups ($P = 0.82$), showing that diazo-4 loading per se has no effect on FF. However, during exposure to UV flash, there is a significant reduction in the diazo-4 group compared with flash alone controls ($P < 0.01$) that is manifest at the first test measurement. The finding that FF appears to be eliminated within a few hundred milliseconds (at the first test IPSC) after initiating photolysis is surprising; we did not expect diazo-4 photolysis to have occurred sufficiently in this time to reduce substan-

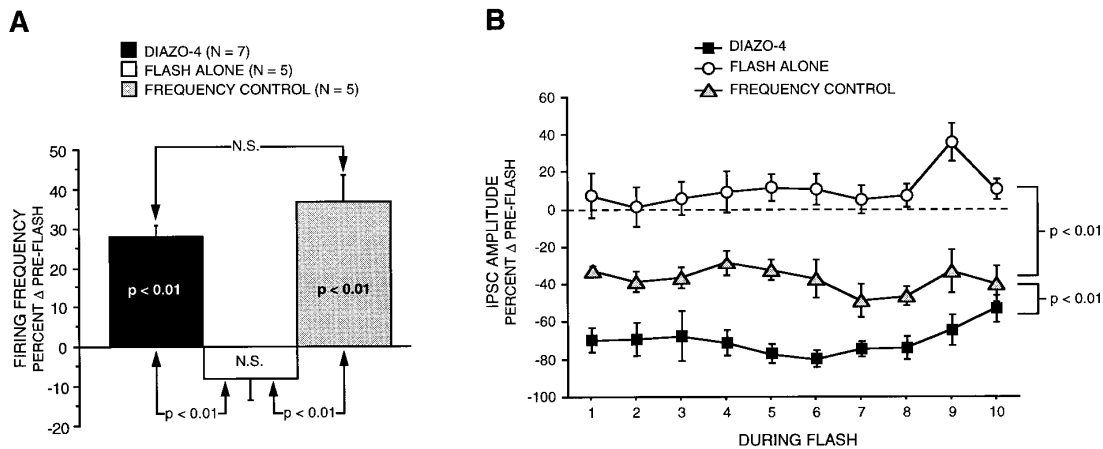


FIG. 5. Effect of increasing firing frequency on frequency facilitation. To examine whether the photolysis-induced change in L30 firing frequency contributes to the reduction of the L30 IPSC, a separate control group was examined in which a step-increase in L30 firing frequency was induced by increasing injected current 2 s after the onset of L30 activation (frequency control). *A*: changes in L30 firing frequency. Data represent the average frequency of the first 10 L30 spikes during flash normalized to the average frequency of the 10 spikes preceding the flash. Both the diazo-4 and frequency control groups exhibited significant increases in firing rate and are not significantly different from each other. *B*: effect of increased firing frequency on facilitation. Amplitudes of the first 10 spikes during the UV flash (or Δ current injection) are plotted; data are normalized to the average of the 10 facilitated IPSCs immediately preceding the flash. A change in frequency alone produced a significant decrease in IPSC amplitude. However, IPSC amplitudes in the frequency control group were still significantly larger than in the diazo-4 group.

tially residual Ca^{2+} (also see below). Interestingly, however, the amplitudes of IPSCs during flash in the diazo-4 group are not significantly different from its initial IPSC measurement ($P = 0.96$), indicating that photolysis reduces the facilitated IPSC back to nonpotentiated levels.

As described above, diazo-4 photolysis also causes an increase in L30 firing frequency, which we analyze further in Fig. 5*A*. To quantify this change, we determined the average firing frequency of the 10 L30 spikes immediately preceding the flash and the first 10 spikes during the flash. Upon photolysis, we observed a significant increase ($28.0 \pm 2.9\%$) in firing frequency in diazo-4-loaded neurons over preflash rates ($n = 7$, $P < 0.01$), whereas flash alone controls exhibited no significant change ($n = 5$, $P = 0.24$). The difference between the diazo-4 and flash alone groups was significant ($P < 0.01$), perhaps demonstrating the action of residual Ca^{2+} on Ca^{2+} -dependent K^+ channels. To examine whether this photolysis-induced change in firing frequency contributes to the observed rapid reduction in the L30 IPSC following photolysis, we performed a separate set of experiments in which we directly induced a change in L30 firing rate by increasing injected current in a step-fashion 2 s after the onset of activation. In five experiments examining this frequency control group, the average change in firing rate was $36.8 \pm 6.7\%$, which was not significantly different from the change observed in the diazo-4 group ($P = 0.51$). Figure 5*B* provides a comparison between the IPSCs during UV exposure in the diazo-4 and flash alone groups (data from Fig. 4*B*), along with the frequency control group. We found that the change in frequency alone can produce a rapid and significant decrease in IPSC amplitude (presumably due to homosynaptic depression) compared with flash alone controls ($P < 0.01$) that may contribute to the rapid decrement in IPSC amplitude on UV exposure observed in the diazo-4 group. However, IPSC amplitudes in the frequency control group were still significantly larger than in the diazo-4 group

($P < 0.01$). These data indicate that, whereas a change in firing frequency may contribute to the photolysis-induced reduction in IPSC amplitude, it cannot account for the total reduction in synaptic efficacy after photoactivation of diazo-4.

