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Postsynaptic Levels of $[Ca^{2+}]_i$ Needed to Trigger LTD and LTP

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Summary

Long-term potentiation (LTP) and long-term depression (LTD) in CA1 pyramidal neurons are both triggered by a postsynaptic rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We used photolysis of postsynaptic caged Ca^{2+} compounds to search for differential thresholds for activation of these processes. Long-lasting potentiation (LLP) resembling LTP, and long-lasting depression (LLD) resembling LTD, were evoked by $[Ca^{2+}]_i$ elevations of comparable magnitude and duration in different cells. No distinctions in threshold for these processes were detectable. LLP was occluded by tetanically induced LTP and blocked by calmodulin inhibition, and LLD was occluded by electrically induced LTD and blocked by phosphatase inhibition.

Introduction

Long-term modifications in synaptic strength are often assumed to be involved in the storage of information in the brain. Long-term potentiation (LTP) and long-term depression (LTD) in the CA1 area of the hippocampus constitute such long-lasting forms of activity-dependent synaptic plasticity, and LTP in particular is widely believed to be involved in learning and memory (Teyler and DiScenna, 1987; Malenka, 1994). Homosynaptic LTP induced by high frequency stimulation (100 Hz for 1–2 s) and homosynaptic LTD induced by low frequency stimulation (1 Hz for 10 min) share many common features. Postsynaptic depolarization, activation of n-methyl-D-aspartic acid (NMDA) receptors, and increase in postsynaptic intracellular Ca^{2+} concentration ($[Ca^{2+}]_{i)}$ are all involved in the induction of both of these phenomena (Mulkey and Malenka, 1992; reviewed by Bliss and Collingridge, 1993; Linden and Connor, 1995). This rise in $[Ca^{2+}]_i$ has been suggested to activate various Ca^{2+}-dependent enzymes: proteases, phosphatases, phospholipases, and protein kinases that would convert the Ca^{2+} induction signal into persistent modification of synaptic transmission (reviewed by Bliss and Collingridge, 1993). Among these enzymes, protein kinases and in particular, Ca^{2+}/calmodulin kinase II (CaMKII; Malenka et al., 1989; Malinow et al., 1989) have captured the most attention in regards to LTP. CaMKII has been suggested to act as a Ca^{2+}-triggered switch (Miller and Kennedy, 1986, Lisman, 1989), and recent studies have shown that the postsynaptic CaMKII activity is necessary and sufficient to generate LTP (Silva et al., 1992; Pettit et al., 1994). As for LTD, recent studies point to the role of phosphatases in its induction, including Ca^{2+}-independent phosphatase 1 and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Lisman, 1989; Mulkey et al., 1993, 1994).

Assuming different affinities of calcineurin and CaMKII for Ca^{2+}, it has been suggested that a modest rise in $[Ca^{2+}]_i$ induces LTD, whereas a higher rise in $[Ca^{2+}]_i$ activates LTP (Lisman, 1989, 1994; Artola and Singer, 1993). The evidence for this hypothesis has been limited and indirect: the higher afferent frequencies needed to induce LTP should cause a greater elevation in postsynaptic $[Ca^{2+}]_i$ (Dunwiddie and Lynch, 1978; Dudek and Bear, 1992); afferent stimulation that generates LTP can generate LTD when postsynaptic neurons are less depolarized, when extracellular $[Mg^{2+}]$ is elevated or NMDA antagonists are present, or by lowering extracellular $[Ca^{2+}]$, all treatments that presumably act solely by reducing Ca^{2+} entry through NMDA receptor channels (Artola et al., 1990; Hirsch and Crepel, 1991; Calabresi et al., 1992; Xie et al., 1992; Mulkey and Malenka, 1992; Lin et al., 1993); and LTP appears to be more sensitive to blockade by postsynaptic injection of Ca^{2+} chelators, suggesting a higher Ca^{2+} affinity for mediators of LTD than for mediators of LTP (Kimura et al., 1990; Yoshimura et al., 1991; Bröcher et al., 1992). Based on this evidence, the hypothesis for how a rise in Ca^{2+} can selectively activate LTD or LTP by acting on processes with distinct thresholds has been widely accepted (Schulman, 1994; Bear and Malenka, 1994; Linden and Connor, 1995; Larkman and Jack, 1995).

