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Postsynaptic Elevation of Calcium Induces Persistent Depression of Developing Neuromuscular Synapses

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Summary

Synaptic activity is known to modulate neuronal connectivity in the nervous system. At developing Xenopus neuromuscular synapses in culture, repetitive postsynaptic application of ACh near the synapse leads to immediate and persistent synaptic depression, which was shown to be caused by reduction of presyaptic evoked transmitter release. However, little depression was found when ACh was applied to the muscle 20 μm or further from the synapse. Fluorescence imaging of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) showed that each ACh pulse induced a transient elevation of myocyte $[Ca^{2+}]_i$ that spread approximately 20 μm. Local photoactivated release of Ca^{2+} from the caged Ca^{2+} chelators nitr-5 or nitrophen in the postsynaptic cell was sufficient to induce persistent synaptic depression. These results support a model in which localized Ca^{2+} influx into the postsynaptic myocyte initiates transsynaptic retrograde modulation of presynaptic secretion mechanisms.

Introduction

Electrical activity plays a critical role in shaping the pattern of synaptic connections in the developing nervous system (Purves and Lichtman, 1985; Constantine-Paton et al., 1990; Van Essen et al., 1990; Goodman and Shatz, 1993). Activity-dependent synaptic modulation is also responsible for several forms of plasticity in the mature nervous system (Bourne and Nicoll, 1993; Bliss and Collingridge, 1993). Although the underlying cellular mechanisms are not yet fully understood, activation of postsynaptic receptors appears to be required in many forms of developmental (Cline et al., 1987; Rabacchi et al., 1992) and adult synaptic plasticity (Collingridge et al., 1983; Harris et al., 1984; Linden and Connor, 1991; Mulkey and Malenka, 1992; Dudek and Bear, 1992; Bourne and Nicoll, 1993). For example, at developing neuromuscular junctions, elimination of polyneuronal innervation depends on activation of postsynaptic ACh receptors (Srihari and Vrbova, 1978; O'Brien et al., 1980). Moreover, Ca^{2+} influx into postsynaptic cells resulting from receptor activation at excitatory synapses appears to be crucial for synaptic modulation in many systems. For example, Ca^{2+} influx is required for the induction of both long-term potentiation (Lynch et al., 1984; Bliss and Collingridge, 1993) and long-term depression in the hippocampus (Mulkey and Malenka, 1992) and cerebellum (Sakurai, 1990; Daniel et al., 1992). In fact, a rise in postsynaptic $[Ca^{2+}]_i$ alone appears to be sufficient to cause a long-lasting potentiation of synaptic transmission (Malenka et al., 1988).

Using Xenopus nerve-muscle cultures, Lo and Poo (1991, 1994) have shown that the efficacy of transmitter release at a developing neuromuscular synapse can be reduced by a repetitive activation of other spinal neurons innervating the same postsynaptic myocyte. This heterosynaptic suppression appears to result from activation of postsynaptic ACh receptors, and may be an early manifestation of synaptic competition between coinnervating nerve terminals, a process that leads to elimination of polyneuronal innervation (Van Essen et al., 1990). To facilitate the study of the cellular mechanisms underlying this form of activity-dependent synaptic modulation, we reduced this model system to a singly innervated myocyte and examined the effect of direct postsynaptic activation of ACh receptors on synaptic efficacy. Repetitive iontophoretic application of ACh pulses to the myocyte, which elicit membrane currents resembling nerve-evoked postsynaptic currents, resulted in synaptic depression similar in extent to that induced by repetitive heterosynaptic stimulation. Furthermore, in both ACh-induced and heterosynaptic depression, the depression was apparently caused by a reduced number of transmitter quanta released from the presynaptic nerve terminal, rather than by changes in postsynaptic ACh responsiveness (Dan and Poo, 1992a, Lo and Poo, 1994). Consistent with Hebb's postulate (Hebb, 1949), coincident pre- and postsynaptic activation blocked the synaptic depression. Influx of Ca^{2+} into the postsynaptic myocyte appears to be necessary for the induction of both heterosynaptic and ACh-induced synaptic depression, since introduction of 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) postsynaptically prevented synaptic depression (Dan and Poo, 1992a; Lo and Poo, 1994).

In the present study, we further characterized the role of postsynaptic Ca^{2+} in the induction of persistent synaptic depression. We found that the depression caused by repetitive ACh pulses required close proximity between the site of ACh application and the synapse. To demonstrate Ca^{2+} influx through ACh receptors and to map the spatial pattern of Ca^{2+} rises resulting from focal ACh iontophoresis, we performed Ca^{2+} imaging experiments. We found that the spatial requirement for the site of ACh application can be accounted for by the localized nature of postsynaptic elevation of $[Ca^{2+}]_i$. Moreover, using photoactivated release of Ca^{2+} from caged Ca^{2+} chelators, we found that elevation of Ca^{2+} alone is sufficient to induce persistent synaptic depression. Together with previous results, these findings provide strong evidence that postsynaptic elevation of Ca^{2+} near the synapse is both necessary and sufficient to induce a persistent presynaptic depression at developing neuromuscular synapses.

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Results

Synaptic Depression Induced by Repetitive ACh Pulses

In 1-day-old cocultures of Xenopus spinal neurons and myotomal myocytes, functional neuromuscular synapses are formed soon after nerve-myocyte contact (Kidokoro and Yeh, 1982, Evers, et al., 1989). Impulse-evoked excitatory postsynaptic currents (EPCs) are readily detected by whole-cell voltage-clamp recording from neurite-contacted myocytes. Innervated spherical myocytes (average diameter 30 μm) were used in all experiments, except those studying distance-dependence (see below). Synaptic efficacy was assayed by measuring the mean amplitude of EPCs elicited by test stimuli applied to the presynaptic neuronal soma at a low frequency (0.05–0.1 Hz). Marked reduction of the mean EPC amplitude was consistently observed immediately following application of a standard episode of 100 ACh pulses to the postsynaptic myocyte surface at a frequency of 2 Hz. The tip of the ACh pipette was placed near the synaptic site, usually on the side of the myocyte facing the culture substratum (Figure 1A). The myocyte was either voltage-clamped throughout the entire experiment, or was recorded under current-clamp during the brief period of ACh application. Results from two typical experiments are illustrated in Figures 1B and 1C. The peak amplitudes of membrane currents induced by ACh pulses were set to be 2–3 nA, which lies within the range of normal EPCs found at these developing synapses. Under current-clamp condition, these ACh pulses usually evoked action potentials in the myocytes (see Figure 1C). The average percent reduction in the mean EPC amplitude within the first 3 min after application of 100 ACh pulses was 59.4 $\pm$ 4.0, and 55.2 $\pm$ 8.7 (SEM, n = 6 and 7) for current-clamp and voltage-clamp conditions, respectively. The extent of synaptic depression induced under these two conditions was not significantly different ($p > 0.05$, t test). This result indicates that membrane depolarization is not required for the induction of synaptic depression. These results are consistent with previous findings in this system (Dan and Poo, 1992a). For later experiments (see below), ACh pulses were applied only under voltage-clamp conditions.

