The Journal of Neuroscience, August 1994, 14(8): 4694–4704

Depression of Developing Neuromuscular Synapses Induced by Repetitive Postsynaptic Depolarizations

Yi-Jiuan Lo,[1] Yi-chun Lin,[1] Dan H. Sanes,[2] and Mu-ming Poo[1]

[1]Department of Biological Sciences, Columbia University, New York, New York 10027 and [2]Center for Neural Science, New York University, New York, New York 10003

Effect of postsynaptic activity on the synaptic efficacy was studied in *Xenopus* nerve–muscle cultures. Repetitive postsynaptic depolarizations induced by injection of current pulses into singly innervated myocytes resulted in significant reduction in the frequency of spontaneous synaptic currents and the amplitude of nerve-evoked synaptic currents at the majority of synapses that showed immature synaptic properties. Repetitive hyperpolarizations and steady depolarizations of similar duration were without effect. The depolarization-induced synaptic depression appeared to result predominately from a reduced ACh secretion from the presynaptic nerve terminal. Buffering the myocyte cytosolic Ca^{2+} at a low level with intracellular loading of a Ca^{2+} buffer, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA), significantly reduced the effect of the depolarizations. Thus postsynaptic electrical activity can regulate the synaptic efficacy of the developing neuromuscular synapses and the regulation may be mediated by retrograde transsynaptic interactions.

[Key words: synaptic competition, synaptogenesis, synaptic plasticity, neuromuscular junction, cell culture,* Xenopus laevis*]

The formation and stability of synaptic connections in the nervous system are influenced by the amount and the pattern of electrical activity (Purves and Lichtman, 1985; Shatz, 1990). In CNS and PNS, activity is known to influence the competition between nerve terminals that coinnervate the same target region, a process of critical importance during the consolidation of mature nerve connections (Hubel and Wiesel, 1965; Benoit and Changeux, 1975; Constantine-Paton et al., 1990). Although the mechanism underlying synaptic competition among coinnervating nerve terminals remains unknown, the involvement of postsynaptic activity has been implicated. Postsynaptic block of ACh receptors with curare reduced the elimination of polyneuronal innervation at developing neuromuscular junctions (Srihari and Vrbova, 1978), and nerve terminals rendered inactive by postsynaptic blockade are at a disadvantage when forced to compete with nearby terminals on the same muscle fibers (Balice-Gordon and Lichtman, 1991). *In vivo* observation of the temporal sequence of the depletion of the postsynaptic ACh receptors and the withdrawal of the presynaptic axons from the developing neuromuscular junctions suggests that the ACh receptors may play an important role in competitive synaptic reorganization (Balice-Gordon and Lichtman, 1993). In the frog nervous system, pharmacological blockade of the NMDA receptors disrupts developing retinotectal connections (Cline et al., 1987), and prevents the normal process of synaptic elimination in the cerebellum (Rabacchi et al., 1992).

Hebb (1949) postulated that the efficacy of a synaptic connection may be influenced by the coincidence of pre- and postsynaptic activities. Extensions of this postulate propose that a synapse may be potentiated or stabilized by synchronous pre- and postsynaptic activity, but weakened by asynchronous activity (Stent, 1973; Brown et al., 1990; Constantine-Paton et al., 1990). Postsynaptic activity alone (in the absence of presynaptic activity) resembles the condition of asynchronous activity, and thus may weaken the synapse. In the present study, repetitive postsynaptic depolarization was induced in innervated myocytes, and the effect on synaptic efficacy was examined.

It has been shown recently that repetitive stimulation of a presynaptic neuron in *Xenopus* nerve–muscle cultures results in functional suppression of synapses made by other neurons innervating the same postsynaptic myocyte (Lo and Poo, 1991, 1994). Similar synaptic depression can be induced at a singly innervated myocyte by repetitive postsynaptic application of ACh (Dan and Poo, 1992). Heterosynaptic suppression can be induced with or without postsynaptic depolarization, but postsynaptic depolarization appeared to slightly enhance the suppression effect (Lo and Poo, 1994). Since one of the consequences of nerve stimulation or ACh application is postsynaptic depolarization, it would be of interest to know whether repetitive postsynaptic depolarizations alone can induce synaptic depression. Systematic studies carried out in the present study showed that significant reduction of both spontaneous synaptic activity and nerve-evoked responses can indeed be induced by repetitive postsynaptic depolarizations at most of the synapses examined. Interestingly, the affected synapses consistently showed functional properties of immature synaptic contacts. Early synaptic activity is believed to play important roles in the maturation of pre- and postsynaptic properties (Kidokoro and Saito, 1988; Stretavan et al., 1988; Pasic and Rubel, 1989). The present result supports the notion that postsynaptic depolarizations induced by active synapses impede the maturation and stabilization of inactive, developing synapses.

Materials and Methods

Culture preparation. Xenopus nerve–muscle culture was prepared as previously reported (see preceding companion article, Lo and Poo, 1994).

Received May 11, 1993; revised Feb. 7, 1994; accepted Feb. 16, 1994.

This work was supported by grants from National Institute of Health (NS 22764) and National Science Foundation (IBW 22106) and Office of Naval Research (N00014-90-J1865).

Correspondence should be addressed to Mu-ming Poo at the above address.

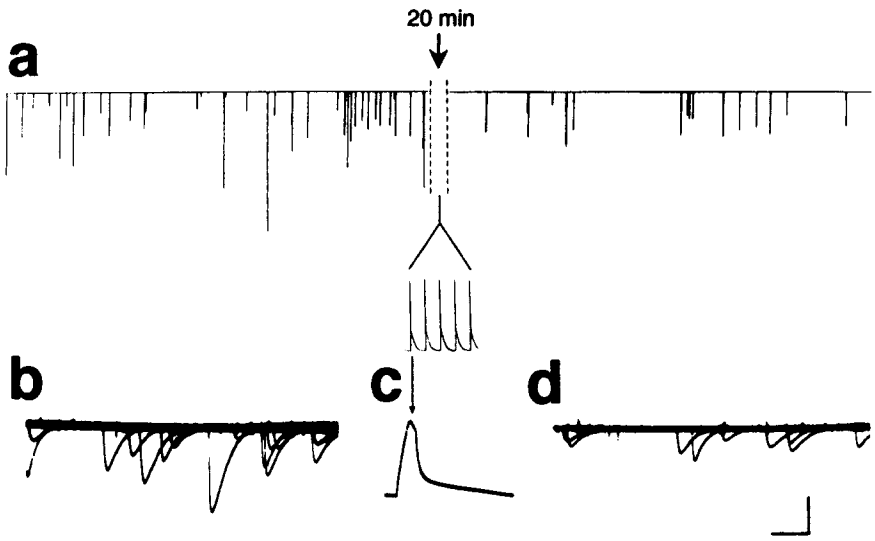

Figure 1. Depression of spontaneous synaptic activity induced by repetitive postsynaptic depolarizations. *a,* The continuous trace depicts whole-cell voltage-clamp recording of the membrane current of a singly innervated myocyte in a 1-d-old *Xenopus* nerve–muscle culture. Spontaneous synaptic currents (SSCs) are shown as downward deflection of the membrane currents (V_h = −70 mV, filtered at 150 Hz). Repetitive depolarizations were induced at a frequency of 6 Hz for a period of 20 min by injection of square-pulse positive currents into the myocyte under the current-clamp condition. Calibration: 100 pA, 30 sec. *b* and *d*, Samples of SSCs at a higher time resolution (filtered at 2.5 kHz) are shown by superimposed oscilloscopic traces for the same number of sweeps before (*b*) and after (*d*) the depolarizations. Calibration: 150 pA, 20 msec. *c*, Sample traces depicting action potentials induced by the injected currents. Calibration: 30 mV, 10 msec.

Isolated myocytes singly innervated by cocultured spinal neurons were used in the present study.

Electrophysiology. Gigaohm-seal whole-cell recording methods followed those described previously (Hamill et al., 1981; Lo and Poo, 1994). Current injection was applied in current-clamp mode through the whole-cell recording pipette. Action potentials resulting from the current injections usually trigger a few muscle contractions at the onset of repetitive stimulation. In the nerve–muscle cultures prepared for the present study, these muscle contractions did not interfere with the stability of whole-cell recording. Initial tests of repetitive stimulation at various frequencies suggested that stimuli with frequencies up to 6–8 Hz can be applied for prolonged period without deleterious effects on the myocyte, as indicated by the maintenance of the same resting membrane potential. Thus a standard stimulation frequency of 6 or 8 Hz was adopted for most of the experiments. For iontophoretic mapping of ACh sensitivity, conventional micropipettes were made and filled with 3 M ACh chloride. The resistance of the ACh pipette was in the range of 100–300 MΩ, and required 2–6 nA braking current. The mean amplitude of membrane current induced by identical pulses of ACh (duration 1 msec) applied at the myocyte surface was used to assay the ACh sensitivity.