If it is assumed that Ca^{2+} level is the only determinant of modification of synaptic transmission, this hypothesis makes a very specific prediction that, as $[Ca^{2+}]_i$ is gradually elevated in a postsynaptic cortical neuron, LTD of synaptic inputs will be activated first, and LTP will be activated only at a distinctly higher $[Ca^{2+}]_i$. In this study, we used photolysis of postsynaptically injected caged Ca^{2+} compounds to test the prediction directly. The goals of this study were to investigate whether a rise in postsynaptic $[Ca^{2+}]_i$ is able to trigger the induction of a long-lasting potentiation (LLP) or depression (LLD) of excitatory synaptic transmission in CA1 pyramidal cells, whether LLP and LLD require different levels of $[Ca^{2+}]_i$ for their induction, and what the relationship is between these forms of long-lasting modifications of synaptic strength and LTP and LTD induced by afferent stimulation.

Results

LLP and LLD Induced by Photolysis of Nitr-5

We first investigated whether a rise of postsynaptic $[Ca^{2+}]_i$ was able to trigger the induction of LLP or LLD and how the amplitude of the Ca^{2+} signal could influence the induction of LLP or LLD. Neurons were filled by perfusion through the patch pipette with 5 mM nitr-5 (Adams et al., 1988) loaded with 2.5 mM Ca^{2+}, and this was photolyzed to increase the $[Ca^{2+}]_i$ in the impaled CA1 pyramidal neuron. Our procedure was to expose slices to a series of photolyzing illuminations, usually beginning with 1 s and increasing until an effect was observed. Figure 1 shows that in two different slices, the photolysis of nitr-5 for 5 or 10 s could lead to either a potentiation (95%, $p < 0.005$, Figure 1A), or a depression

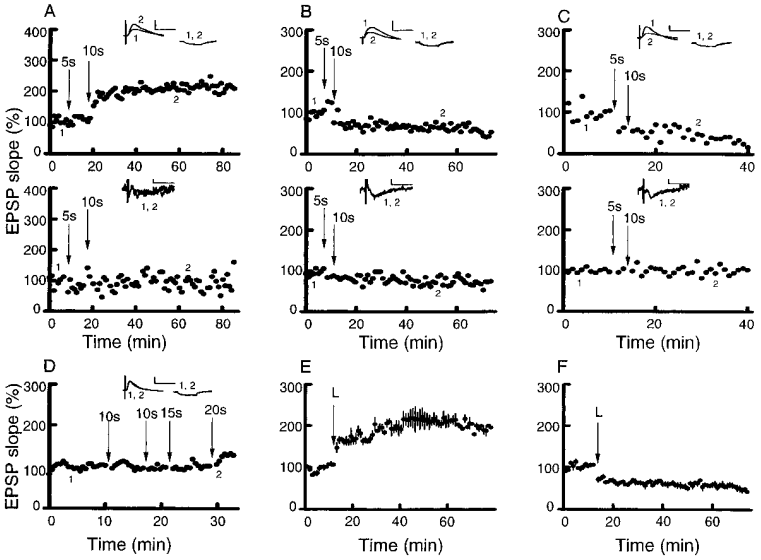

Figure 1. Photolysis of Postsynaptic Nitr-5 Can Enhance or Depress Synaptic Transmission

In (A)–(C) and in Figures 5 and 6, graphs of the maximum rising slope of the intracellular excitatory postsynaptic potential (EPSP, top) recorded in somata of CA1 pyramidal neurons and the maximum rising slope of the simultaneously recorded extracellular EPSP (bottom) in the stratum radiatum are displayed. Each point represents the average of all responses made during a 60 s interval to 0.1 Hz stimulation, normalized to responses at the beginning of the experiment. Insets in each panel show raw and smoothed data traces for EPSP and extracellular EPSP, respectively, taken before and after photolysis of nitr-5. The calibration bars show 10 mV and 50 ms for intracellular EPSPs in (A–D), and 0.1 mV (A), 0.2 mV (B), 0.4 mV (C), and 20 ms for extracellular EPSPs. The input resistances of the cells are also monitored as negative deflections to −30 pA pulses. The four cells in Figures (A)–(D) were located at the surface of the slice and held at a membrane potential of −76 mV (A), −77 mV (B), −79 mV (C), and −81 mV (D).