AUGMENTATION. AUG is a short-term enhancement in synaptic transmission that is induced by activating L30 neurons at rates of 6–12 Hz for 5 s. This typically produces a synaptic enhancement lasting ~ 60 s (Fischer and Carew 1993). In these experiments, we were interested in investigating both the dependency of AUG on residual Ca^{2+} via photolysis-induced Ca^{2+} buffering as well as examining whether diazo-4 injection alone can affect the induction of AUG. To this end, each experiment was performed in two parts: we first examined augmentation of the L30 to L29 IPSC before diazo-4 injection (baseline augmentation), then examined AUG again at the same synapse 10 min after injection. In each part of the experiment, we first obtained a preactivation measurement of the L30 to L29 IPSC, and then tested every 10 s following L30 activation for ≤ 60 s (data for preactivation measures were discussed in Fig. 2). UV light was flashed for 3 s in the diazo-4 group before the 20-s postactivation test; the flash terminated 5 s before testing. Baseline augmentation trials were not exposed to the UV flash, so a separate flash alone group also was examined (with comparable procedures to the diazo-4 group). Data from these experiments are summarized in Fig. 6.

An example of results from a typical experiment is shown in Fig. 6*A* in which the *top traces* are from the baseline augmentation trial and the *bottom* are the same synaptic connection examined 10 min after diazo-4 injection. In both cases, AUG is observed at the 10-s posttest. Photolysis of diazo-4 before the 20-s test reduces the L30 to L29 IPSC back to preactivation levels (depicted by the dashed line). Results from seven experiments are summarized in Fig. 6*B*,