Effect of ACh Pulses on Miniature End-Plate Currents

Our previous work has suggested that synaptic depression induced by ACh iontophoresis was due to presynaptic reduction of evoked ACh release, rather than a decreased postsynaptic ACh sensitivity (Dan and Poo, 1992). In the present study we further examined the effect of repetitive ACh application on the postsynaptic sensitivity by comparing the amplitude distribution of spontaneous miniature end-plate currents (MEPCs) before and after ACh application. We found no change in the mean MEPC amplitude after ACh pulses had induced a marked reduction of mean EPC amplitude. The mean MEPC amplitude after ACh application was 101.5% $\pm$ 3.3% (SEM, n = 10) of the control value. The distribution of MEPC amplitudes exhibited no significant change after synaptic depression was induced ($p < 0.05$, Kolmogorov-Smirnov test, data not shown, but see Figure 6). Furthermore, there was no significant difference in frequency before and after ACh iontophoresis (102% $\pm$ 8% of control values, SEM, n = 5). Together with our previous results using quantal analysis (Dan and Poo, 1992), these data support the notion that the synaptic depression is expressed as a presynaptic modification, rather than a postsynaptic change.

Dependence on the Site of ACh Application

The dependence of synaptic depression on the site of ACh receptor activation was examined using synapses made between Xenopus spinal neurons and spindle-shaped myocytes. In order to estimate the distance between the site of ACh application and the synapse, we have selected innervated spindle-shaped myocytes on which the sites of neurite contact are clearly restricted to one end of the spindle (see Figures 2A and 2B). In each experiment, the iontophoretic ACh pipette was placed at two different positions on the myocyte surface along the long axis of the myocyte. As depicted in Figure 2C, when 100 ACh pulses were first applied at a distance of about 70 μm from the synaptic site, no effect on synaptic efficacy was observed. The same 100 ACh pulses, when applied again at a region within 10 μm of the synapse, induced a marked reduction in the mean EPC amplitude. Figure 3 summarizes results from 24 experiments using spindle myocytes in which the distance between the site of ACh application and the synapse could be estimated. No significant synaptic depression was found when ACh pulses were applied at a distance of more than 30 μm, while significant depression was induced in all cases in which ACh pulses were applied within 10 μm of the synapse. This distance dependence indicates that the cellular mechanism underlying the induction of synaptic depression is relatively localized to the site of ACh application. This result is consistent with the previous finding that the activity-induced heterosynaptic depression between two coinnervating nerve terminals can occur only when the two synapses are in close proximity (Lo and Poo, 1991), although in the latter case the distance between the coinnervating synapses was less precisely determined.

Profile of ACh-Induced Ca^{2+} Influx

An immediate consequence of ACh application is the activation of postsynaptic nicotinic ACh channels and subsequent Ca^{2+} influx (Decker and Dani, 1990). Previous studies have shown that the induction of synaptic depression by repetitive ACh application requires a rise in postsynaptic Ca^{2+}, since cytoplasmic loading of BAPTA completely abolished the effect of the ACh pulses (Dan and Poo, 1992a). If Ca^{2+} is indeed the primary signal for the induction of depression, a spatially restricted Ca^{2+} elevation may account for the distance-dependence of the depression described above. This hypothesis was tested by direct examination of the cytosolic Ca^{2+} changes induced by iontophoretic ACh application at the surface of spindle-shaped myocytes, using

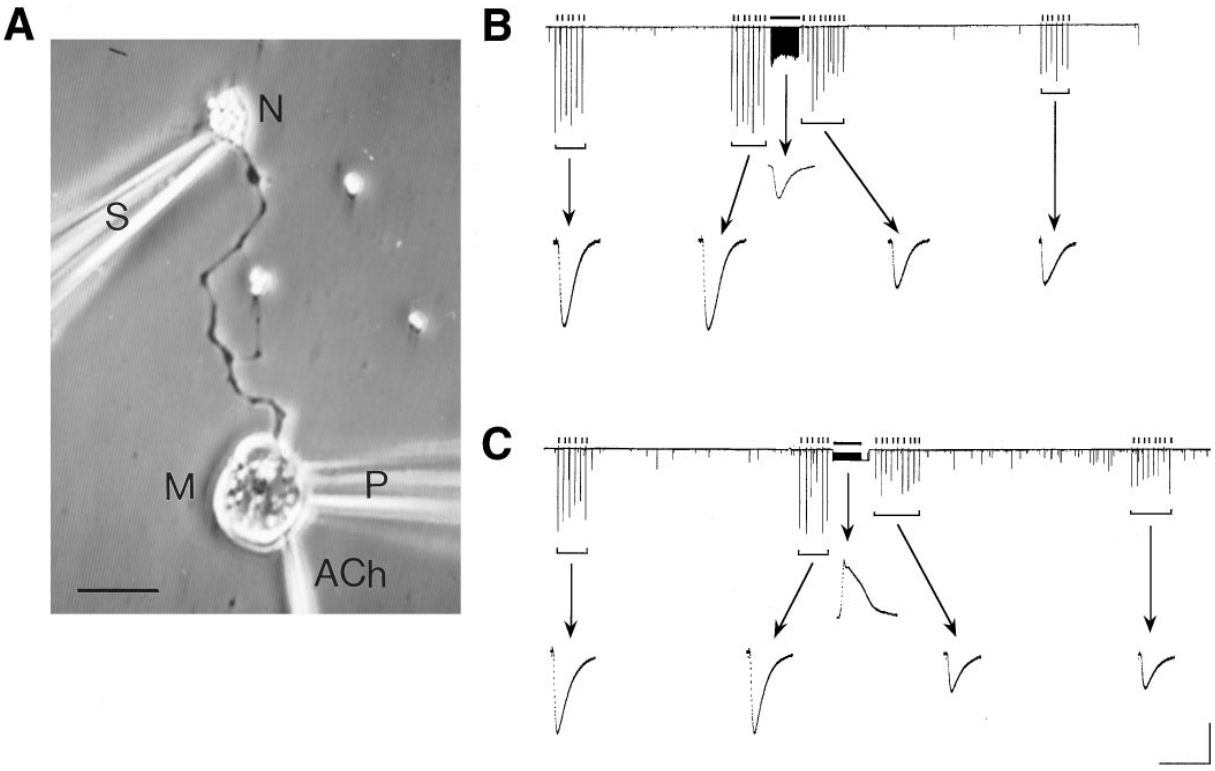

Figure 1. Synaptic Depression Induced by Repetitive Iontophoretic Application of ACh