In this and all the following figures, at the time marked by the arrows, the slices were exposed to ultraviolet light for the durations indicated in seconds on the graphs. In (A)– (C), the cells were perfused with an internal solution containing 5 mM nitr-5 and 2.5 mM Ca^{2+}. The estimated maximal $[Ca^{2+}]_i$ elicited by the first flash was 275 nM in (A)–(C), if we assume that the recorded synapses were 50 μm deep. After the second flash, we estimate that $[Ca^{2+}]_i$ reached 470 nM in (A), 715 nM in (B) and (C). If the synapses were right at the surface of the slices, the estimated peak $[Ca^{2+}]_i$ levels are 360 nM in (A), (B), and (C) for the first flash and 670 nM in (A) and 965 nM in (B) and (C) for the second flash.

(D) Control experiment in which a cell was perfused with the standard internal solution and exposed to ultraviolet light.

(E and F) Summary of experiments in which the photolysis of nitr-5 was carried out. Long recordings (>40 min) were obtained in five cells that showed LLP and in five that showed LLD, and the results were averaged for these graphs. In these summary graphs and those in Figures 3–6, vertical lines through the points represent standard errors.

(39%, $p < 0.005$, Figure 1B). These stable modifications in synaptic strength lasted for at least 60 min, and so are referred to as long-lasting potentiation (LLP) and long-lasting depression (LLD). These effects were encountered only in perfused cells. Indeed, the extracellular field potentials that were always monitored simultaneously with the intracellular excitatory postsynaptic potential (EPSP) showed no change during all the experiments (Figures 1A and 1B). We also ensured that the light by itself induced no photosensitive reaction in the perfused cell (Figure 1D). Similar LLP (mean ± SD: 56% ± 32%, n = 18) and LLD (average: 38% ± 15%, n = 17) were observed in other slices regardless of the membrane potential of the recorded cell (from −86 mV to −76 mV) and of the age of the rat between 11 and 22 days (Figures 1E, 1F, and 2). A few cells (7 among 42) exhibited no change in synaptic transmission after 5–10 s light exposure.

Photolysis of nitr-5 for shorter durations (1 s) did not strongly affect synaptic transmission in any of the cells we studied. In eight slices, 1 s light exposures had no effect, while in five cells a slight increase in transmission was observed, and in four cells a slight decrease was seen. These effects were usually too small to be statistically significant. Thus, 1 s exposures generate $[Ca^{2+}]_i$ rises that are near to or below thresholds for generating LLP and LLD, while 5–10 s exposures are sufficient to have clear effects. Moreover, of the 18 cells showing LLP to 5–10 s of light, 5 were initially tested with briefer exposures; none of them responded to the first photolysis (see Figure 1A). These results suggest that the induction of LLP and LLD require a similar minimum level or threshold rise in $[Ca^{2+}]_i$.

As $[Ca^{2+}]_i$ was not monitored in these experiments, we do not know the exact magnitudes of $[Ca^{2+}]_i$ elevation produced by nitr-5 photolysis. However, from measurements of the photolysis efficiency of our light source,

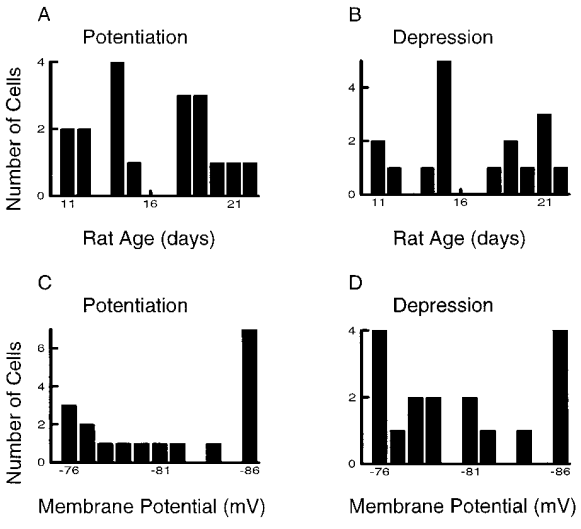

Figure 2. Modification of the Synaptic Transmission Depends neither on the Age of the Rat nor on the Membrane Potential of the Cell

Histograms showing the number of cells exhibiting either potentiation or depression after photolysis of nitr-5 as a function of the age of the rat (A), or of the membrane potential of the cell (B). The total numbers of cells displaying potentiation or depression were 18 and 17, respectively.