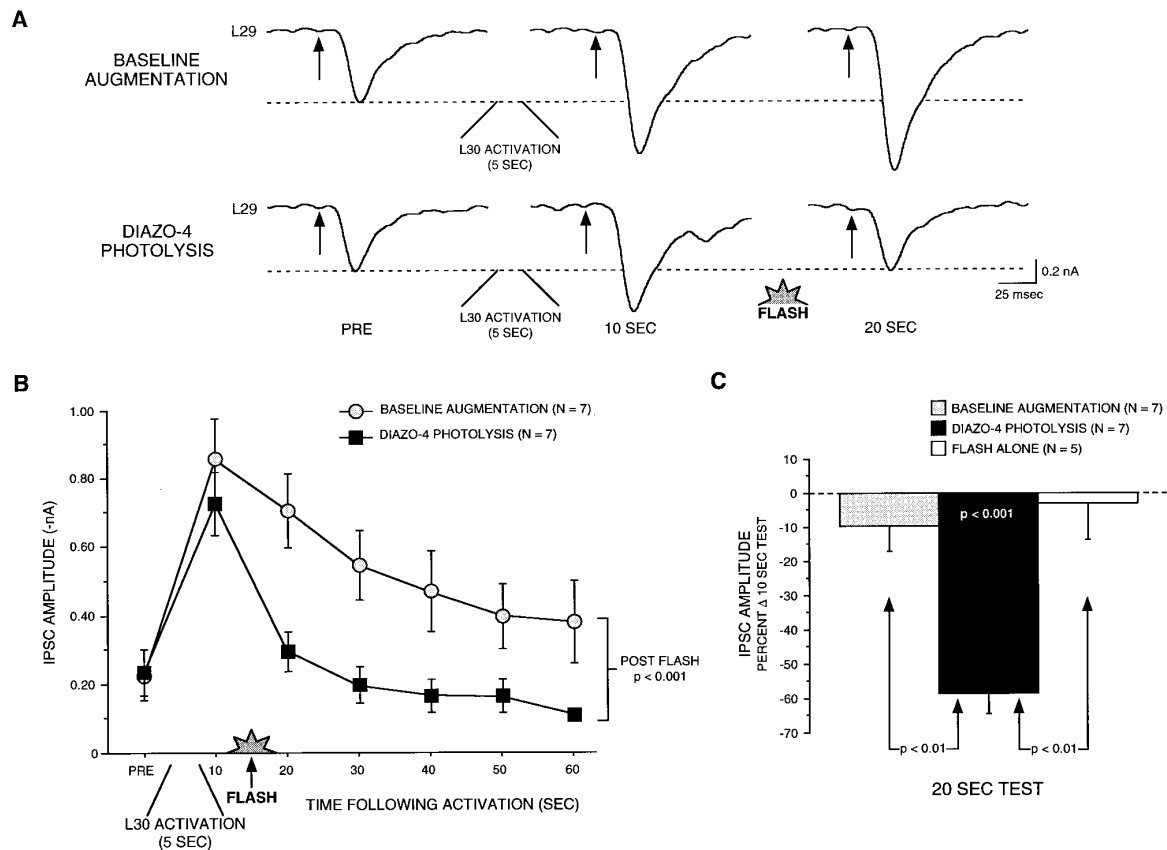


FIG. 6. Diazo-4 photolysis reduces the augmented L30 IPSC. Augmentation (AUG) was induced by activating L30 neurons at rates of 6–12 Hz for 5 s. After an initial characterization of AUG (baseline augmentation trials), L30 neurons were injected iontophoretically with diazo-4. Ten minutes later, AUG was again induced. UV light was flashed for 3 s between the 10- and 20-s postactivation measures. Flash terminated 5 s before the 20 s test. *A*: recordings from a voltage-clamped L29 neuron; \rightarrow , times of L30 presynaptic spikes. *Top*: baseline augmentation trial; *bottom*: from the same neuron after diazo-4 injection. In both trials, AUG is observed at the 10-s posttest. UV flash before the 20 s posttest in the diazo-4 trial reduces the IPSC back to preactivation levels (---). *B*: summary data from 7 experiments performed as in *A*; IPSC amplitudes are plotted. Significant differences were observed for postflash measures between the baseline augmentation and diazo-4 photolysis groups. Further, postflash measures for the baseline augmentation group remained significantly elevated over their preactivation levels up to the 60-s test, illustrating typical AUG as well as a more consistent component likely representing PTP. Conversely, all of the postflash tests in the diazo-4 group were not different from preflash levels. *C*: comparison of the reduction in the augmented L30 IPSC between baseline augmentation and diazo-4 photolysis trials, along with a separate flash alone group. Data represent 20-s postactivation IPSCs normalized to their respective augmented 10-s postactivation IPSCs. Reduction in the diazo-4 group was significantly greater than in both the flash alone and baseline augmentation groups.

in which we plot preactivation measures, as well as measures from the 10- to 60-s postactivation tests. No significant difference was found in the amount of AUG between the two conditions, as measured in the 10-s posttests ($P = 0.39$); thus diazo-4 loading per se has no effect on this form of STE. However, IPSC amplitudes in the diazo-4 condition after flash are significantly lower than in the baseline augmentation condition ($P < 0.001$). Further, in the diazo-4 condition, there was no significant difference between preactivation measures (PRE) and the first postflash test (20 s; $P = 0.28$), indicating that photolysis reduces the augmented synapse back to initial preactivation levels. Likewise, subsequent postflash tests (30- to 60-s tests) were not significantly different from preactivation measures. Conversely, in the baseline augmentation condition, the amplitude of the L30 to L29 IPSC remains elevated from preactivation levels up to the 60-s test ($P < 0.05$, all time points), illustrating typical AUG for this synaptic connection, as

well as a more persistent component probably representing PTP (further explored below). An additional illustration of the reduction of the augmented synapse by diazo-4 photolysis is provided in Fig. 6C, which compares the amount of reduction at the 20-s test relative to the augmented synapse (10-s test). Also included are data from the separate flash alone control group ($n = 5$). After diazo-4 photolysis, we observed a significant reduction ($58.5 \pm 6.0\%$) in the L30 to L29 IPSC from the 10-s test ($P < 0.001$). Conversely, only a marginal (nonsignificant) reduction was observed in both the baseline augmentation group ($-12.3 \pm 12.5\%$; $P = 0.36$) and the flash alone group ($-2.9 \pm 10.9\%$; $P = 0.80$). Further, there were significant between-group differences in comparing the diazo-4 group with both the flash alone and baseline augmentation groups ($P < 0.01$, both comparisons). These data indicate that neither the normal time course of decrement of AUG nor the UV flash (with accompanying L29 photoresponse) can account for the observed