Results

Effects of repetitive depolarizations on spontaneous synaptic activity

Cultures of embryonic nerve and muscle cells were prepared from *Xenopus* embryos. One day after cell plating, many spinal neurons establish functional synapses with cocultured myotomal myocytes (Xie and Poo, 1986; Evers et al., 1989). Spontaneous synaptic activity was assayed by whole-cell recording of spontaneous synaptic currents (SSCs) from the innervated myocyte, with the holding voltage set at the resting membrane potential (range, −65 to −80 mV). To examine the effect of postsynaptic depolarizations, recording of SSCs was interrupted for a period of 20 min, during which the whole-cell recording was switched to current-clamp mode and pulses of positive currents were repetitively injected into the myocyte through the recording pipette. In a typical experiment, as shown in Figure 1, we used square-pulse currents of 0.8 nA amplitude and 5 msec duration at a rate of 6 Hz, resulting repetitive firing of myocyte action potential. The peak amplitude of the induced action potentials was in the range of 70–100 mV. Myocyte membrane currents were recorded for about 8 min before and after the 20 min period of repetitive depolarizations. In this case, both the frequency and the mean amplitude of SSCs were found to be significantly reduced after the depolarizations.

The effect of repetitive depolarizations on the SSC frequency was first analyzed. Figure 2*a* depicts changes in SSC frequency observed in 17 experiments using a single episode of depolarizations at 6–8 Hz for 20 min: 11 cases showed significant reduction, one showed significant increase (all at $p < 0.05$, rank sum test), and five remained unchanged. For control experiments in which no current was injected into the myocyte over the 20 min period, we found no significant change in the SSC frequency in all five cases (Fig. 2*b*).

The change in the SSC amplitude was then analyzed by comparing the amplitude distribution before and after the depolarization. A significant shift of the distribution toward smaller amplitudes was found in 8 of 17 experiments ($p < 0.05$, rank sum test), six cases showed no change ($p > 0.05$), and three cases showed significant shift toward larger amplitude ($p < 0.05$). Among the eight cases that showed amplitude reduction, six cases also exhibited reduction of SSC frequency. Including all 17 cases, the average mean amplitude after depolarizations was 80 ± 11% (±SEM) of the predepolarization value (see Fig. 3). Interestingly, we found there was no apparent correlation between the extent of depression in the frequency and amplitude of SSCs (coefficient = 0.29), suggesting separate mechanisms underlie these two effects. The observed reduction of SSC amplitude showed no recovery over the duration of these experiments (up to 30 min postdepolarization).

In a series of experiments we studied the dependence of the depression effect on the number of depolarizing current pulses applied to the innervated myocytes. As shown in Figure 4*a*, the percentage reduction of mean SSC frequency increased with the total number of current pulses. At 100 pulses, three out of five cases showed significant reduction of SSC frequency. On the average, a reduction of 15.4 ± 8.8% (±SEM, $n = 5$) was observed after 900 pulses and the reduction increased to 30.0 ±

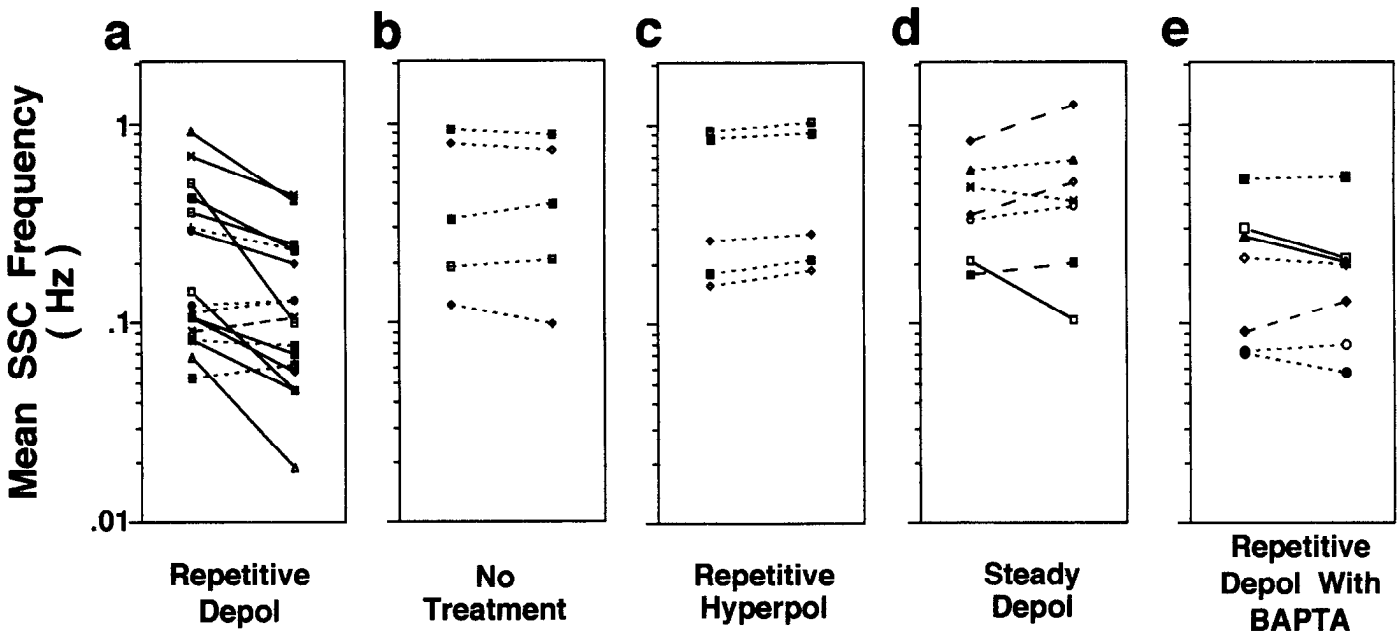

Figure 2. Changes in the frequency of spontaneous synaptic currents (SSCs) under various conditions. *a*, Repetitive depolarizations were induced in the myocytes at a frequency of 6–8 Hz for 20 min by postsynaptic injection of positive current pulses of 5 msec duration. The frequency of SSCs (events/minute) was measured over 10 min periods immediately before and after the depolarizations. Two groups of frequency values obtained were subjected to rank sum test to determine whether there was significant difference and that were averaged within the group to determine the mean SSC frequency before and after depolarizations. Data point represents mean SSC frequency, and the line connects data from the same cell. *Solid lines* indicate synapses that showed significant reduction in the SSC frequency (at $p < 0.05$). *Dashed lines* show significant increase (at $p < 0.05$) and *dotted lines* show no significant change. *b*, Control recordings for which no current was injected into the myocyte during the 20 min period. *c*, Negative currents (square pulses of 5 msec duration) were injected into the myocyte to induce repetitive hyperpolarizations of amplitudes 70–100 mV for 20 min at a frequency of 6 Hz. *d*, Steady depolarization of the myocyte was produced by voltage clamping the myocyte membrane potential at 0 mV for 20 min. *e*, The same as *a* except that the recording pipette contained 5 mM BAPTA and 2 mM Ca^{2+}.

8.0% (±SEM, $n = 17$) after 7200 pulses. On the other hand, the reduction in the mean SSC amplitude became significant only after about 3000 pulses (Fig. 4*b*), again suggesting different mechanisms may be involved in the reduction of SSC amplitudes.

Effects of repetitive hyperpolarization and steady depolarization on spontaneous activity

Whether the depression of spontaneous synaptic activity depends on the polarity of postsynaptic potential changes was examined by injecting negative current pulses into innervated myocytes. The duration of the current pulses was 10 msec and the amplitude was adjusted to a level that produced hyperpolarizations in the range of 70–100 mV. For repetitive hyperpolarizations induced at 6 Hz for 20 min, we found no significant change in SSC frequency in all five cases examined ($p > 0.05$, rank sum test, see Fig. 2*c*). The average percentage change in either the mean frequency or the mean amplitude was not significant (Fig. 3).