(A) Phase-contrast microscopic image of an isolated neuromuscular synapse in a 1-day-old Xenopus culture. A spherical myocyte (M) innervated by a cocultured spinal neuron (N). The membrane current of the myocyte was recorded by a whole-cell patch-clamp recording pipette (P), the spinal neuron was stimulated at the soma by an extracellular stimulating pipette (S), and the tip of the iontophoretic ACh pipette (ACh) was placed at the surface of the myocyte on the side facing the glass substratum. Scale bars represent 30 μm.

(B) Continuous traces represent the membrane current recorded from an innervated myocyte under voltage-clamp ($V_c = -70$ mV, filtered at 150 Hz). Evoked postsynaptic currents (EPCs) were elicited at the times marked by the short vertical lines. An episode of 100 ACh pulses was applied (at 2 Hz) to the myocyte at the time marked by the horizontal bar. Computer-averaged traces of EPCs and ACh-induced membrane currents are shown below at a higher time resolution (filtered at 2.5 kHz) for the recording periods indicated by the arrows. Scale bars represent slow traces, 3 nA, 2 min; fast traces, 3 nA, 30 ms.

(C) Similar experiment as that shown in (B), except that the recording was changed to current-clamp configuration during ACh application. The trace below shows a depolarization and action potential triggered by an ACh pulse. Scale bars represent 50 mV, 50 ms for ACh-induced depolarizations.

fluorescence imaging. The culture was incubated in solution containing 10 μM Fluo-3–AM for 1 hr. Myocytes were then whole-cell voltage-clamped at −110 mV. Fluo-3 fluorescence in the myocyte was monitored before and after iontophoretic ACh application at one end of the myocyte. We observed a transient and local elevation of fluorescence that lasted for 250–350 ms after each ACh pulse application. An example of the local fluorescence increase is shown in Figure 4. Quantitative measurement of the profile of fluorescence intensity showed that the maximal amplitude of the fluorescence increase at the site of ACh application was 212% ± 26% (SEM, n = 5) of the control intensity, prior to the ACh application. At the time of the maximal fluorescence increase, the mean half-width of the fluorescence profile was 13.2 ± 3.1 μm (n = 5). In three experiments, in which the holding voltage of the whole-cell recording was changed from −110 to −70 mV to reduce the driving force on Ca^{2+}, we observed a reduction in the change of fluorescence for identical ACh applications. This was consistent with Ca^{2+} influx, occurring primarily through ACh receptors.

Depression Induced by Postsynaptic Photoactivated Release of Ca^{2+}

Previous studies have shown that a rise in postsynaptic $[Ca^{2+}]_i$ is required for the induction of the long-term synaptic depression by either repetitive ACh application or by tetanic heterosynaptic stimulation (Dan and Poo 1992a; Lo and Poo, 1994). Whether Ca^{2+} elevation by itself is sufficient to induce synaptic depression in the absence of receptor activation was tested using photoactivated release of Ca^{2+} from photolabile Ca^{2+} chelators. In the first set of experiments, myocytes were loaded with nitr-5 through the whole-cell recording pipette. After control recording of EPCs, the myocyte was exposed to a 2 s steady UV light (with an illumination spot size 20 μm in diameter, centered on the synapse), and evoked synaptic responses were assayed immediately and at regular intervals after the exposure to UV