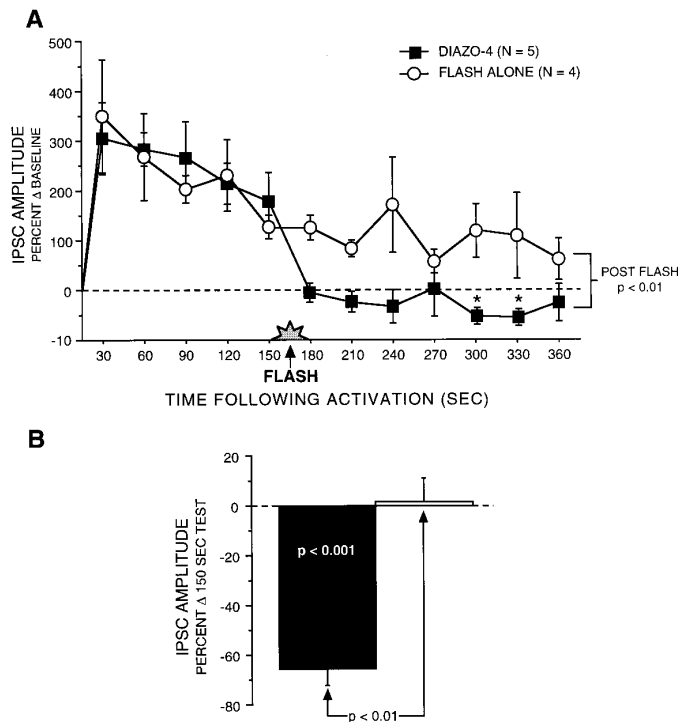


FIG. 7. Diazo-4 photolysis reduces the potentiated L30 IPSC. To induce posttetanic potentiation (PTP), L30 neurons were activated at rates of 4–8 Hz for 60 s. Postactivation measures were obtained at 30-s intervals following activation. PTP was characterized in 2 separate groups: diazo-4 injected neurons and noninjected flash alone controls. UV light was flashed for 3 s between the 150- and 180-s tests; the flash terminated 5 s before the 180-s test. *A*: summary data plotting postactivation IPSC amplitudes normalized to their respective preactivation measures (average of 2 preactivation measures). Significant differences were observed for postflash measures between the diazo-4 photolysis and flash alone groups. Measures for the flash alone group remained significantly elevated over preflash levels (---) up to the 360-s test. Conversely, all of the postflash tests in the diazo-4 group were not different from preflash levels with the exception of the 300- and 330-s tests (*, $P < 0.05$). *B*: comparison of the reduction in the potentiated L30 IPSC at the 180-s postactivation test (postflash). Data are normalized to their respective potentiated 150-s postactivation IPSCs (preflash). The reduction in the diazo-4 group was significantly greater than in the flash alone group.

reduction in the augmented synapse on photoactivation of the chelator.

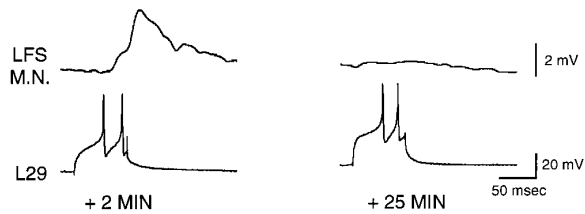
POSTTETANIC POTENTIATION. PTP is a longer-lasting form of enhancement in synaptic transmission than AUG, with a time course of minutes. We found that PTP can be induced in L30 neurons by activating them at rates of 3–5 Hz for 1 min; this typically produces a synaptic enhancement lasting ~6 min (Fig. 7). In these experiments, the L30 to L29 IPSC was measured at 30-s intervals after L30 activation. The dependence of PTP on residual Ca^{2+} was investigated by diazo-4 photolysis 3 min after L30 activation (180-s test), a time at which the contribution of AUG to synaptic enhancement is minimal. As in experiments examining AUG, a 3-s exposure to UV light was used to photolyze diazo-4; the flash terminated 5 s before the 180-s test. A separate set of control experiments (flash alone) also was performed. Data from these experiments are summarized in Fig. 7A, which depicts postactivation IPSC amplitudes normalized to their respective preactivation measures (average of 2 preactivation

measures). There was no significant difference in the amount of PTP between the diazo-4 and flash alone groups ($P = 0.74$), indicating once again that diazo-4 loading per se does not effect STE. However, after diazo-4 photolysis (180 s), we observed a significant reduction in the diazo-4 group compared with flash alone controls ($P < 0.01$). As in FF and AUG experiments, the potentiated IPSC was reduced to preactivation levels after diazo-4 photolysis; there was no significant difference between preactivation measures and the first postflash test (180 s; $P = 0.56$). Of the remaining postflash tests, only the 300- and 330-s tests were significantly different from preactivation measures ($P < 0.05$). Figure 7B compares the amount of reduction in the potentiated IPSC between the flash alone and diazo-4 groups at the first postflash test (180 s); data are normalized to the potentiated IPSC from the preceding test (150 s). In the diazo-4 group, we observed a significant reduction ($65.3 \pm 7.1\%$) in the L30 to L29 IPSC from the 150-s test ($P < 0.001$). Conversely, we only observed a modest, nonsignificant reduction ($1.5 \pm 9.4\%$) in the flash alone group ($P = 0.88$). Also, there was a significant difference between the diazo-4 and flash alone groups ($P < 0.01$). These data indicate that neither the normal time course of decrement of PTP nor the UV flash alone can account for the observed reduction in the potentiated synapse.