knowledge of the concentrations, Ca^{2+} loading, and affinities of nitr-5 and its photoproduct, measurements of Ca^{2+} removal processes in pyramidal cell dendrites (Regehr and Tank, 1992; Spruston et al., 1995; Yuste and Denk, 1995), and assuming 2 mM of native cytoplasmic buffer with 20 μM affinity, we can estimate (Landò and Zucker, 1989; Marrion et al., 1991) that 1 s light exposures elevate $[Ca^{2+}]_i$ to about 175 nM (from a resting level of 140 nM), 5 s light exposures elevate $[Ca^{2+}]_i$ to about 300 nM, and subsequent 10 s exposures raise $[Ca^{2+}]_i$ to about 500–1000 nM. The range of estimated values for the second light exposure depends on the interval between exposures, since longer intervals allow more time for Ca^{2+} released by the first photolysis to be pumped out of the cell (and off nitr-5 and its photoproduct, since free and bound Ca^{2+} are in equilibrium). Our estimates also depend on whether synapses occur at the surface of the slice or 50 μm deeper. Since we select surface pyramidal cells for recording and study only rapidly-rising EPSPs that are electrically near the soma, it is unlikely that the synapses we study are very deep. Thus, our results indicate that both LLD and LLP can be achieved by $[Ca^{2+}]_i$ reaching a minimal level of about 300–500 nM. The results are not consistent with widely different minimal thresholds for induction of LLD and LLP.

The results leave open the possibility that LLP and LLD have similar $[Ca^{2+}]_i$ induction thresholds, with the threshold for LLP induction slightly higher than that for LLD. In that case, in slices in which only a 5 s photolysis of nitr-5 induced LLD, a successive 10 s photolysis should reverse this and lead to LLP. However, in the six slices in which this experiment was done, the sequence LLD–LLP to successively larger increases in $[Ca^{2+}]_i$ was never observed (Figure 1C), suggesting that LLP does not simply arise from a slightly larger change in $[Ca^{2+}]_i$.

Effects of DM-Nitrophen Photolysis on Synaptic Transmission

Nitr-5 photolysis for 5–10 s should raise the $[Ca^{2+}]_i$ to 0.3–1.0 μM, with a half-width above resting levels of 5 to 20 s. The rising phase of the change in $[Ca^{2+}]_i$ depends on the duration of photolysis, and its falling phase is determined by the removal of Ca^{2+} by cell pumps, slowed from its normal rate (Regehr and Tank, 1992; Spruston et al., 1995; Yuste and Denk, 1995) by the extra buffering capacity of nitr-5 and its photoproduct (Landò and Zucker, 1989).

To investigate a wider range of durations and amplitudes of $[Ca^{2+}]_i$ in the induction of LLP or LLD, we used photolysis of DM-nitrophen, another caged Ca^{2+} compound that can elicit briefer rises in $[Ca^{2+}]_i$ to brief light exposures, and larger elevations to long exposures, than are achievable with nitr-5 (Kaplan and Ellis-Davies, 1988; Zucker, 1993; 1994).

Cells were perfused with 2.5 mM DM-nitrophen partially loaded with 1.25–2 mM Ca^{2+} and photolyzed with successive light exposures, usually beginning with 100 or 250 ms and sometimes followed by exposures of 0.5–5 s. The initial short exposures lead to reversible rises in $[Ca^{2+}]_i$ of about 1 μM that last little more than the duration of the light exposure, as Ca^{2+} rebinds rapidly to unphotolyzed chelator (Zucker, 1993, 1994); additional longer exposures lead to much higher rises in $[Ca^{2+}]_i$ (>5 μM) that are pumped out by the cell within a few seconds (Regehr and Tank, 1992; Spruston et al., 1995; Yuste and Denk, 1995). We tested 13 cells: 7 exhibited a prolonged depression and 6 showed no change in synaptic transmission to an initial short reversible rise in $[Ca^{2+}]_i$. In the cells in which no depression developed to a brief light exposure, photolysis was continued for an additional 0.5–5 s to raise the $[Ca^{2+}]_i$ to higher levels for longer times: in one of these cells, the test EPSP displayed an LLD. None of the cells ever exhibited an LLP. In 3 of the 7 cells that displayed LLD to the initial light exposure, additional light exposures up to 1 s long were presented: none of these cells showed a reversal or reduction in LLD or any tendency to display LLP.