In experiments studying the effect of repetitive depolariza-

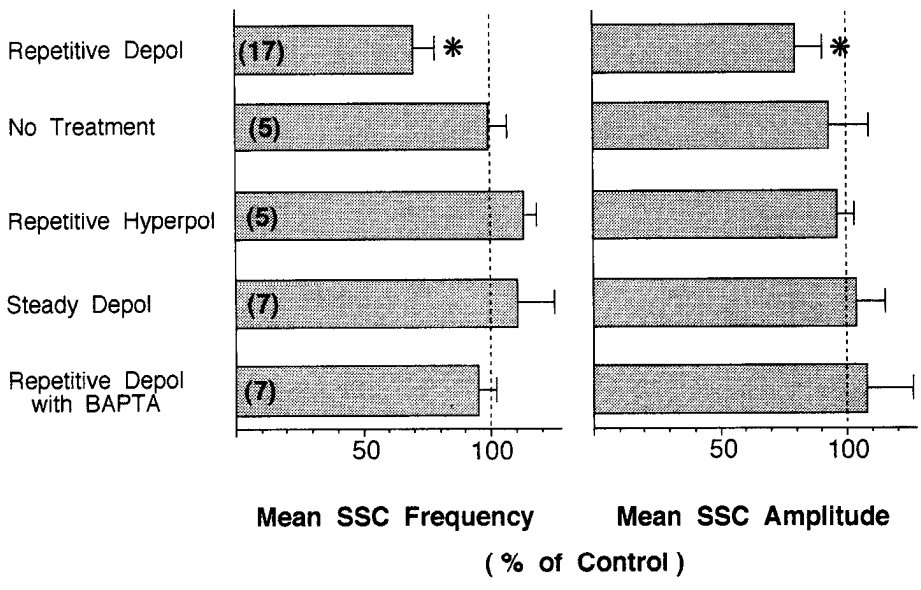

Figure 3. Effects of various types of postsynaptic stimulation on the frequency and amplitude of SSCs. The percentage values refer to average values of SSC frequency or mean SSC amplitude after the experimental treatment, normalized to the value prior to the treatment at the same cell. The same data set as that shown in Figure 2. *Repetitive Depol, No Treatment, Repetitive Hyperpol, Steady Depol,* and *Repetitive Depol with BAPTA* correspond to experimental conditions described for Figure 2*a–e*, respectively. Error bars represent SEM ($n = 5$–17). ANOVA test of the entire data set suggested significant difference in the subgroups (at $p = 0.02$ and 0.06 for mean SSC frequency and amplitude, respectively). *, values significantly different from the no treatment control by paired *t* test ($p < 0.05$).

tions, pulsed currents were used in order to initiate action potentials in the myocytes. To determine whether action potentials are required to produce the depression, steady depolarization was applied to myocytes by voltage clamping the membrane potential at 0 mV for a duration of 20 min. Many myocytes showed irreversible loss in their membrane potential after 20 min of steady depolarization, and large leakage currents prevented reliable assay of SSCs after the voltage clamp was set back to the original resting potential. However, in all seven cases where relatively stable membrane potential at the original level was restored after the steady depolarization, we found no reduction in the frequency of SSCs in three cases, a significant increase in three cases, and a significant reduction in one case (Figs. 2*d*, 3).

Persistence of depression in spontaneous synaptic activity

The depolarization-induced depression of SSCs appeared to be relatively long-lasting. Figure 5 depicts changes in SSC frequency with time in three separate experiments. We found SSC frequency remained relatively constant in the absence of postsynaptic depolarizations (Fig. 5*a*). Persistent depression was observed after 20 min of repetitive depolarizations at 6 Hz. When repeated episodes of depolarizations were applied, the effect appeared to accumulate until a steady level of depression was reached (Fig. 5*b,c*). No recovery of SSCs was observed for as long as stable recording could be made (up to 1 hr). Figure 5*d* depicts a composite graph of the SSC frequency with time obtained by normalizing all frequency values against the mean frequency observed prior to repetitive depolarizations from nine experiments that showed significant suppression. Persistent depression was apparent for the 15 min period after the depolarization.

Effects of repetitive depolarizations on evoked synaptic responses

The amplitude of impulse-evoked synaptic currents (ESCs) was measured by low-frequency (0.033 Hz) extracellular suprathreshold stimulation of the presynaptic neuron at the soma before and after repetitive induction of postsynaptic depolarizations. After 100 pulses of current injection (at 2 Hz), we observed a significant reduction of mean ESC amplitude of 20.4 ± 5.8% (±SEM, $n = 12$; $p < 0.005$, t test), as compared to the control predepolarization level. After 20 min of repetitive depolarizations (7200 pulses at 6 Hz), a reduction of 41.2 ± 4.6% (±SEM, $n = 25$; $p < 0.0005$, t test) was observed. Large variation in the extent of depression was due to the lack of effect on a minority of synapses examined. In 25 cases of prolonged (20 min) repetitive depolarizations, 6 showed no significant reduction in the mean ESC amplitude. Three examples of recordings that showed significant depolarization-induced depression of ESCs are shown in Figure 6. We noted that the reduction of evoked responses was accompanied by a reduction in the SSC frequency in most cases (14 of 19) and SSC amplitude in some cells (9 of 19), although the extent and time course in the reduction of spontaneous activity varied greatly. However, there was no apparent correlation between the extent of changes in SSC frequency or amplitude with changes in ESC amplitude. For example, in the case shown in Figure 6*b*, recovery of the evoked response was observed within 5 min after the depolarization, while little recovery was observed for spontaneous events.

In a series of experiments we studied the depression of ESCs after various number of depolarization pulses were applied. As

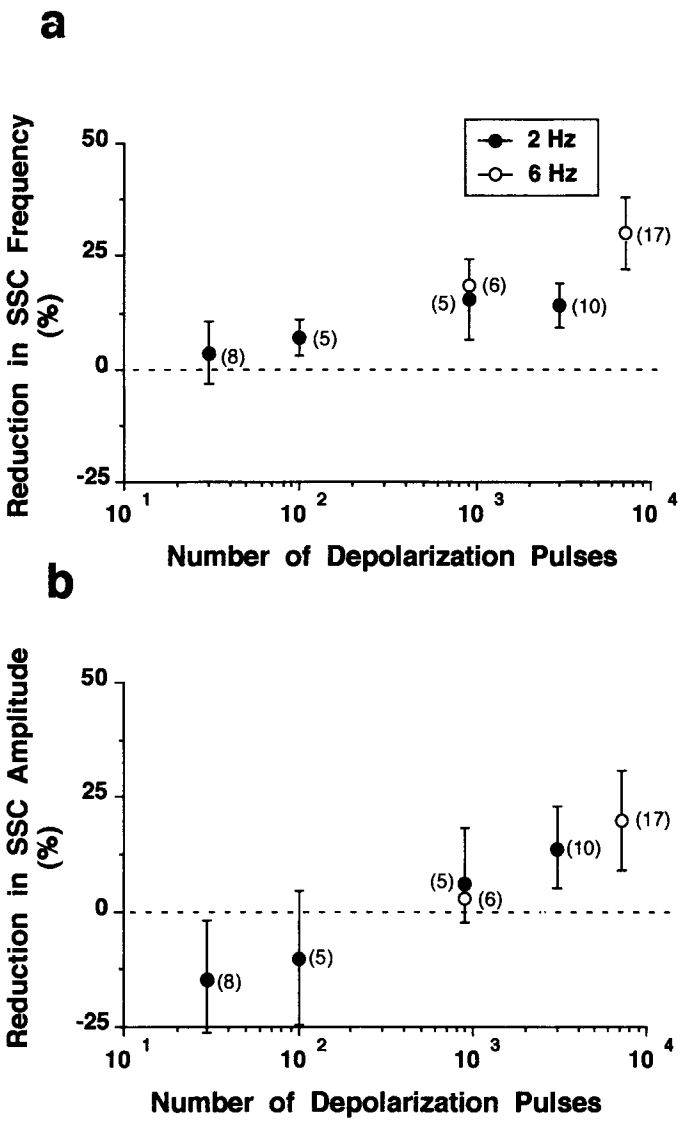

Figure 4. Reduction in mean SSC frequency (a) and mean SSC amplitude (*b*) following different numbers of depolarization pulses. Current pulses ranging from 10 to 7200 were applied to singly innervated myocytes at a frequency of 2 or 6 Hz. Percentage of reduction from control values (set as 100%) was determined within the first 10 min following the depolarization. The error bars refer to SEM, and the *number* associated with each bar refers to the number of cells examined.

shown in Figure 7, a small but significant reduction of ESC amplitude (20.2 ± 8.7%, ±SEM, $n = 6$) was found after 30 pulses of depolarizing currents but not hyperpolarizing currents, and the effect increased with the number of the current pulses from 10 to 7200. It may be noted that there was large variation in the extent of depression in different cells. For example, at 100 depolarization pulses, 6 out of 12 cases showed significant reduction in ESC amplitudes, while the rest (including the case shown in Fig. 6*a*) showed no significant change.