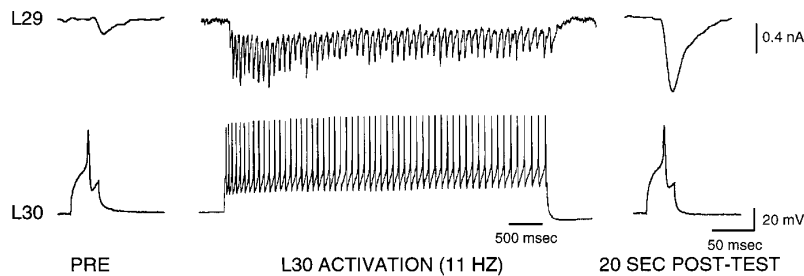
Postsynaptic changes in $[Ca^{2+}]_i$ are not required for the induction of short-term synaptic plasticity

The results described above are consistent with the hypothesis that STE depends on residual presynaptic Ca^{2+} for its maintenance. Recent work has suggested the possibility that STE of *Aplysia* sensory to motor neuron synapses also may require a rise in postsynaptic $[Ca^{2+}]_i$ for its induction (Bao et al. 1997; Cui and Walters 1994; Lin and Glanzman 1994). To examine this possibility for FF, AUG, and PTP at the L30 to L29 synapse, we performed a series of experiments in which we first loaded the L29 neurons with the Ca^{2+} buffer BAPTA (Sigma, St. Louis, MO) before L30 activation. BAPTA loading was accomplished by penetrating L29 neurons with a beveled microelectrode containing 50 mM BAPTA in 3 M KCl. The efficacy of BAPTA loading was monitored by examining the monosynaptic connection between the L29 neuron and a LFS motor neuron at 2- to 5-min intervals, using a current pulse that elicited two spikes in the L29 neurons (to produce paired-pulse facilitation). A typical response to BAPTA injection is illustrated in Fig. 8A, which shows recordings from the L29 to LFS synapse 2 and 25 min after penetration of the L29 neuron with the BAPTA electrode. Typically, the EPSPs were no longer detectable within 25 min of penetration. Once the synaptic potentials in LFS neurons were blocked, the BAPTA electrode was removed, and the L29 interneuron was placed under voltage clamp. This is illustrated in Fig. 8B, which depicts the same L29 neuron as in Fig. 8A, now serving as the postsynaptic cell to examine FF and AUG in L30. Under these conditions, both FF and AUG are produced readily, as illustrated by the significant enhancement of the L30 to L29 IPSC both during and 20 s after L30 activation. A quantitative summary of six experiments examining AUG is shown in Fig. 8C, along with an independent control group

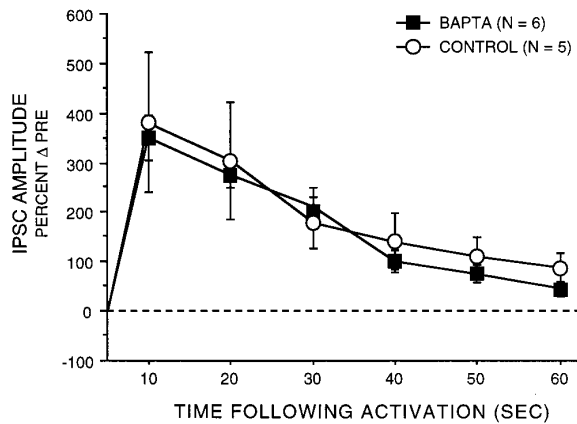
A BAPTA INJECTION



B AUGMENTATION



C



(no BAPTA). No difference was observed in either the amplitude or time course of AUG between the BAPTA group and controls ($P = 0.72$). In these same experiments, we measured FF as the 10th evoked IPSC in the spike train and also found no difference between the BAPTA group and controls ($P = 0.27$; data not shown). A separate series of experiments was carried out to examine a possible postsynaptic contribution to PTP. These results are shown in Fig. 9, which demonstrates that PTP of the L30 to L29 IPSC is also not affected by preloading postsynaptic L29 neurons with BAPTA ($P = 0.68$). These data indicate that a postsynaptic elevation in $[Ca^{2+}]_i$ is not required for induction of STE at the L30 to L29 inhibitory synapse.