As DM-nitrophen binds Ca^{2+} with an affinity of 5 nM at pH 7.2 in the absence of Mg^{2+}, the resting cellular Ca^{2+} should be unphysiologically low in the solutions used to perfuse cells: 5–20 nM. We checked whether this low resting $[Ca^{2+}]_i$ could have affected synaptic transmission. EPSPs that were recorded in six cells perfused with DM-nitrophen were stable for at least 30 min. We further investigated whether this low-resting $[Ca^{2+}]_i$ could account for our inability to induce LLP. As the initial level of $[Ca^{2+}]_i$ before photolysis depends on the total concentrations of Ca^{2+}, Mg^{2+}, DM-nitrophen, ATP, and native buffers (Delaney and Zucker, 1990), we increased the resting $[Ca^{2+}]_i$ by adding Mg^{2+} to the DM-nitrophen. We perfused 13 cells with the standard internal solution to which was added DM-nitrophen (2.5 mM), $CaCl_2$ (1.25 mM or 1.8 mM), and $MgCl_2$ (1.25 mM or 1.4 mM). The resting $[Ca^{2+}]$, checked with a Ca^{2+}-selective electrode, ranged from 20 to 160 nM, depending on the amount of Mg^{2+} added and the concentration of EGTA present in the internal solution (0–0.2 mM). Still, no LLP could be recorded in any of these cells, even when 10 mM glutathione was added to prevent toxic effects from photolyzed products of DM-nitrophen (Kaplan and Somlyo, 1989). Two of the cells exhibited LLD to the first brief, or to a subsequent longer photolysis. In the remaining 11 cells, the EPSP remained relatively stable, even in the 7 cells subjected to a second, more prolonged photolysis.

In summary, photolysis of DM-nitrophen generated only LLD in some hippocampal CA1 pyramidal cells, whether the photolysis induced a brief (<1 s) modest (about 1 μM) $[Ca^{2+}]_i$ elevation or a more prolonged (several seconds) larger $[Ca^{2+}]_i$ elevation (calculated as over 5 μM), and regardless of the initial resting level of $[Ca^{2+}]_i$. Since the inability to potentiate synaptic potentials might reflect some deleterious side effect of DM-nitrophen, all remaining experiments were performed using nitr-5.

LTP and LTD Occlude LLP and LLD, Respectively

We next tested whether the long-lasting modifications of synaptic transmission following a rise in $[Ca^{2+}]_i$ shared common processes with electrically induced LTP and LTD. We first investigated whether LTP (or LTD) could occlude LLP (or LLD). To approach this question, we designed two kinds of experiments. In the first, two separate afferent pathways were stimulated alternatively at 0.1 Hz. This provided a control for the pathway