Involvement of postsynaptic Ca^{2+} elevation

In Purkinje cells of acute cerebellar slices, elevation of cytosolic Ca^{2+} resulting from depolarizing voltage pulses has been shown to induce a reduction of spontaneous inhibitory currents (Llano et al., 1991). In *Xenopus* nerve–muscle cultures, Ca^{2+} influx appears to be responsible for the induction of heterosynaptic

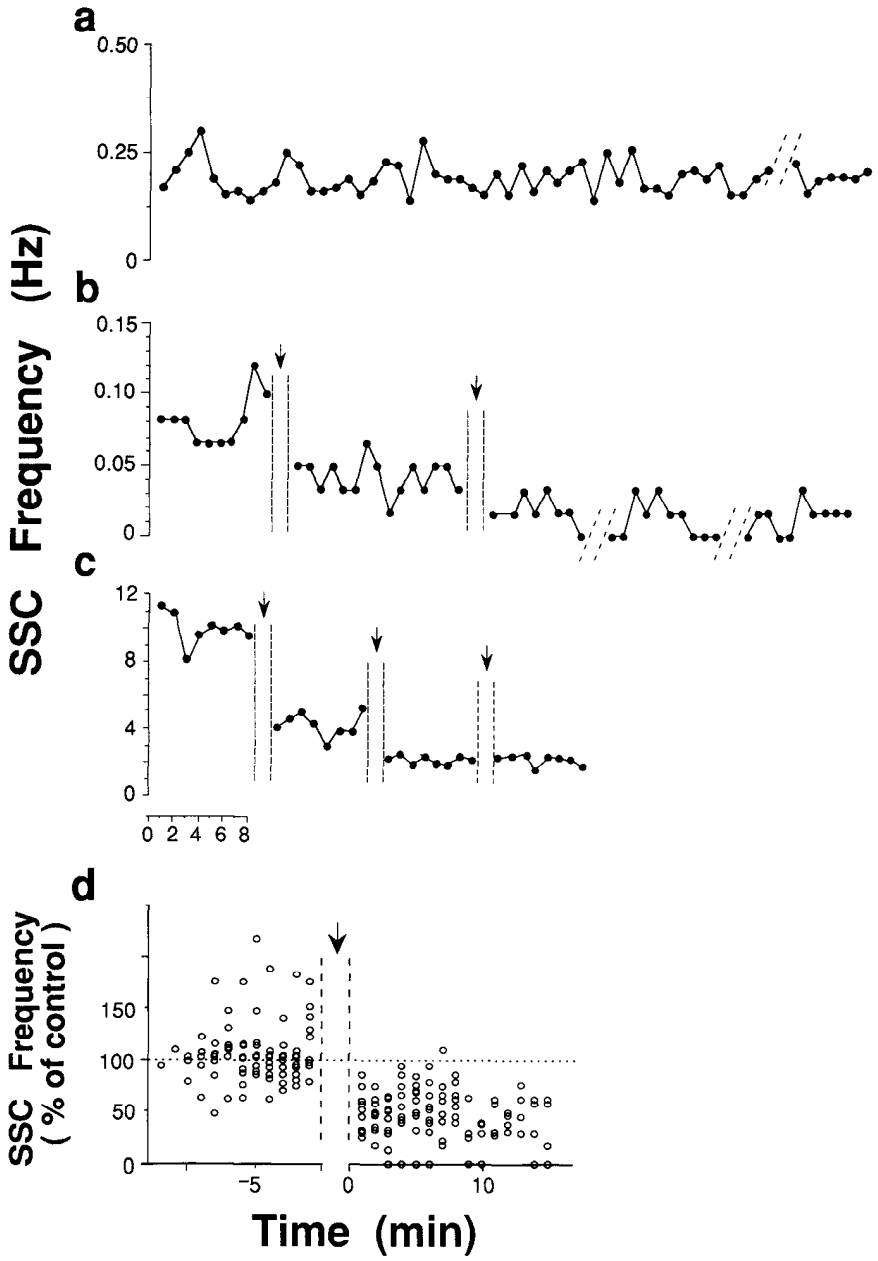

Figure 5. Time course of depolarization-induced depression in SSC frequency. *a* Control recording from a synapse for a duration of about 70 min, showing the stability of SSC frequency in the absence of repetitive depolarizations. *b* and *c*, The same 20 min episode of depolarizations (5 msec pulse, 6 Hz) was repeated two or three times during the course of the experiment (at time marked by the *arrow*), resulting progressive reduction in the SSC frequency. The effect of depolarizations appeared to accumulate and reached a steady level after two episodes of depolarizations. No recovery of the frequency was observed for as long as the recording could be made (1 hr in *c*). Data were omitted for 20 min intervals marked by the *tilted dashed lines*. *d*, Normalized plot of the changes in SSC frequency with time for nine synapses that showed significant depression after repetitive depolarizations. For each synapse, the data for the frequency (events/min) were normalized against the mean frequency of the same synapse during the entire recording period prior to the depolarizations.

suppression, since the latter was absent when myocyte Ca^{2+} level was buffered at a low level with BAPTA (Lo and Poo, 1994). In the present study, Ca^{2+} influx through the voltage-dependent Ca^{2+} channels due to postsynaptic depolarizations could also be responsible for the induction of synaptic depression. This possibility was tested by filling the whole-cell recording pipette with 5 mM BAPTA and 2 mM Ca^{2+} in order to buffer the Ca^{2+} concentration of the myocytes at 100 nM (see Lo and Poo, 1994). The average frequency of SSCs after 20 min depolarizations at 6 Hz was 93 ± 9.8% (±SEM, $n = 7$) and the mean ESC amplitude was 81 ± 14.3% (±SEM, $n = 9$) of the predepolarization control values, respectively. Although the reduction in the ESC amplitude persisted relative to the control values, the percentage reduction was significantly smaller than that found in the absence of BAPTA ($p < 0.05$, t test).

Correlation of depolarization-induced depression with synapse maturity

Although the majority of synapses studied were significantly affected by repetitive depolarizations, a number of cases showed no or opposite effect on either spontaneous or evoked synaptic currents. To understand the cause of this variability, we have examined the correlation between the depression effect and the characteristics of synaptic currents observed prior to the depolarizations. As shown in Figure 8, *a* and *b*, we found a negative correlation between depolarization-induced depression in SSC frequency with the predepolarization mean amplitude of the SSCs (correlation coefficient = −0.55). A weaker positive correlation was observed between the depression and the initial SSC frequency (coefficient = 0.40). The average value of the