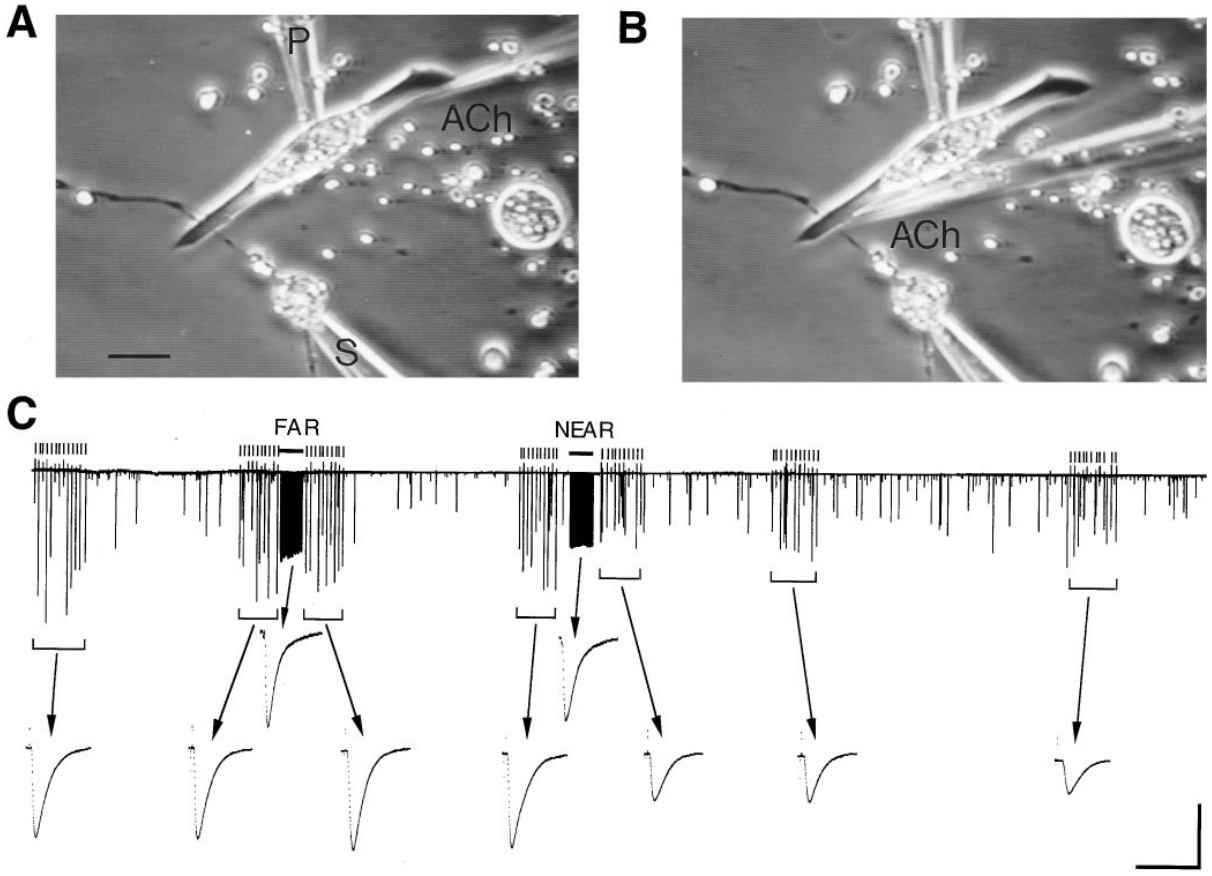

Figure 2. Dependence of Synaptic Depression on the Position of ACh Application

(A and B) Phase-contrast microscopic images of spindle-shaped myocytes innervated by cocultured spinal neurons in 1-day-old Xenopus cultures. The presynaptic neuron possesses a single, unbranched neurite that intersected the myocyte at one end of the spindle so that the site of synaptic contact was clearly restricted to a small region of the myocyte surface. The tip of the iontophoretic ACh pipette (ACh) was placed at two different positions on the myocyte surface along the long axis of the spindle, and the distance between the sites of ACh application and the synapses could be readily estimated. (P) and (S) are the patch-clamp pipette and the extracellular stimulating pipette, respectively. Scale bar represents 20 μm.

(C) An example of the recording showing the distance-dependence of synaptic depression. The experimental protocol was similar to that described for Figure 1, except that two episodes of ACh pulses were sequentially applied (under voltage-clamp condition) at two different positions on the myocyte surface. After the first episode of 100 ACh pulses was applied at a distance of more than 70 μm (far) from the synapse, no depression was observed. A similar episode of 100 ACh pulses, when applied within 10 μm from the synapse (near), induced a marked reduction of the EPC amplitude. Scale bars represent slow trace, 2 nA, 3 min; fast traces, 2 nA, 30 ms.

light (see Figure 5). We observed significant synaptic depression, with an average 56% ± 9% (SEM, n = 13) reduction in the mean EPC amplitude within 5 min after the light exposure. As shown in Figure 5B, the depression was relatively persistent, lasting for as long as stable recordings could be made (up to 1 hr after photolysis). Control experiments using the same exposure to UV light on postsynaptic myocytes not loaded with nitr-5 showed that there was no detectable photodamage to the cell (Figure 5B). Similar experiments using DM-nitrophen loading also indicated that elevation of Ca^{2+} in the myocyte can also cause synaptic depression of evoked responses. The average EPC amplitude was reduced 5 min after a single UV flash by 69% ± 18% (SEM, n = 3), as compared to the control pre-photolysis value. In 2 cells not loaded with DM-nitrophen but exposed to the same UV flash there was no apparent change in the EPC amplitude, with an average EPC amplitude, 5 min after light exposure, 123% and 91% of the control value, respectively. In two separate experiments, spindle-shaped myocytes innervated by cocultured spinal neurons at one end of the myocyte were loaded with nitr-5. The myocytes were illuminated with a UV spot (20 μm in diameter) centered at about 50 μm from the synapse for a duration of 2 or 16 s. No apparent change in synaptic efficacy was observed. At 5 min after UV exposure, the mean EPC amplitude was 99% and 92% of that found during the control period.

Analysis of miniature excitatory postsynaptic currents (MEPCs) from nitr-5 loaded cells indicated that there was no significant change in the frequency, rise time, decay time, or the mean amplitude of the MEPCs at synapses that showed significant reduction of the mean EPC amplitude after UV photolysis (Figure 6A). Furthermore, there was no significant difference in the amplitude distribution of the MEPCs of these cells before and

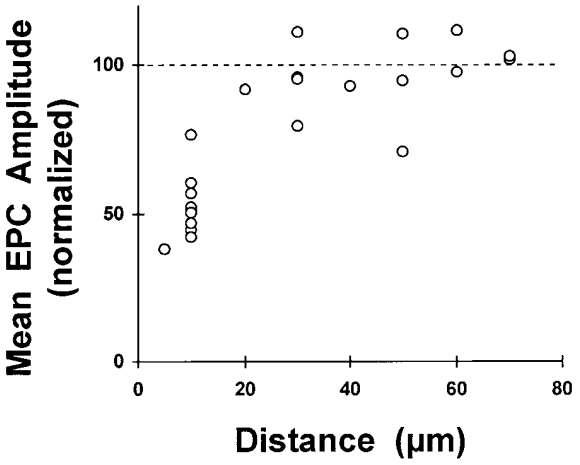

Figure 3. Summary of Distance-Dependence of Synaptic Depression

Each data point represents the result from a single experiment. The mean EPC amplitude immediately after a standard episode of ACh application was normalized against the control mean EPC amplitude before ACh application, and the normalized EPC amplitude was plotted against the estimated distance between the site of ACh application and the synapse.

after the photolysis (Figure 6B). These results suggest that the synaptic depression was due to a change in the presynaptic ACh release machinery, rather than in the postsynaptic ACh sensitivity.

Presynaptic Coactivation Prevents Depression Induced by Postsynaptic Elevation of Ca^{2+}

Long-term synaptic depression induced by tetanic heterosynaptic stimulation or by repetitive ACh application is known to satisfy a Hebbian rule, namely, it can be prevented by synchronous coactivation of the presynaptic neuron (Lo and Poo, 1991; Dan and Poo, 1992a). If the synaptic depression induced by postsynaptic elevation of Ca^{2+} through photolysis of caged compounds is due to the same mechanism as that induced by synaptic or ACh activation of the myocyte, we would expect that presynaptic coactivation during the period of postsynaptic elevation of Ca^{2+} should prevent synaptic depression. This was confirmed by the following experiment. Presynaptic neurons were stimulated to fire action potentials at a rate of 2 Hz for a 2 s period. When the tetanic stimulation was applied at the same time as UV exposure of the nitr-5 loaded myocyte (using the same protocol as that described in Figure 5), we observed no significant effect on the amplitude of EPCs, as shown by low frequency test stimuli. However, at the same synapse, an identical 2 s photolysis applied at a later time in the absence of presynaptic stimulation resulted in immediate and persistent synaptic depression. Results from six synapses are summarized in Figure 7.