DISCUSSION

The two principal conclusions from this study are STE in L30 neurons requires residual presynaptic Ca^{2+} for its maintenance and L30 STE does not require the elevation of postsynaptic $[Ca^{2+}]_i$ for its induction. We will discuss each of these basic findings in turn.

FIG. 8. Postsynaptic bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) does not alter augmentation. The efficacy of BAPTA injections was assessed by examining the synapse between L29 neurons and LFS siphon motor neurons using a paired-pulse protocol. When synaptic transmission was abolished, L29 was placed under voltage clamp and used as the postsynaptic neuron for measurement of L30 augmentation (examined as in Fig. 5). *A*: recordings from a hyperpolarized LFS motor neuron (*top*) and a L29 interneuron (*bottom*). Records are 2 and 25 min after penetration of the L29 with a BAPTA-containing microelectrode. *B*: voltage-clamp recording of the same L29 neuron as in *A* (*top*); *bottom*: L30 neuron. Twenty seconds after L30 activation, the amplitude of the L30 IPSC is increased substantially over PRE levels, demonstrating normal AUG. Normal frequency facilitation is also observable in the L30 activation record. *C*: summary AUG data from experiments in which BAPTA was first injected in the postsynaptic neuron compared with separate control experiments. Data are normalized to their respective preactivation measures. No significant difference was obtained between the 2 groups.

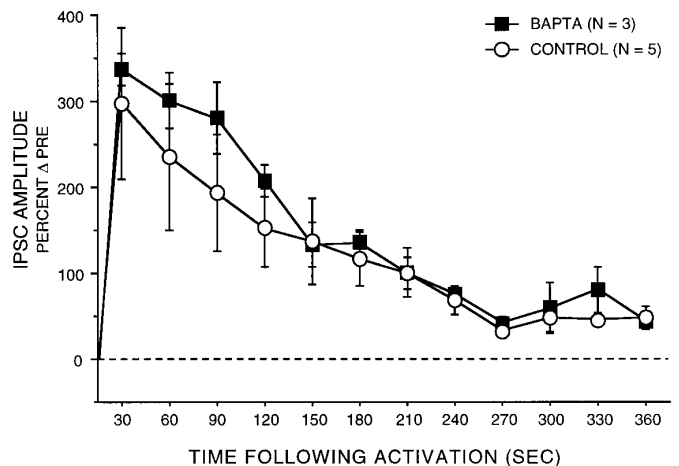


FIG. 9. Postsynaptic BAPTA does not alter posttetanic potentiation. Summary potentiation data from experiments in which BAPTA was first injected in the postsynaptic neuron (as in Fig. 8) are plotted along with separate control experiments. Data are normalized to their respective preactivation measures (average of 2 preactivation measures). There was no significant difference between the 2 groups.

Activity-dependent potentiation requires residual Ca²⁺ for its maintenance

We have shown that STE in the L30s is abolished on photoactivation of the Ca²⁺ buffer diazo-4 after the induction of plasticity. All three forms of L30 STE examined (FF, AUG, and PTP) exhibit similar dependencies on residual Ca²⁺, with photolysis of diazo-4 decreasing the amplitude of the enhanced L30 to L29 IPSC back to preactivation levels. This return to baseline may be related to the observation that photoactivation of the chelator does not affect basal transmission, suggesting that although the concentration of buffer is high enough to adequately buffer the residual Ca²⁺ pool contributing to STE, it is not sufficiently high to buffer the Ca²⁺ transients coupled to neurotransmitter release (Adler et al. 1991). These results are consistent with and extend our previous observations that the induction of STE in L30 neurons depends on the level of [Ca²⁺]_o during cell activation (Fischer and Carew 1994). Also, they are consistent with the large body of evidence demonstrating a role for presynaptic residual Ca²⁺ in short-term plasticity (reviewed in Fisher et al. 1997; Magleby 1987; Zucker 1989, 1996). Further, our results are in general agreement with those obtained by Kamiya and Zucker (1994), who used the related compound diazo-2 to examine the dependency of facilitation, augmentation, and potentiation on presynaptic residual Ca²⁺ at the crayfish neuromuscular junction (see below). However, we should emphasize, as will be discussed later, that whereas our results demonstrate the requirement for residual Ca²⁺ in maintenance of L30 STE, they do not address how or where Ca²⁺ might be acting to produce its effects.