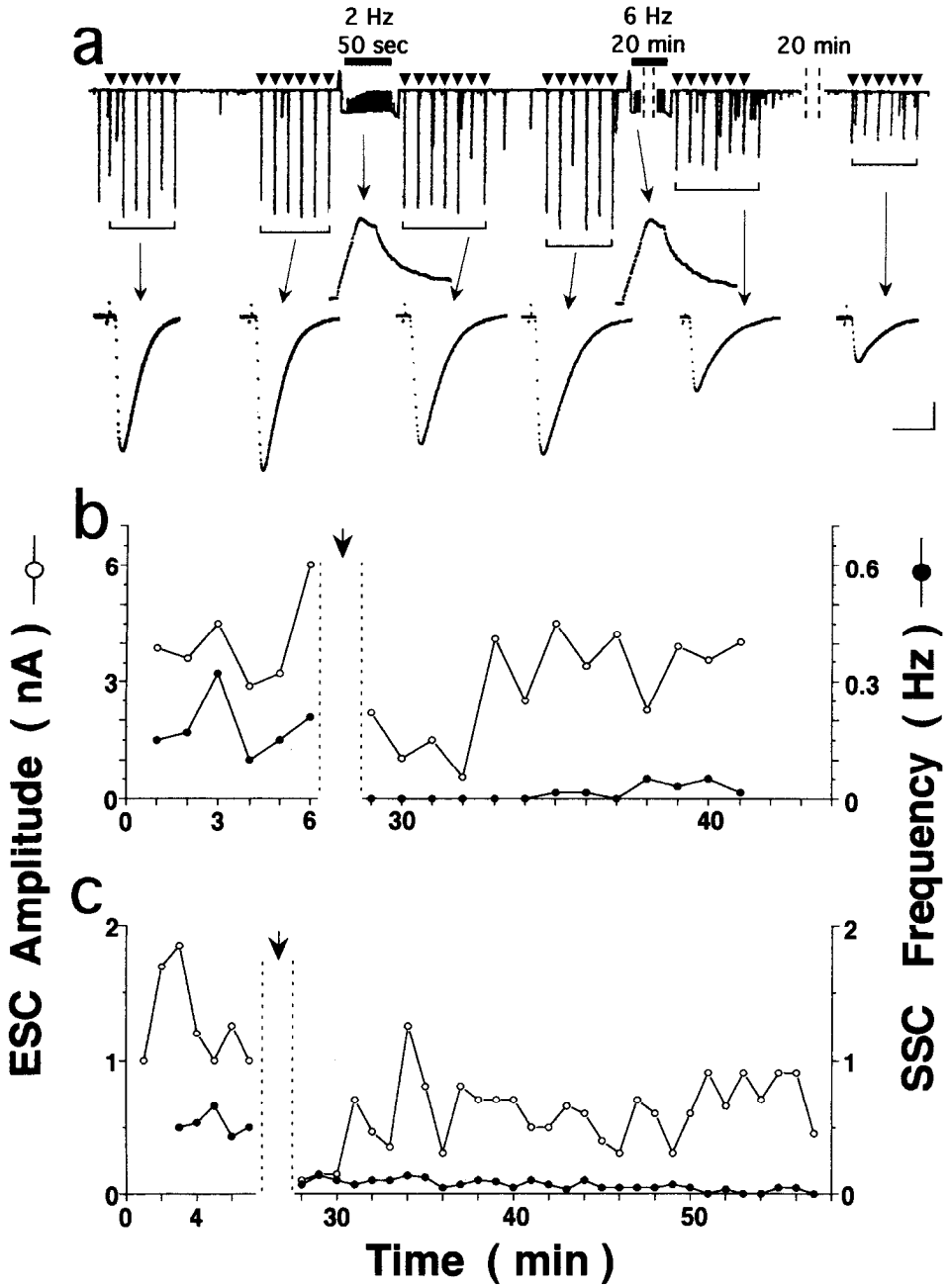

Figure 6. Evoked synaptic currents (ESCs) were reduced in amplitude after repetitive depolarizations. *a*, The continuous trace depicts the membrane current recorded from an innervated myocyte ($V_h = -70$ mV, filtered at 150 Hz). The presynaptic neuron was stimulated to fire action potentials and ESCs recorded in the myocyte were shown as downward deflections at the times marked by the *triangles*. Randomly occurring downward deflections are SSCs. Calibration: 1 nA, 1 min. The recording was interrupted for two intervals during which 100 and 7200 depolarization pulses, respectively, were applied. Shown below at higher time resolution are computer-averaged ESCs before and after the depolarizations (calibration: 1 nA, 5 msec), and myocyte membrane depolarizations during repetitive pulse application (calibration: 25 mV, 10 msec). *b* and *c*, Results from two experiments in which only one episode of repetitive depolarizations (20 min at 6 Hz) was applied. The mean ESC amplitude (*open circles*) and the mean frequency of SSCs (*solid circles*) were plotted before and after repetitive depolarizations. Each point represents the mean value over 1 min interval. Partial recovery of ESCs was observed in *b*, while persistent depression of both ESCs and SSCs was observed in *c*.

mean SSC amplitude of 6 cases that showed no suppression in SSC frequency was 0.34 ± 0.07 nA (±SEM), while that of the 11 cases showing depression was 0.14 ± 0.02 nA (±SEM). The difference was statistically significant ($p < 0.05$, t test). Moreover, we noticed that all synapses that showed depression in SSC frequency exhibited a simple skewed distribution of SSC amplitudes with predominantly small events, as illustrated in Figure 9*a*, while all cases that showed no change or increases in frequency exhibited many events at higher amplitudes (Fig. 9*b*). A cumulative frequency plot of the SSC amplitude distribution for all cases studied is presented in Figure 9*c*. Statistical significant difference (at 0.05 level, Kolmogorov-Smirnov test) was found between the values for cases that showed significant depression and that showed no change. Increases in SSC amplitudes are known to accompany synaptic maturation in these cultures (Kidokoro, 1984; Lu et al., 1992). On the other hand, no correlation of the synaptic maturation with the frequency of SSCs has been established in previous studies. The present results thus are consistent with the idea that less mature synapses are more susceptible to depression by repetitive postsynaptic depolarizations.

The extent of depolarization-induced depression of evoked responses appears to correlate with either the mean amplitude or the relative fluctuation of the predepolarization ESC amplitudes, as indicated by the coefficient of variation (SD/mean). As shown in Figure 8, *c* and *d*, the correlation coefficients were −0.50 and 0.43, respectively. Since maturation of these synapses is accompanied by an increase in the mean amplitude and a reduction of amplitude fluctuation (Buchanan et al., 1989; Evers et al., 1989; Lu et al., 1992), the above correlation again

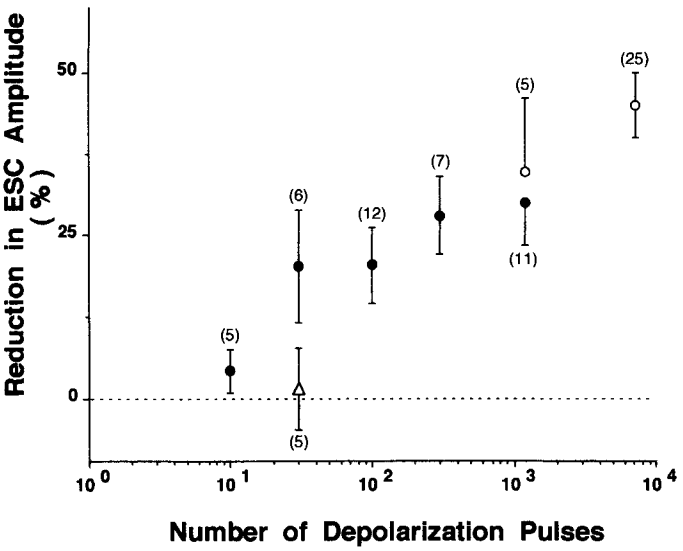

Figure 7. Depression of ESCs after different numbers of depolarizing pulses applied to postsynaptic myocyte. Percentage of reduction in the mean ESC amplitude is plotted against the pulse number, which ranges from 10 to 7200 pulses and were applied at 2 or 6 Hz. Data for 2 and 6 Hz pulses are represented by *solid* and *open symbols*, respectively. The *triangle* depicts result from experiments in which hyperpolarization pulses were applied. The error bars refer to SEM, and the *number* associated with each bar refers to the number of cells examined.

suggests that less mature synapses are more susceptible to modulation by postsynaptic depolarizations.

Effect of repetitive depolarizations on myocyte ACh sensitivity

Electrical activity of the muscle cell is known to downregulate the density of muscle surface ACh receptors (Berg and Hall, 1975; Schuetze and Role, 1987). It is possible that the observed effect of repetitive depolarizations on the amplitude of synaptic currents was due to a reduction of muscle ACh sensitivity. Lower ACh sensitivity could reduce spontaneous ACh-induced muscle responses to a level below the resolution of our recording, resulting an apparent reduction in SSC frequency. This possibility was examined by measuring the effect of repetitive depolarizations on the membrane current induced by focal iontophoresis of ACh on the extrasynaptic surface of the innervated myocyte. We found no significant change in ACh-induced responses at randomly selected spots on the myocyte surface after repetitive depolarizations of the myocyte for 20 min at 6 Hz. An example of the result is shown in Figure 10. The average ACh-induced response was 99.0 ± 6.6% (±SE, n = 5) of the control value before the depolarization. Although an effect of depolarizations on subsynaptic ACh sensitivity was not excluded, the present result suggests that depolarization paradigms used in the present study did not induce a global reduction of myocyte surface ACh sensitivity.