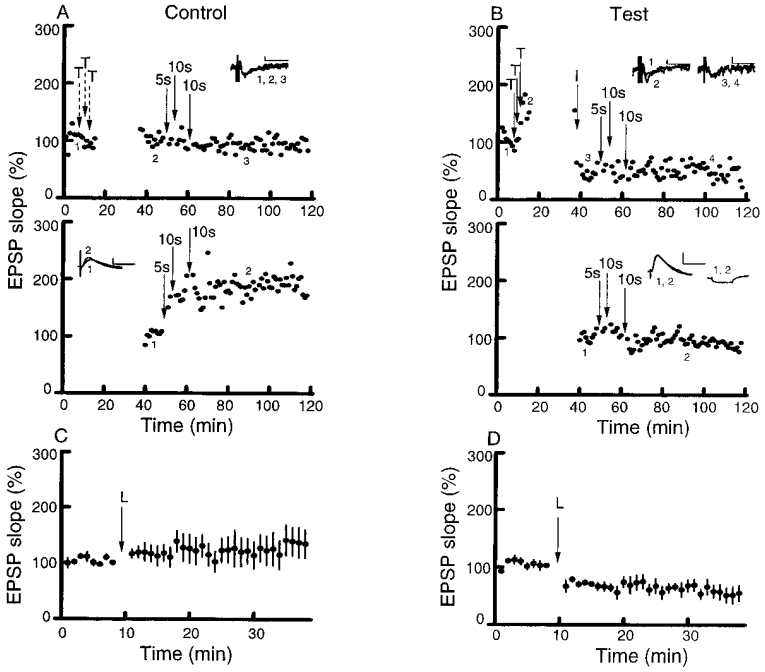

Figure 3. Tetanic LTP Occludes Photoactivated LLP

Two stimulating electrodes alternately activate independent fiber bundles at 0.1 Hz. Whole-cell recording electrode contains 5 mM nitr-5 and 2.5 mM Ca^{2+}. LTP was generated in one pathway (B, 'Test,' top) with three sets of two tetani (T, 100 Hz) separated by 20 s, whereas the 'Control' pathway was not potentiated (A, top). A whole cell recording was obtained from a surface cell approximately 37 min after the beginning of the experiment and held at −84 mV. The intensity of stimulation in the prepotentiated pathway was reduced at the time marked by the arrow (I). In this figure and in Figure 4, graphs of the maximum rising slope of the extracellularly recorded EPSP are shown in the top panels, and the maximum rising slope of the simultaneously recorded intracellular EPSP recorded in the soma of a CA1 pyramidal neuron are shown in the bottom panels (A and B). The calibration bars show 10 mV and 50 ms for intracellular EPSPs and 0.2 mV and 20 ms for extracellular EPSPs. The estimated maximal $[Ca^{2+}]_i$ evoked by the first flash was 275 or 360 nM, by the second flash 785 nM or 1.1 μM, and 425 or 420 nM by the third flash, assuming that the recorded synapses were 50 μm deep or at the surface of the slices, respectively. Photolysis of nitr-5 was performed in similar experiments on seven cells. Summary graphs of the effects of photolysis on the intracellular EPSP in the control and test (prepotentiated) pathways are shown in (C) and (D), respectively, for averaged responses from all seven cells.

upon which the effects of nitr-5 photolysis following conditioning stimulation were examined (Figure 3). After recording a stable 5–10 min baseline in the extracellular EPSPs, several tetani (100 Hz lasting 1 s) were applied to one pathway to induce a saturated LTP that was recorded on the extracellular field potentials of this pathway (Figure 3B, top), but had no effect on the untetanized pathway (Figure 3A, top). Following this, a whole-cell patch–clamp recording was established, and the electrical stimulus intensity was usually reduced to prevent action potentials in the recorded cell (Figure 3B, top). After recording a stable intracellular baseline for 5–10 min to 0.1 Hz stimulation, the effects of photolysis of nitr-5 were examined on each pathway.

In the example of Figure 3, nitr-5 photolysis produced LLP in the untetanized pathway (Figure 3A, bottom), whereas no potentiation, and perhaps even a small depression, was obtained in the prepotentiated pathway (Figure 3B, bottom). We have performed experiments of this sort on seven slices. Nitr-5 photolysis produced potentiation in three slices, depression in two, and no effect in two, in the untetanized pathway. In the prepotentiated pathway, we observed potentiation in zero slices, depression in five, and no effect in two. The key result is that LLP was never found in the prepotentiated pathway, whereas it occurred in the unstimulated pathway. These patterns were significantly different (χ^2 = 7.5, d.f. = 2, $p < 0.025$), as were the averaged responses from the unstimulated and prepotentiated pathways (Figures 3C and 3D).

The second experimental design was analogous, except that low frequency stimulation (1 Hz) was sent to one pathway during 10 min to produce LTD. In the example illustrated in Figure 4, LTD was recorded on the extracellular field potential of the test pathway (Figure 4B, top), whereas no modification was seen on the control pathway (Figure 4A, top). The rise in $[Ca^{2+}]_i$ following photolysis of nitr-5 induced LLP in the predepressed pathway (Figure 4B, bottom), whereas LLD was recorded in the control pathway (Figure 4A, bottom). In the five slices in which this kind of experiment was carried out, nitr-5 photolysis produced in the unstimulated pathway depression in three slices, potentiation in one, and no effect in 1. In the predepressed pathway, we observed potentiation in all 5 slices, and never saw depression. These patterns were significantly different (χ^2 = 20.0, d.f. = 2, $p < 0.01$), as were the averaged responses from the unstimulated and predepressed pathways (Figures 4C and 4D).

These results show that photoactivated LLP (or LLD) could be occluded by tetanic LTP (or electrically induced LTD), suggesting that these phenomena share common processes of induction and/or expression. They also show that a given uniform rise in postsynaptic $[Ca^{2+}]_i$ can induce opposite reactions in two different afferent pathways. In all cases, effects were elicited by 5 or 10 s of nitr-5 photolysis, with estimated $[Ca^{2+}]_i$ elevations of 0.3–1.0 μM. In most cells, a 5 s photolysis (300 nM $[Ca^{2+}]_i$ elevation) was sufficient to evoke the observed response, and subsequent larger $[Ca^{2+}]_i$ elevations had little additional effect.

Effects of Calmodulin and Phosphatase Inhibition on Response to a Rise in $[Ca^{2+}]_i$

We next asked whether LLP and LLD initiated by nitr-5 photolysis share biochemical pathways involved in the generation of tetanically induced LTP and electrically induced LTD.