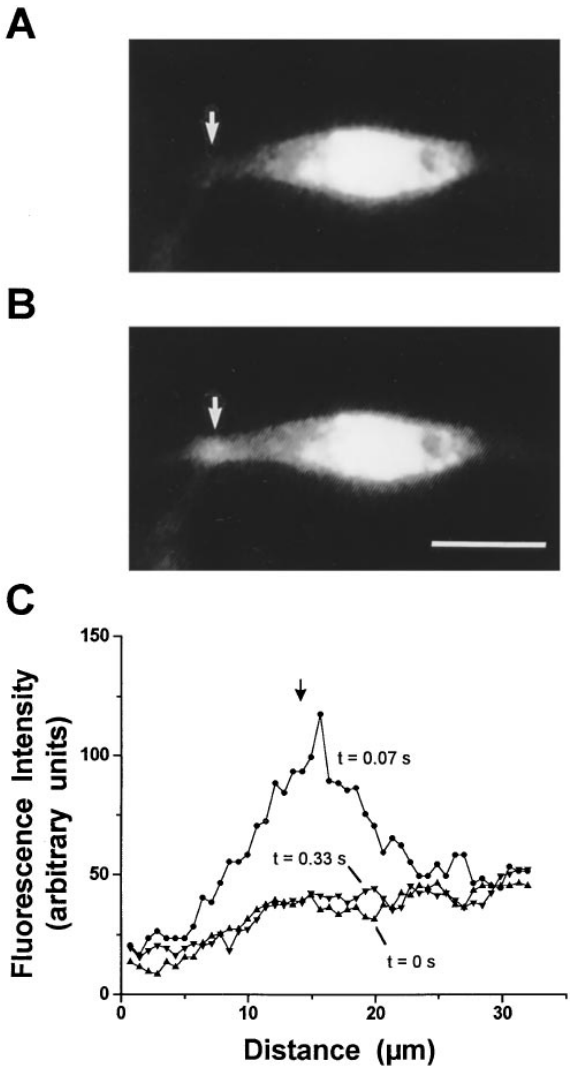

Figure 4. Localized Ca^{2+} Influx Induced by Iontophoretic ACh pulses

Effect of ACh application on the myocyte Ca^{2+} level was monitored with Ca^{2+}-sensitive fluorescent dye Fluo-3.

(A) Fluorescence image of a spindle-shaped myocyte at resting state.

(B) The same myocyte 0.07 s after application of a pulse of ACh near one end of the myocyte (marked by the white arrow). Note the localized increase in fluorescence near the site of ACh application. Bar represents 30 μm.

(C) Quantitative measurements of the fluorescence intensity along a line parallel to the axis of the spindle. Only a 35 μm region centered at the site of ACh application is shown. The fluorescence intensities measured at three time points (t = 0, 0.07, 0.33 s) after the application of an ACh pulse are shown. The arrow marks the location of ACh iontophoresis. This myocyte showed a relatively high background fluo-3 fluorescence that did not change throughout the experiment.

Discussion

To determine the role of postsynaptic Ca^{2+} in long-term synaptic depression induced by postsynaptic activity at developing neuromuscular junctions (Lo and Poo, 1992; 1994; Dan and Poo, 1992a), we have examined the dependence of ACh-induced synaptic depression on the proximity of ACh application to the synaptic site, the spatial profile of postsynaptic Ca^{2+} influx induced by localized ACh application, and the effect of direct elevation of postsynaptic $[Ca^{2+}]_i$.

ACh-induced synaptic depression requires postsynaptic elevation of Ca^{2+}, since preventing changes in myocyte $[Ca^{2+}]_i$ by loading BAPTA into the postsynaptic

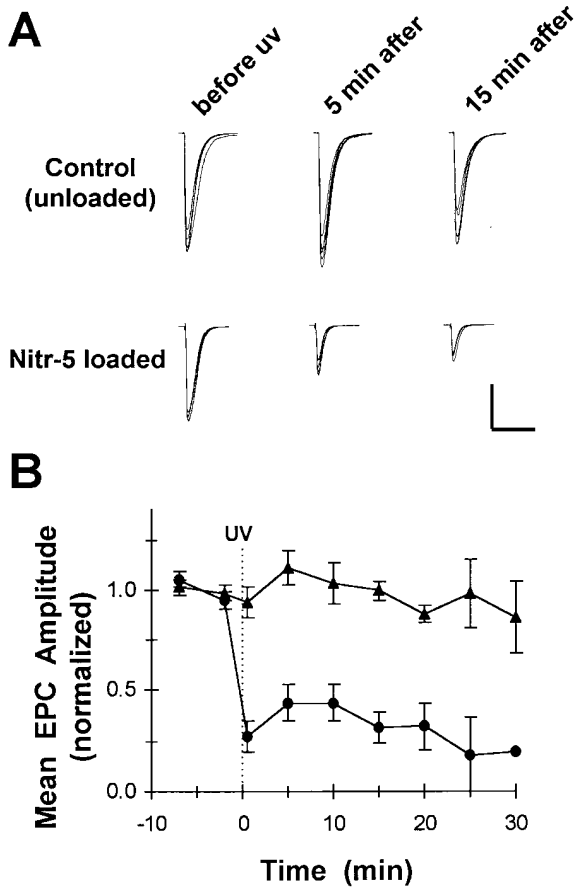

Figure 5. Synaptic Depression Induced by Photoactivated Release of Ca^{2+} in the Postsynaptic Myocyte

(A) Traces shown are samples of recordings of EPCs from innervated myocytes, using a whole-cell recording pipette containing 8.4 mM nitr-5 (Nitr-5 loaded) or containing no nitr-5 (Control (unloaded)), at three different times (2 min before, 5 and 15 min after UV illumination). Scale bars represent 2 nA, 50 ms.

(B) Summary plot of all nitr-5 photolysis experiments. The mean amplitude of EPCs evoked by 5–6 test stimuli observed at 5-min intervals was normalized to the mean EPC amplitude during the entire pre-photolysis control period for each cell. Data from 13 cells using pipettes containing nitr-5 (circles) and 17 control cells (triangles) with no nitr-5 added in the pipette. Recording period after UV illumination was variable (from 5–30 min). Error bars show SEM.