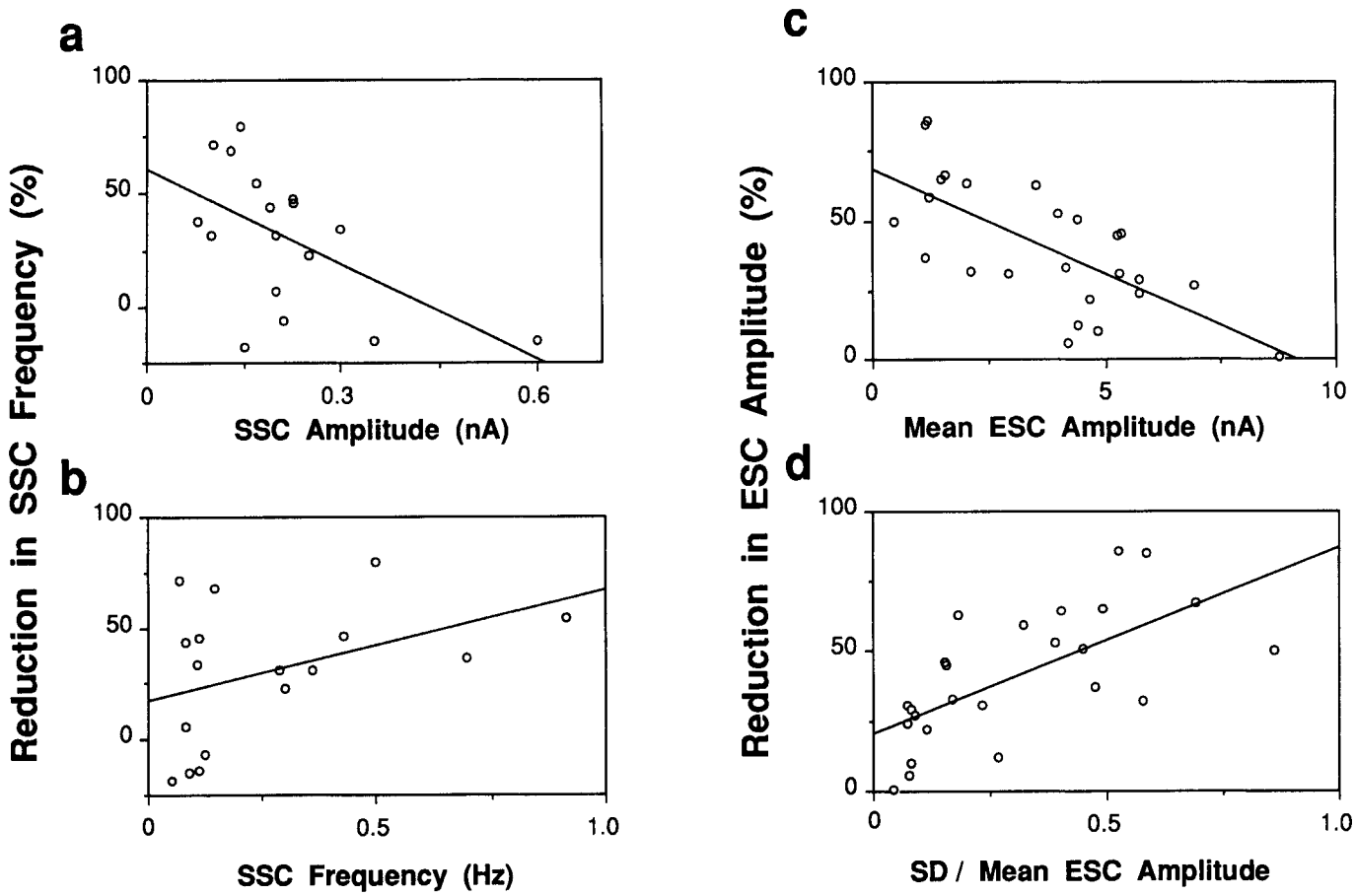

Figure 8. Correlation of the depolarization effect with the synaptic characteristics. *a* and *b*, The percentage reduction in SSC frequency after the depolarizations was plotted against the predepolarization mean SSC frequency or mean SSC amplitude observed for each synapse. The *solid lines* represent the best-fit lines for all points, with linear correlation coefficient of −0.55 and 0.40, respectively, in *a* and *b*. *c* and *d*, The percentage reduction in the mean ESC amplitude after the repetitive depolarizations was plotted against the predepolarization mean ESC amplitude (*c*) or the coefficient of variation (SD/mean) of the ESC amplitude (*d*). Correlation coefficient was −0.50 and 0.43, respectively, in *c* and *d*.

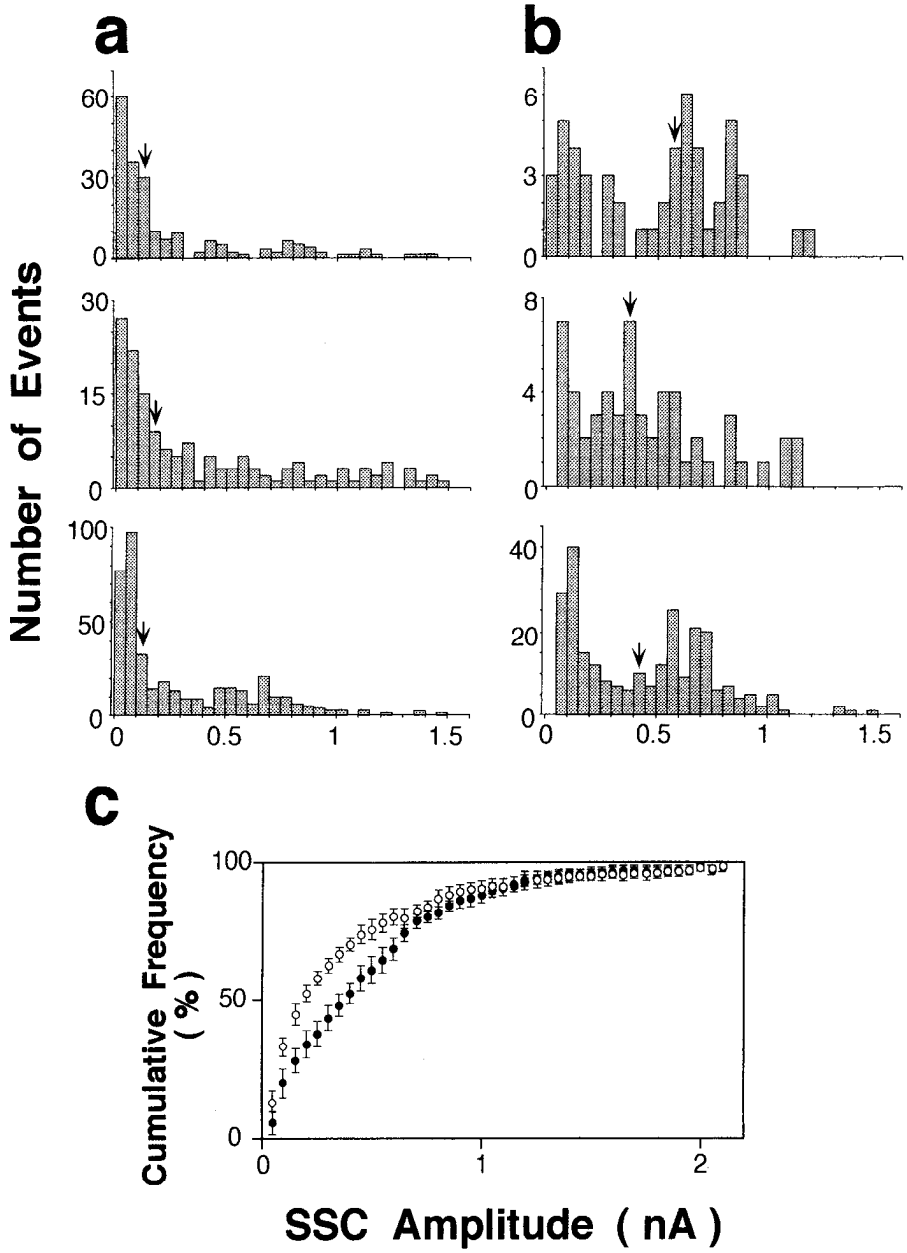

Figure 9. Amplitude histograms of SSCs from two groups of synapses. *a* and *b*, Examples of synapses that showed significant depression and no depression in SSC frequency, respectively, after repetitive depolarizations (6 Hz, 20 min) were applied. Note the distinct skewed distribution in *group a* and appearance of events at higher amplitudes in *group b*. The *arrow* marks the median amplitude. *c*, Comparison of amplitude distribution between all synapses in the "depression" and "no depression" groups. Cumulative frequency is defined as the percentage of total events exhibiting amplitudes below a given amplitude value. The data points depict the average frequency values for 11 and 6 synapses in the "depression" (*open circles*) and "no depression" (*solid circles*), respectively.

Fluctuation analysis of the ESC amplitude

Analysis of the fluctuation of the ESC amplitude could help to determine whether the observed synaptic depression was due solely to a change in the quantal size, for example, resulting from a reduction of postsynaptic density and/or sensitivity of ACh receptors, or whether changes in presynaptic release mechanisms are involved. As described previously (Lo and Poo, 1994), if the depression is due to a reduction of the quantal size, the coefficient of variation (CV) of ESC amplitudes should remain unchanged after induction of synaptic depression. We have measured the CV of ESC amplitudes before (CV_b) and after (CV_a) synaptic depression induced by repetitive depolarizations of the myocyte (6 Hz for 20 min). As shown in Figure 11, the ratios of CV squared (CV_b^2/CV_a^2) deviate significantly from unity (dashed line) in 14 experiments for which sufficient number of ESC events were collected for the analysis. Thus, the depression cannot be a result of a simple reduction of the quantal size, and a reduction in quantal content of the ESCs is likely to be involved. Whether this is due to a reduction of the number of available quanta and/or the probability of quantal release remained to be determined.