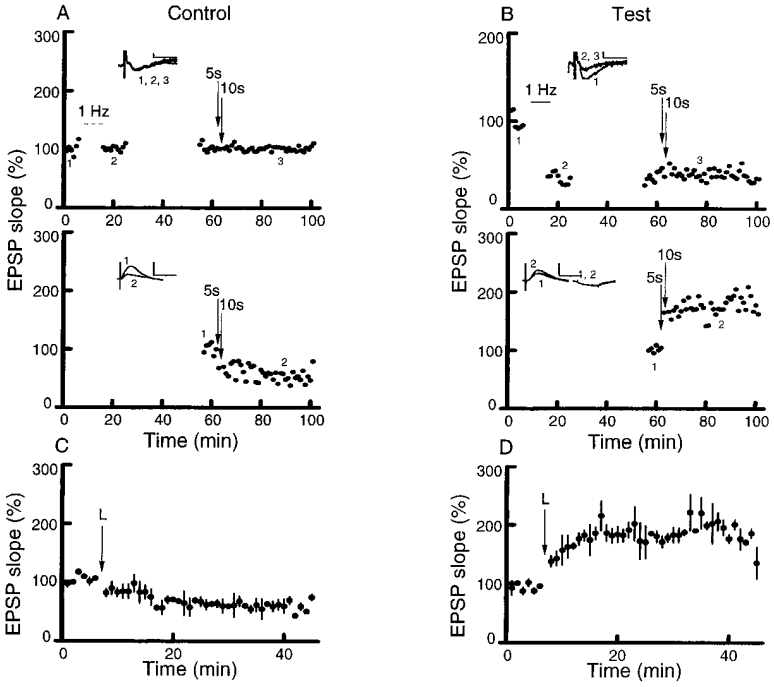

Figure 4. Electrically Induced LTD Occludes Photoactivated LLD

Two pathways were stimulated alternately at 0.1 Hz. LTD was generated in the 'Test' pathway (B) with a low frequency stimulus (1 Hz) for 10 min, whereas the 'Control' pathway was not depressed (A). A seal was formed onto a cell at the surface of the slice approximately 55 min after the beginning of the experiment and held at −76 mV. The estimated maximal [Ca^{2+}]$_i$ achieved during the first flash was 275 or 360 nM, and 785 nM or 1.1 μM during the second flash, assuming that the recorded synapses were 50 μm deep or at the surface of the slices, respectively. Photolysis of nitr-5 was performed in similar experiments on five cells. Summary graphs of the effects of photolysis on the intracellular EPSP in the control and test (predepressed) pathways are shown in (C) and (D), respectively, for averaged responses from all five cells.

In one set of experiments, 2.5 μM calmidazolium, a calmodulin antagonist that has been reported to block LTP (Malenka et al., 1989), was introduced into the whole-cell pipette with 5 mM nitr-5. After photoactivation of nitr-5, a cell that had been perfused with calmidazolium showed LLD (69%, Figure 5A). This result could not be explained by a simple effect of calmidazolium on synaptic transmission, because a stable baseline could be recorded in four cells for 30 min (Figure 5C). No LLP could be recorded after photoactivation of nitr-5 in ten cells perfused with calmidazolium. Figure 5D shows the distribution of the effects (potentiation, no effect, and depression) following photoactivation of nitr-5 in a control population of cells and in the population of ten cells perfused with calmidazolium, among which three cells presented a depression (Figure 5B, mean ± SD, 32% ± 14%). These distributions are statistically different ($p < 0.01$).

Another set of experiments used microcystin-LR, an impermeant protein phosphatase inhibitor that has been reported to inhibit LTD (Mulkey et al., 1993). After photoactivation of nitr-5, a cell that had been loaded with 10 μM of this compound exhibited LLP (63%, Figure 6A). A long-lasting potentiation was generated in three other cells (Figure 6B, 51% ± 18%, n = 4). Control experiments were performed in five cells to ensure that microcystin-LR (10–20 μM) had no effect on synaptic transmission (Figure 6C). No LLD could be recorded after photoactivation of nitr-5 in 10 cells perfused with microcystin-LR (Figure 6D). The distribution of the effects (potentiation, no effect, and depression) following the photoactivation of nitr-5 in the population of cells loaded with microcystin-LR (10–20 μM) was significantly different from that of the control population ($p < 0.01$).