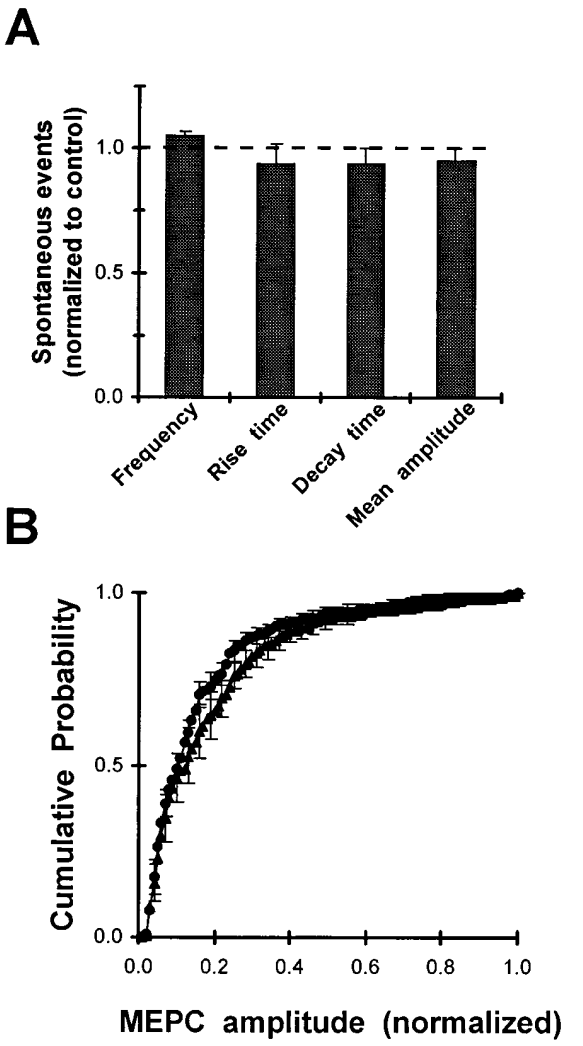

Figure 6. Properties of MEPCs before and after synaptic depression

(A) The frequency, mean rise time, mean decay time, and mean amplitude of the MEPCs for each synapse were determined before and after UV-induced synaptic depression in experiments shown in Figure 5. The values after UV photolysis were normalized by those recorded at the same synapse before UV exposure. No significant change was observed in any of the parameters.

(B) The amplitude distribution of MEPCs observed during a 5 min period before (triangles) and after (circles) UV photolysis. For each synapse, the MEPC amplitude was normalized to the maximal amplitude of MEPCs for that synapse. Cumulative probability refers to the fraction of total MEPC events with amplitude smaller than a given amplitude. Data from four cells with a high number of MEPC events were analyzed. Error bars show SEM. For clarity, bars were shown for 1/3 of the data points.

myocyte completely abolished ACh-induced synaptic depression (Dan and Poo, 1992a). We found that synaptic depression induced by ACh also requires that the point of ACh application and the synaptic site be close. No depression was seen if the iontophoresis site and the synapse were greater than 20 μm apart. Using fluo-3 fluorescence imaging, we have observed a localized elevation of postsynaptic $[Ca^{2+}]_i$ immediately after each iontophoretic ACh pulse, while the muscle membrane was voltage-clamped at −110 mV. Thus, the Ca^{2+} influx is likely to result from the opening of ACh channels, rather than from the opening of voltage-dependent Ca^{2+} channels or Ca^{2+} release from internal stores. In the absence of the voltage-clamp, depolarization-induced Ca^{2+} elevation can be readily observed as a strong flash of fluo-3 fluorescence over the entire myocyte after each ACh pulse (data not shown). Moreover, the observation of a reduced Ca^{2+} elevation when the clamping voltage was reduced from −110 to −70 mV is consistent with a reduced driving force for Ca^{2+} influx through ACh channels. At the time of the maximal fluorescence increase, the mean half-width of the fluorescence profile was 13 μm. Since the distance of internal Ca^{2+} spread and the proximity between synapse and iontophoresis pipette required for depression are similar, localized Ca^{2+} influx

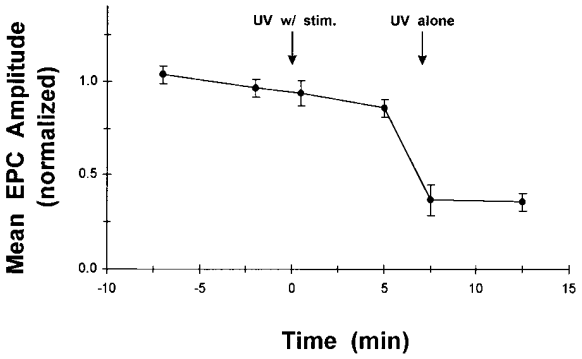

Figure 7. Synaptic Depression Induced by Photoactivated Release of Ca^{2+} in the Postsynaptic Myocyte Can Be Prevented by Presynaptic Costimulation

Postsynaptic myocytes were loaded with nitr-5, and UV photolysis was applied in the same manner as that described in the legend of Figure 5. During the first 2 s of photolysis, the presynaptic neuron was stimulated to fire action potentials at a frequency of 2 Hz. No significant change in the mean EPC amplitude was observed following the photolysis. The same photolysis applied approximately 7 min later in the absence of presynaptic stimulation resulted in significant depression. The EPC amplitude was normalized as that described in Figure 5, and data points represent average values from six experiments. Error bars show SEM.

might not only be required, but may in fact be the primary signal responsible for inducing the synaptic depression.

We directly tested the role of a postsynaptic Ca^{2+} elevation in inducing depression using photolabile chelators of Ca^{2+}. These experiments demonstrated that localized Ca^{2+} elevation is sufficient to induce synaptic depression. We also found that a rise in Ca^{2+} at a region approximately 50 μm from the synapse was ineffective. From models of nitr-5 photolysis in cells that include provisions for localized photolysis, diffusion of Ca^{2+} and nitr-5, and Ca^{2+} extrusion (Land and Zucker, 1989), we estimate that a 2 s exposure to UV light elevated $[Ca^{2+}]_i$ from 145 nM to 400 nM, with a half width of about 2 s. In contrast, simulations of DM-nitrophen photolysis (Land and Zucker, 1994) suggest that flash photolysis of DM-nitrophen elevated $[Ca^{2+}]_i$ from 5 nM to a peak level of about 200 μM for 2 ms, dropping to 15 μM in 6 ms, 3 μM in 40 ms, 500 nM in 125 ms, 100 nM in 250 ms, and to a final level of 20 nM in 500 ms. Surprisingly, both photolysis protocols were effective in inducing depression when Ca^{2+} elevation occurred at the synaptic site. These results from photolysis experiments further indicate that a localized rise in Ca^{2+} near the synapse is required for inducing depression. This implies that not only is the spread of elevated Ca^{2+} restricted, as shown by the imaging experiment, but downstream effectors are also spatially restricted.