Three interactive components of synaptic enhancement generally are recognized across different species, each of which is dependent on presynaptic [Ca²⁺]_i (Delaney and Tank 1994; Fisher et al. 1997; Magleby 1987; Zengel and Magleby 1982; Zengel et al. 1980; Zucker 1989, 1996). These three components are primarily distinguished by their time course of decay, and each has a different activation requirement: *facilitation*, which requires the least activity for induction (1 or a few action potentials) and decays within 1 s; *augmentation*, which requires at least a few seconds of activation for its induction and decays with a time course of a few to tens of seconds; and *posttetanic potentiation*, which is the longest-lasting of the three, typically requires higher frequency activation for up to a few minutes and decays with a time course of minutes. In this paper, we induced STE using activation protocols similar to those that we used in our previous experiments examining the functional and behavioral significance of L30 synaptic enhancement (Fischer and Carew 1993–1995). We have chosen to use similar terms as used in other systems to describe L30 STE simply to refer to fast (FF), intermediate (AUG), and longer-lasting (PTP) forms of enhancement in L30 neurons. Whereas the use of these terms can be justified by both the activation requirements and decay kinetics of each process, differences in the forms of STE described for L30 and those described in other systems certainly may exist and remain to be explored.

PTP is one such process in which differences between L30 neurons and neurons in other systems may exist, particularly in the activation requirements for the induction of this

type of enhancement. This is suggested by a significant difference between our results and those of Kamiya and Zucker (1994), who examined the effects of diazo photolysis on PTP at the crayfish neuromuscular junction. In the crayfish, PTP is induced by activating motor neurons at rates of 20–50 Hz for 5–10 min (Delaney et al. 1989; Kamiya and Zucker 1994; Mulkey and Zucker 1992), which is considerably more vigorous than the activation protocol used to induce PTP in L30 neurons (3–5 Hz for 1 min) even though in both systems potentiation lasts for minutes. These differences in cell activation requirements may lead to both quantitative and qualitative differences in ion loading. Consistent with this notion is the observation by Kamiya and Zucker (1994) that PTP (but not augmentation) exhibits recovery back to potentiated levels within 30 s of diazo photolysis. This recovery was proposed to reflect a subsequent rise in [Ca²⁺]_i, which then could saturate the photoproduct and reestablish potentiation. The source of this increase in [Ca²⁺]_i is from leakage of Ca²⁺ that was loaded into presynaptic mitochondria during cell activation (Kamiya and Zucker 1994; Mulkey and Zucker 1992; Tang and Zucker 1997). We did not see a similar recovery of potentiation after photolysis in our experiments. This may be due to lower levels of Ca²⁺ loading with our less-vigorous activation protocol and/or a difference in how changes in [Ca²⁺]_i are handled by the cell (i.e., buffering or extrusion). Another possible reason is that the photoproduct in our experiments is of sufficient concentration that it would not become saturated by a secondary rise in [Ca²⁺]_i and continues to buffer any Ca²⁺ that might be released from intracellular stores. To address these possibilities, it would be of interest to use a more vigorous activation protocol for inducing enhancement in the L30s, then to examine whether this protocol induces either quantitative or qualitative differences in PTP and whether this potentiation also might exhibit a similar recovery from photolysis as was observed in crayfish.

Our results demonstrate that residual Ca²⁺ after activation is necessary for the maintenance of L30 STE. They do not, however, demonstrate how or where this Ca²⁺ may be acting to have its effect. A number of studies have illustrated that residual Ca²⁺ interacts with other molecular processes to produce enhancement so that STE may be thought of as a calcium-driven reaction (Atluri and Regehr 1996; Bittner and Schatz 1981; Blundon et al. 1993; Delaney and Tank 1994; Kamiya and Zucker 1994; Landau et al. 1973; Osanai et al. 1996; Regehr et al. 1994; Zengel and Magleby 1980; Zengel et al. 1994). The rapid control of [Ca²⁺]_i buffering capability afforded by the diazo series of photolabile chelators, which upon photolysis have similar rapid Ca²⁺ binding rates as their parent compound BAPTA (Adams and Tsien 1993; Adams et al. 1989), has provided a useful tool for addressing this issue. For example, Kamiya and Zucker (1994) were able to obtain sufficiently rapid photolysis to demonstrate that facilitation is abolished within 10 ms of photolysis, whereas the time from photolysis to the reduction of AUG and PTP was considerably longer, having in both cases a time constant of ~350 ms. These data suggest that facilitation acts at a separate molecular site, with faster Ca²⁺ kinetics than AUG and PTP. The identity of the molecular site(s) of action for residual Ca²⁺ is currently unknown. The results of Kamiya and Zucker (1994) further indicate

that all three forms of synaptic plasticity are caused by residual Ca^{2+} continuing to act at sites distinct from those causing exocytosis, rather than by aftereffects arising from the brief local high Ca^{2+} occurring near Ca^{2+} channels after action potential activity. If the latter were true, removal of residual Ca^{2+} after the induction of synaptic enhancement would be without effect. Our present results lead to the same conclusion.