Discussion

In dually innervated *Xenopus* myocytes in culture, repetitive stimulation of one input resulted in persistent suppression of synaptic efficacy of the unstimulated input (Lo and Poo, 1991, 1994). Repetitive iontophoretic application of ACh on singly innervated myocytes also resulted in synaptic depression similar to that induced by stimulation of one input on dually innervated myocyte (Dan and Poo, 1992). In all these studies, ACh receptor activation and associated Ca^{2+} influx appear to play a major role in the induction of synaptic depression. However, under physiological conditions, postsynaptic receptor activation is al-

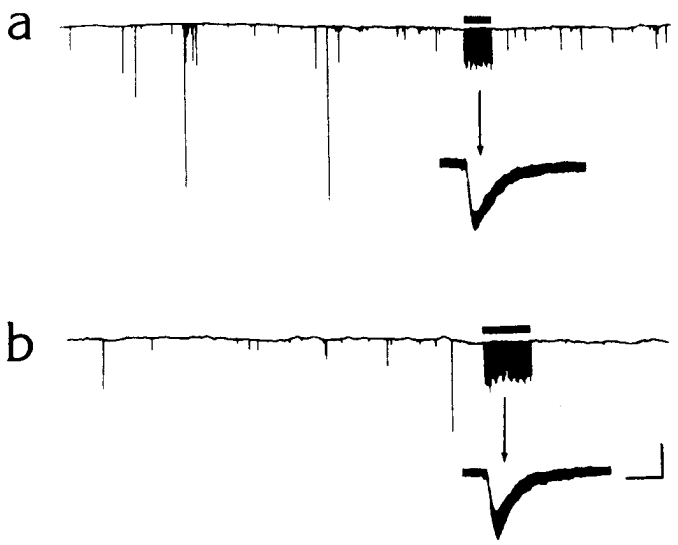

Figure 10. Iontophoretic mapping of ACh sensitivity of the myocyte before (*a*) and after (*b*) repetitive depolarizations. The experiment was similar to that shown in Figure 1 except that focal ACh iontophoresis was also used to induce ACh responses at the myocyte surface, in order to assay the myocyte ACh sensitivity. Square ACh pulses of the same amplitude (2 nA), duration (1 msec), and frequency (1 Hz) were applied during the interval marked by the *solid bar*. *Insets* depict 20 superimposed oscilloscopic traces of ACh-induced currents at a higher time resolution. Calibrations: *continuous trace*, 300 pA and 25 sec; *insets*, 150 pA and 10 msec.

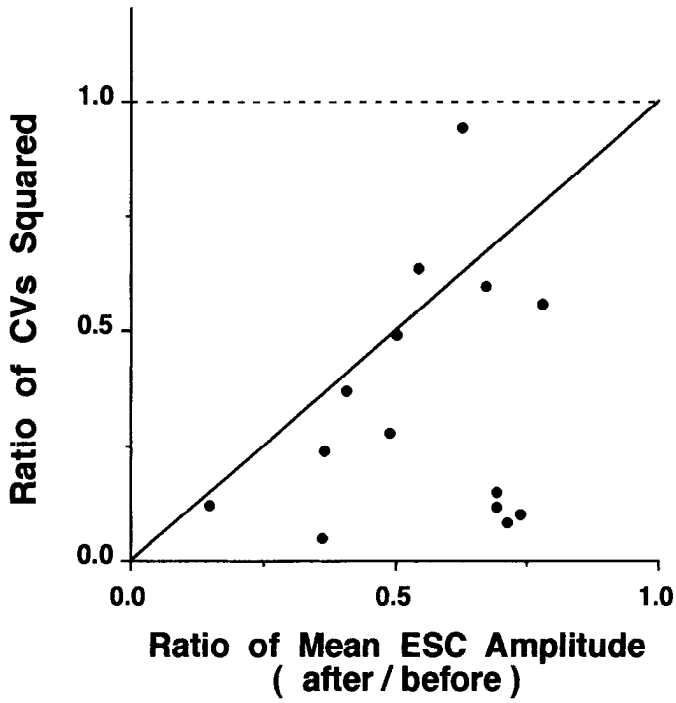

Figure 11. Analysis of the amplitude fluctuation of ESCs before and after depolarization-induced synaptic depression. The ratio of coefficients of variation (CV) squared before and after depression, that is, CV_b^2/CV_a^2, was plotted against the ratio of mean ESC amplitude after the depolarizations to that before the depolarization. Each point represents data from one experiment. *Dashed line* represents theoretical prediction for pure postsynaptic changes in synaptic responses, and the *solid diagonal line* represents the prediction for pure presynaptic changes in transmitter secretion.

ways accompanied by membrane depolarization and the opening of voltage-dependent Ca^{2+} channels in the plasma membrane. Moreover, in elongated muscle fibers, receptor activation and associated Ca^{2+} influx are restricted to the endplate region, while muscle depolarization propagates throughout the entire muscle fiber via action potentials. In multiply innervated neonatal muscle fibers, direct stimulation of the muscle, which presumably did not activate ACh receptors, accelerates the synapse elimination (Thompson, 1983). If synaptic depression is a prelude to synapse elimination, a depolarization-dependent synaptic depression could facilitate synapse elimination at sites relatively distant from the active synapses.

The main finding of the present study is the reduction in spontaneous and evoked synaptic currents after repetitive depolarizations of the postsynaptic myocyte. This is not due to deleterious effect of current injections, since similar hyperpolarization pulses were not effective. The viability of the myocyte was also suggested by the stability of myocyte membrane potential during and after repetitive depolarizations.

Two factors may account for the variability in synaptic depression following repetitive depolarizations. First, there may be heterogeneity in the neuronal types among the presynaptic neurons examined (Bixby and Spitzer, 1984). Although a great majority of neurons in these cultures are cholinergic and capable of forming functional synapses with cocultured myocytes (Xie and Poo, 1986), the SSC frequency at different synapses can vary by two orders of magnitude, suggesting intrinsic differences in the secretory activity among these spinal neurons. Such differences may contribute in part to the differences in their response to muscle depolarizations. Second, the status of maturation of various synapses may differ. The SSCs of early developing synapses show skewed amplitude distributions and small mean amplitudes, and their ESCs exhibit higher fluctuation. These synapses appear to be most affected by the repetitive depolarizations (Fig. 9). Indeed, our analysis of ESC fluctuation (Fig. 11) showed that synapses with higher fluctuation exhibited more pronounced synaptic depression after depolarization.

Depolarization-induced depression could result from a decreased postsynaptic ACh receptor density/sensitivity, a reduced ACh secretion from the presynaptic neuron, a change in synaptic morphology, or a combination of these factors. The finding that iontophoretically induced ACh responses at the myocyte surface remained unchanged suggests that a global decrease in the ACh receptor density/sensitivity seems unlikely. Since subsynaptic ACh receptors are not accessible to iontophoresis assay (because of the hindrance of presynaptic neurites), it is possible that the density/sensitivity of junctional receptors was selectively reduced by depolarizations. We have observed significant reduction of SSC frequency after 100 pulses of depolarization, but significant reduction of SSC amplitude was observed only when the number of depolarization pulses was more than 3000. Even for prolonged repetitive depolarizations at 7200 pulses, there was substantial fraction of cells (5 of 11) for which the reduction in SSC frequency was not accompanied by any significant change in the mean SSC amplitude. It should also be noted that no change in the SSC amplitude was detected in experiments using heterosynaptic stimulation at 100 pulses (see preceding companion article, Lo and Poo, 1994). Results from the analysis of ESC amplitude fluctuation have suggested changes in presynaptic release mechanisms after

depression, either a reduction in available quanta or reduced probability of secretion. Nevertheless, we cannot exclude the possibility that the depression was caused by purely morphological changes of the synapse, for example, a selective withdrawal of some of the nerve terminal branches, or by a selective elimination of some postsynaptic ACh receptor clusters. The reduction of the mean amplitude of SSCs and the significant shift of amplitude distribution toward smaller sizes in some cells suggest that there may be a reduction of subsynaptic ACh sensitivity, a reduced content of ACh contained in the quantal unit, a reduced release probability of larger quanta, or a selective withdrawal of terminal branches responsible for their secretion. Different mechanisms responsible for the effects on the frequency and amplitude of SSCs may operate after different duration of depolarizations. For example, reduction of postsynaptic ACh sensitivity may appear after presynaptic changes had occurred, thus reduction of SSC amplitude was observed only after prolonged repetitive depolarizations.

The finding that steady depolarization was not effective in producing synaptic depression suggests that initiation of action potentials in the myocyte may be required for the observed effect. An immediate consequence of repetitive action potentials is the opening of voltage-dependent ion channels in the muscle membrane and the resulting flow of cations across the membrane. It is well known that extracellular accumulation of K^+ after repetitive impulse activity may significantly affect the membrane potential and signaling processes in the nervous system (Kuffler et al., 1966). Because of the easy access of diffusion space in these monolayer cultures, membrane depolarization resulting from extracellular K^+ accumulation is likely to be less significant than that occurring *in vivo*. Depolarization of nerve terminal induced by K^+ accumulation is clearly not the cause of reduced frequency of spontaneous release, since elevating extracellular K^+ was found to markedly increase the SSC frequency (Tabti and Poo, 1990). The accumulation of K^+ within the synaptic cleft, however, may exert other actions on the nerve terminal which are not directly related to membrane depolarization. Neurites in cell cultures have been shown to exhibit negative chemotropic response to elevated concentrations of extracellular K^+ (Sussdorf and Camponot, 1986). Withdrawal of some of the terminal branches from the muscle surface could account for the present result as discussed above.