The cellular mechanisms involved in the induction and the expression of synaptic depression induced by UV photolysis of caged Ca^{2+} chelators appear to be the same as those involved in the ACh-induced depression. In both cases, the depression can be prevented, by costimulation of the presynaptic neuron during the period of postsynaptic activation. There is a possibility that costimulation delayed the onset of synaptic depression. More extensive studies on the mechanism by which coactivation affects the induction of synaptic depression remain to be performed. Another significant similarity between the forms of depression is that both appear to be expressed through presynaptic modifications. As was demonstrated previously, and is true here as well, the present protocol of ACh iontophoresis induced only a transient and small receptor desensitization (see Figure 3 of Dan and Poo, 1992a; Figures 1 and 2). This receptor desensitization could not account for the persistence and the extent of synaptic depression, and therefore does not explain the lack of depression when ACh iontophoresis or UV photolysis is performed far from the synapse. Furthermore, synaptic depression induced by ACh iontophoresis and by UV-activated postsynaptic elevation of Ca^{2+} is expressed as a reduction in the EPC amplitude without any changes in either the kinetics, amplitude or frequency of the MEPCs. Postsynaptic changes, such as persistent receptor desensitization and changes in the ACh channel properties would be accompanied by changes in the amplitude and/or the kinetics of the MEPCs. Another possible postsynaptic modification is the "silencing" of discrete clusters of postsynaptic receptors, which could result in changes in MEPC frequency without affecting their amplitude or kinetics. However, depression induced either by ACh iontophoresis or photolysis of caged calcium compounds did not produce any of these changes. Therefore, the most likely site of depression is presynaptic, through a reduced number of quanta released by each action potential. It may be noted that such presynaptic modification does not necessarily result in a change in MEPC frequency (that remained constant in the present study), as in the case of a presynaptic modulation that leads to reduced Ca^{2+} influx during the action potential without changing the resting Ca^{2+} level.

The synaptic depression reported here shows some interesting similarities and differences from hippocampal and cerebellar long-term depression (LTD). Like the neuromuscular depression reported here, there is evidence suggesting that hippocampal LTD (Bolshakov and Siegelbaum, 1994), but not cerebellar LTD (Ito, 1989), is expressed presynaptically. This implies that neuromuscular depression involves retrograde signaling, similar to that suggested for long-term synaptic changes in the hippocampus (Williams et al., 1989; Bohme et al., 1991; Schuman and Madison 1991; Zhuo et al., 1993; Stevens and Wang, 1993; but see Nicoll and Malenka, 1995). However, at least under certain conditions, hippocampal LTD appears to require coincident presynaptic activity (Bolshakov and Siegelbaum, 1994), while neuromuscular depression does not require coactivation. On the contrary, synchronous pre- and postsynaptic activation prevents depression (Lo and Poo, 1991; Dan and Poo, 1992a, present study).

It has been suggested that long-term activity-dependent synaptic changes are mediated by Ca^{2+}-dependent enzymes, including calpain (Lynch and Baudry, 1984; Staubli et al., 1988; Oliver et al., 1989), Ca^{2+}/calmodulin-dependent protein kinase (Lisman, 1985; Miller and Kennedy, 1986; Malinow et al., 1989), protein kinase C (Hu et al., 1987; Akers et al., 1986; Malinow et al., 1989), and phosphatases (Mulkey et al., 1993). Downstream actions of these proteins may lead to production and/or secretion of retrograde factors from the postsynaptic cell

that modulate the presynaptic release mechanism. For a different form of neuromuscular depression, namely that induced by long-term repetitive postsynaptic depolarizations induced by current injections, there is evidence that nitric oxide (NO) may be the retrograde signal (Wang, et. al., 1995). However, efforts in demonstrating involvement of NO in ACh-induced or heterosynaptic depression have so far been unsuccessful (Y. Lo and M. P., unpublished data). Elevation of Ca^{2+} in the postsynaptic cell may also directly trigger exocytosis of vesicles containing prepackaged retrograde modulators (Dan and Poo, 1994; Cash and Poo, 1995), as suggested by recent findings of Ca^{2+}-dependent secretion from muscle cells and fibroblasts (Dan and Poo, 1992b; Girod et al., 1995; Poo et al., 1995). How elevated postsynaptic calcium results in retrograde modulation and how coincident presynaptic activity protects the synapse from this modulation are key issues yet to be addressed.

Previous studies in these Xenopus cultures have shown that tetanic stimulation of one of two synaptic inputs on a myocyte was ineffective in inducing heterosynaptic depression when the two synapses were separated by about 50–75 μm (Lo and Poo, 1991). The present study provides further quantitative information concerning the spatial requirement for postsynaptic activation in the induction of synaptic depression. Synapse elimination at developing neuromuscular junctions is known to be a competitive process between coinnervating nerve terminals (Betz et al., 1990). Interestingly, elimination of polyneuronal innervation in vivo was mostly observed among closely-opposed nerve terminals on a muscle fiber, and stable endplates coexisting on a single muscle fiber are always separated by a distance (Gordon et al., 1974; Brown et al., 1976; Kuffler et al., 1977). Recent morphological studies of the elimination process at the level of a single junction have shown that depletion of postsynaptic ACh receptors precedes the withdrawal of presynaptic nerve terminals, suggesting an important role for ACh receptors in the structural modulation of the synapse (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993, 1994). Our results provide direct evidence for the importance of Ca^{2+} elevation resulting from ACh receptor activation in the functional modulation of developing synapses. These similarities in ACh receptor involvement and spatial requirements for postsynaptic activation support the notion that the synaptic depression observed here may underlie synapse elimination.