Postsynaptic calcium is not necessary for induction of STE in L30 neurons

Short-term changes in synaptic transmission are generally believed to be presynaptic in origin, as opposed to long-term changes such as long-term potentiation (LTP), which can have a clear postsynaptic component (Bliss and Collingridge 1993; Rison and Stanton 1995). However, recent experiments examining short-term potentiation of synaptic transmission at sensory neuron (SN) to motor neuron (MN) synapses in *Aplysia* have revealed a novel postsynaptic component for the induction of STE. In these experiments, the induction of potentiation was abolished by either strong postsynaptic hyperpolarization or BAPTA injection. These studies suggest that a postsynaptic increase in $[\text{Ca}^{2+}]_i$ may be necessary to induce STE at this synapse (Bao et al. 1997; Cui and Walters 1994; Lin and Glanzman 1994). In the present study, we examined whether a similar requirement for a postsynaptic rise in $[\text{Ca}^{2+}]_i$ exists at the L30 to L29 synapse by first injecting BAPTA into the L29 neurons at sufficient concentrations to eliminate neurotransmitter release from the L29s. This treatment had no effect on the induction of FF, AUG, or PTP, indicating that L30 STE does not involve a postsynaptic Ca^{2+} -dependent mechanism. Additionally, in the presence of exogenous buffer, any potential rise in postsynaptic Ca^{2+} that might occur with L30 activation would be significantly slower (Tank et al. 1995), which should be reflected in an alteration of the temporal characteristics of STE. We observed no difference in the time course of either the induction or expression of STE in the L30s compared with controls. The lack of a postsynaptic contribution to L30 STE is perhaps not surprising, because the L30 to L29 synapse is inhibitory and unlikely to produce a significant increase in postsynaptic Ca^{2+} . This is further discussed below.

The differences between our results and those examining the SN to MN synapse raise several interesting mechanistic and functional possibilities. First, they may indicate that inhibitory synapses in *Aplysia* follow different rules for potentiation than do excitatory synapses. This may be necessary because inhibitory synapses would not experience a concomitant postsynaptic depolarization with presynaptic activation. This possibility could be investigated by examining PTP at other synapses in *Aplysia*. The L29 interneurons would be an informative postsynaptic cell for such an analysis, because they are a common postsynaptic target for both siphon SNs as well as the L30s (Hawkins et al. 1981a). The L29s also would be an intriguing presynaptic candidate to examine whether a requirement for postsynaptic depolarization is a common property for excitatory neurons in *Aplysia*. This is because they readily exhibit PTP onto the same siphon MNs that have been used to demonstrate postsynaptic effects on

PTP induction in SNs (Cui and Walters 1994; Frost et al. 1988) as well as being an integral component in mediating sensitization of the siphon withdrawal reflex (Frost et al. 1988; Hawkins et al. 1981b). Thus we are currently carrying out experiments in which L29 neurons will serve as either a pre- and postsynaptic cell.

A second possibility is that a postsynaptic requirement for potentiation in *Aplysia* SNs may represent a specialized form of short-term enhancement that is perhaps unique to these cells. This may reflect some unique adaptation providing a Hebbian-like mechanism that is important for learning-related changes in the defensive withdrawal circuits. For example, it recently has been shown that injecting BAPTA into siphon MNs blocks the long-term synaptic enhancement normally seen in SN to MN synapses after a cellular analogue of classical conditioning of siphon withdrawal (Murphy and Glanzman 1996). From this perspective, the long-term enhancement exhibited by *Aplysia* SN to MN synapses may represent a unique or rudimentary form of LTP that is observed at vertebrate synapses, with a key difference being the relationship between short-term enhancement (PTP) and LTP in each system. LTP-like enhancement in *Aplysia* SNs and vertebrate neurons appears to be similar in that postsynaptic hyperpolarization or Ca^{2+} buffer injection prevents its induction. However, the enhancements differ in that these manipulations do not abolish PTP in vertebrate neurons (Malenka et al. 1988, 1992) as they do in *Aplysia* SNs (Bao et al. 1997; Cui and Walters 1994; Lin and Glanzman 1994). These differences thus provide an intriguing framework for a comparative analysis of the underlying molecular mechanisms of activity-dependent synaptic enhancement in each system.

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