In addition to K^+ efflux, influx of Ca^{2+} ions through voltage-dependent Ca^{2+} channels could trigger Ca^{2+}-dependent cytosolic events in the myocyte that eventually result in a retrograde modulation of the presynaptic nerve endings. In Purkinje neurons of acute cerebellar slices, an elevation of Ca^{2+} resulting from depolarizing voltage pulses has been shown to induce a reduction of spontaneous inhibitory synaptic currents (Llano et al., 1991). In *Xenopus* nerve–muscle cultures, iontophoretic application of ACh pulses at postsynaptic myocytes is also effective in inducing persistent synaptic depression at nerve–muscle synapses, which appears to be due to the influx of Ca^{2+} through ACh channels (Dan and Poo, 1992). Similar dependence of Ca^{2+} influx was also observed for heterosynaptic suppression induced by tetanic stimulation of neurons coinnervating the same myocyte (Lo and Poo, 1994). It is thus likely that Ca^{2+} influx through voltage-dependent Ca^{2+} channels is responsible for triggering the depolarization-induced depression reported here. This contention is supported by the finding in the present study that buffering myocyte cytosolic Ca^{2+} at a low level significantly reduced the depolarization-induced effects. If postsynaptic Ca^{2+} is the sole determinant of the synaptic depression, the elevation of myocyte Ca^{2+} by depolarizations is apparently lower than that induced by ACh receptor activation. For the same frequency (2 Hz) and total number of stimuli (100), the level of depression induced by depolarizations is significantly lower than that induced by heterosynaptic stimulation (Lo and Poo, 1994) or by repetitive iontophoretic application of ACh on the postsynaptic myocyte (Dan and Poo, 1992).

Induction of spontaneous secretion of ACh is one of the earliest events during the formation of neuromuscular synapse (Xie and Poo, 1986). The developmental significance of this early ACh secretion is now beginning to be understood. Because of the high input resistance of the embryonic muscle cell (Chow and Poo, 1984), spontaneous ACh secretion at developing synapses produces large postsynaptic potentials, many of which are capable of inducing action potentials and contraction of the muscle cell (Xie and Poo, 1986). It has been shown that the differentiation of muscle striations in *Xenopus* myocytes is regulated by the ACh-induced contraction during the first few days of synaptogenesis (Kidokoro and Saito, 1988). Muscle activity is also known to regulate the biochemical and contractile properties of the innervated muscle cells (Salmons and Sreter, 1976). It is likely that such regulation starts soon after nerve–muscle contact, and the spontaneous release of ACh as well as other trophic factors plays a crucial role in the regulation of synaptic specializations. Results from the present study suggest that repetitive postsynaptic depolarizations that accompanying the muscle activity will exert a negative influence on the ACh secretion from the innervating nerve terminal. If the secreting nerve terminal is capable of protecting itself from the effect of postsynaptic depolarizations induced by its own secretion, then depolarization-induced depression could contribute in the synaptic competition whereby other terminals on the same postsynaptic cell become suppressed. Since depolarization-induced effects appear to be most pronounced at synapses of immature properties, these effects may contribute more to synaptic competition during the initial period of synaptogenesis.

References

Balice-Gordon RJ, Lichtman JW (1991) Motor nerve terminals are selectively eliminated from regions of neuromuscular junctions focally blocked with α-bungarotoxin. Soc Neurosci Abstr 17:514.3.

Balice-Gordon RJ, Lichtman JW (1993) *In vivo* observations of pre- and post-synaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. J Neurosci 13:834–855.

Benoit P, Changeux JP (1975) Consequences of tenotomy on the evolution of multi-innervation in developing rat soleus muscle. Brain Res 99:354–358.

Berg BK, Hall ZW (1975) Increased extrajunctional acetylcholine sensitivity produced by chronic postsynaptic neuromuscular blockade. J Physiol (Lond) 244:659–676.

Bixby JL, Spitzer NC (1984) The appearance and development of neurotransmitter sensitivity embryonic spinal neurons *in vitro*. J Physiol (Lond) 353:143–155.

Brown TH, Kairiss EW, Keenan CL (1990) Hebbian synapses: biophysical mechanisms and algorithms. Annu Rev Neurosci 13:475–512.

Buchanan J, Sun Y, Poo M-m (1989) Studies of nerve-muscle interactions in *Xenopus* cell culture: fine structure of early functional contacts. J Neurosci 9:1540–1554.

Chow I, Poo M-m (1984) Formation of electrical coupling between embryonic *Xenopus* muscle cells in culture. J Physiol (Lond) 346: 181–194.

Cline HT, Debski E, Constantine-Paton M (1987) NMDA receptor antagonist desegregates eye-specific stripes. Proc Natl Acad Sci USA 84:4342–4345.

Constantine-Paton M, Cline HT, Debski E (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. Annu Rev Neurosci 3:129–154.

Dan Y, Poo M-m (1992) Hebbian depression of isolated neuromuscular synapses *in vitro*. Science 256:1570–1573.

Evers J, Laser M, Sun Y, Xie Z, Poo M-m (1989) Studies of nerve-muscle interactions in *Xenopus* cell culture: analysis of early synaptic currents. J Neurosci 9:1523–1539.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. Pfluegers Arch 391:85–100.

Hebb HO (1949) The organization of behavior, p 62. New York: Wiley.

Hubel DH, Wiesel TN (1965) Binocular interaction in striate cortex of kittens reared with artificial squint. J Neurophysiol 28:1041–1059.

Kidokoro Y (1984) Two types of miniature endplate potentials in Xenopus nerve muscle cultures. Neurosci Res 1:157–170.

Kidokoro Y, Saito M (1988) Early cross-striation formation in twitching *Xenopus* myocytes in culture. Proc Natl Acad Sci USA 85:1978–1982.

Kuffler SW, Nicholls JG, Orkand RK (1966) Physiological properties of glial cells in the central nervous system of amphibian. J Neurophysiol 29:768–787.

Llano I, Leresche N, Marty A (1991) Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. Neuron 6:565–574.

Lo YJ, Poo M-m (1991) Activity-dependent synaptic competition *in vitro*: heterosynaptic suppression of developing synapses. Science 254: 1019–1022.

Lo YJ, Poo M-m (1994) Heterosynaptic suppression of developing neuromuscular synapses in culture. J Neurosci 14:4684–4693.

Lu B, Greengard P, Poo M-m (1992) Exogenous synapsin I promotes functional maturation of developing neuromuscular synapses. Neuron 8:521–529.

Pasic TR, Rubel EW (1989) Rapid changes in cochlear nucleus cell size following blockade of auditory nerve electrical activity in gerbils. J Comp Neurol 283:474–480.

Purves D, Lichtman JW (1985) Principles of neural development, pp 289–312. Sunderland, MA: Sinauer.

Rabacchi S, Bailly Y, Dehaye-Bouchaud N, Mariani J (1992) Involvement of the *N*-methyl-D-aspartate (NMDA) receptors in synapse elimination during cerebellar development. Science 256:1823–1825.

Salmons S, Sreter FA (1976) Significance of impulse activity in the transformation of skeletal muscle type. Nature 263:30–34.

Schuetze SM, Role LW (1987) Developmental regulation of nicotinic acetylcholine receptors. Annu Rev Neurosci 10:403–457.

Shatz CL (1990) Impulse activity and the patterning of connections during CNS development. Neuron 5:745–756.

Srihari T, Vrbova G (1978) The role of muscle activity in the differentiation of neuromuscular junctions in slow and fast chick muscles. J Neurocytol 7:529–540.

Stent GS (1973) A physiological mechanism for Hebb's postulate of learning. Proc Natl Acad Sci USA 70:997–1001.

Stretavan DW, Shatz CJ, Stryker MP (1988) Modification of retinal ganglion cell axon morphology by prenatal infusion of tetrodotoxin. Nature 336:468–471.

Sussdorf WS, Campenot RB (1986) Influence of the extracellular potassium environment on neurite growth in sensory neurons, spinal cord neurons and sympathetic neurons. Dev Brain Res 25:43–52.

Tabti N, Poo M-m (1990) Spontaneous synaptic activity at developing neuromuscular junctions. In: Progress in brain research, Vol 84 (Aquilonius S-M, Gillberg P-G, eds), pp 63–72. Amsterdam: Elsevier.

Thompson W (1983) Synapse elimination in neonatal rat muscle is sensitive to pattern of muscle use. Nature 302:614–616.

Xie Z, Poo M-m (1986) Initial events in the formation of neuromuscular synapse. Proc Natl Acad Sci USA 83:7069–7073.