Experimental Procedures

Culture Preparation

Cultures were prepared according to procedures reported previously (Spitzer and Lamborghini, 1976; Anderson et al., 1977; Tabti and Poo, 1991). Briefly, the neural tube and the associated myotomal tissue from 1-day-old embryos (stage 20–24, Nieuwkoop and Faber, 1967) were dissociated in Ca^{2+}- and Mg^{2+}-free Ringer's solution supplemented with EDTA, plated on clean glass coverslips, and incubated at room temperature (20–22°C) for 1 day prior to the experiment. The culture medium consisted of 50% (vol/vol) of Leibovitz medium (GIBCO), 1% (vol/vol) of fetal calf serum (GIBCO), and 49% (vol/vol) of Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, and 10 mM Hepes, pH 7.3). For stability of long-term recordings, innervated spherical myocytes were used in most experiments. These myocytes showed identical functional characteristics to spindle-shaped or extended myocytes in the same culture (Evers et al., 1989). Myocytes are readily innervated by cocultured spinal neurons, and the synapses showed functional and structural features (Buchanan et al., 1989) similar to those of developing neuromuscular synapses in Xenopus embryos (Kullberg et al., 1977). Spindle-shaped myocytes with neurite contact sites clearly localized to one end of the cell were used for studying the dependence of synaptic depression on the proximity of the site of repetitive ACh application.

Electrophysiology

Recordings of synaptic currents were made with the gigaohm-seal whole-cell recording method (Hamill et al., 1981; Evers et al., 1989). Presynaptic extracellular suprathreshold stimulation was applied with heat-polished patch pipettes at the neuronal soma under loose-seal conditions, following procedures previously described (Sun and Poo, 1987). Synaptic efficacy was assayed by recording 5–12 nerve-evoked postsynaptic currents (EPCs) at a low frequency (0.05–0.1 Hz). Only recordings that showed no significant changes in access resistance were used for analysis. We used intermittent test pulses in order to reduce the total number of stimuli applied, since higher number of stimuli more often results in damage of these fragile developing neurons. The filling solution in the whole-cell recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2). All recordings were made with a patch clamp amplifier (Axopatch-1D, Axon). For experiments using caged Ca^{2+} chelators (see below), the pipette filling solution consisted of the above without $MgCl_2$. The data were stored after digitization (Neurodata 384) into a videotape recorder for later playback onto a storage oscilloscope (5113, Tektronix) or an oscillographic recorder (Gould RS3200), and for analysis by a microcomputer.

Iontophoretic Application of ACh

Microelectrodes (resistance 200–350 M Ω) filled with 3M ACh chloride (Sigma) were used for iontophoretic application of ACh (Poo, 1982). Braking currents of 4–5 nA were used to prevent ACh leakage from the pipette, and constant iontophoretic current pulses of amplitude 10–20 nA and 2 ms duration were delivered through a microelectrode amplifier (Getting) to the ACh pipette that was positioned at the myocyte surface. These pulses normally induced inward currents in the myocyte similar to EPCs, with amplitudes in the range of 2–3 nA. In all experiments using spherical myocytes, the tip of the ACh pipette was placed near the synapse, usually on the side of the myocyte facing the culture substratum (Figure 1A). In experiments studying the distance-dependence of synaptic depression, spindle-shaped myocytes innervated at one end were used, and the ACh pipette was placed at different positions on the myocyte surface along the long axis of the spindle to vary the distance between the synapse and the site of ACh application.

Fluorescence Imaging of Ca^{2+}

The Ca^{2+}-sensitive dye fluo-3-pentaacetyloxymethyl ester (fluo-3/AM; Molecular Probes Incorporated) was used to monitor changes in cytosolic Ca^{2+} concentration (Minta et al., 1989) in the myocyte upon ACh application. Myocytes were incubated with fluo-3/AM (10 μM) for 1 hr at room temperature and extensively washed with fresh culture medium before examination with an inverted fluorescence microscope (Nikon Diaphot). An oil-immersion objective (40X) was used with an intensified CCD camera (Quantex, QX-100) operating at a fixed gain, and the images were recorded on regular videotapes for later analysis. Typical fluorescein filters (excitation wavelength 420–460 nm and barrier filter 520–560 nm) were used together with a neutral density filter to adjust the level of excitation light. In order to optimize conditions for measuring local fluorescence changes, ACh pulses were applied at the flattened region of extended spindle-shaped myocytes, where the surface to volume ratio is the highest. Fluorescence image of the myocyte was digitized by using an image frame grabber (Model 151, Imaging Technology Incorporated) as an array of 512 × 480 pixels with 256 grey levels. To achieve temporal resolution, single frames were captured for quantitative analysis. A straight line was drawn along the long axis of spindle-shaped myocytes, and the fluorescence intensity of pixels along the line was measured. The background fluorescence, measured at nearby

cell-free areas, was subtracted from each measurement. The nonlinearity of the CCD camera is negligible under the resolution of the present study, and the extent of photobleaching of Fluo-3 fluorescence was less than 5% in a typical experiment.

Photoactivated Release of Ca^{2+}

Postsynaptic myocytes were loaded with 8.4 mM nitr-5 (50% Ca^{2+} loaded) through a conventional whole-cell patch electrode. After a control period of greater than 5 min the myocyte was exposed to a 2 s steady illumination of light provided by a 75 W Xenon lamp focused through the epifluorescence port of a Nikon Diaphot Microscope with a 400 DCLP dichroic mirror and a UG1 filter. A spot 20 μm in diameter was focused on the cell using a 40X water immersion objective, exposing approximately 30% of the cell. In some experiments, cells were loaded with 8.4 mM DM-nitrophen and exposed to a single flash of light focused as above but generated using a XF-10 high energy flash-lamp system (Hi-Tech Scientific). The photolysis efficiencies of our light sources were measured by determining the amount of time (or number of flashes) required to photolyze half the DM-nitrophen in small droplets under mineral oil that contained 5 mM DM-nitrophen, 2.5 mM Ca^{2+}, and 2 mM fluo-3 (Molecular Probes), and were uniformly illuminated. A sudden rise in fluo-3 fluorescence indicated that half the DM-nitrophen was photolyzed, and the photolysis rates of Ca^{2+}-free and Ca^{2+}-bound DM-nitrophen were calculated as 26% and 65% per flash respectively, as described in Zucker (1993). The Ca^{2+}-free and Ca^{2+}-bound photolysis rates of nitr-5 were estimated as 14% and 42% per s, respectively from measurements of the relative photolysis rates of nitr-5 and DM-nitrophen (R.S.Z., unpublished data).

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