These results suggest that LLP shares common processes of induction, expression, or both with electrically induced LTP, while LLD shares common processes of induction, expression, or both with electrically induced LTD. They confirm the essential role of calmodulin-dependent enzymes in LTP and of protein phosphatases in LTD.

Discussion

LLP and LLD Have Similar [Ca^{2+}]$_i$ Induction Thresholds

Our results show that photolysis of the caged Ca^{2+} compound nitr-5 perfused into postsynaptic CA1 pyramidal neurons can induce either LLP or LLD. The induction was performed by elevating postsynaptic [Ca^{2+}] to about 0.5 μM for about 10 s, as estimated by models described previously (Landò and Zucker, 1989; Marrion et al., 1991). When the duration of light exposure was too short to elicit a sufficient rise in [Ca^{2+}]$_i$, no modification in the synaptic efficiency could be detected. Longer durations of light exposure elicited induction of LLP or LLD with equal frequency. Few cells were unaffected. These failures could be due to a failure of nitr-5 to perfuse some neurons, due to resealing of the patch pipette, to an inability of the studied synapses to exhibit a long-term plasticity (perhaps due to washout; see Kato et al., 1993), or to a failure to elevate [Ca^{2+}]$_i$ sufficiently due to Ca^{2+} pumps, cellular buffers, or depth of the stimulated synapse and consequent dimness of the photolysis light.

It might seem that these factors also contributed to the variability of responses to photolysis. Although they probably cause the absolute elevation of [Ca^{2+}]$_i$ elicited by a given light exposure to vary from cell to cell, they should not influence whether depression or potentiation are observed first, as [Ca^{2+}]$_i$ is elevated in steps. A given light exposure might have somewhat different effects among cells, but the effect on any one cell should be

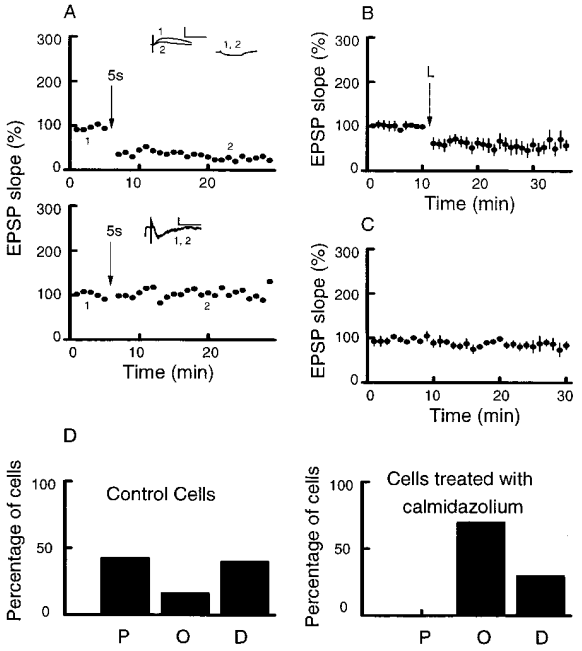

Figure 5. Photoactivation of Nitr-5 Is Unable to Enhance Synaptic Transmission in Cells Perfused with Calmidazolium

(A) A whole-cell recording was made from a surface cell, held at a membrane potential of −76 mV and perfused with the standard internal solution containing 2.5 mM nitr-5, 1.25 mM Ca^{2+}, and 2.5 μM calmidazolium (DMSO, 0.025%). EPSPs to stimulation of an afferent pathway at 0.1 Hz were recorded. Photoactivation of nitr-5 induced LLD. In this figure and in Figure 6, the calibration bars show 10 mV and 50 ms for intracellular EPSPs, and 0.5 mV and 20 ms for extracellular EPSPs. In both figures, the estimated maximal $[Ca^{2+}]_i$ produced was 275 or 360 nM if we assume that the observed synapses were 50 mm deep or at the surface of the slices, respectively.

(B) Averaged responses from three experiments in which photolysis of nitr-5 induced LLD in cells perfused with 2.5 μM calmidazolium.

(C) Control experiment. Four cells were perfused with the same solution as the cell shown in (A). But in this case, no photoactivation of nitr-5 was performed. The EPSPs remained stable for 30 min.

(D) Histograms showing the percentage of cells exhibiting a potentiation (P), no effect (O), or a depression (D) after photoactivation of nitr-5 in the control population of 42 cells, or in a population in which 10 cells have been perfused with 2.5 μM calmidazolium.

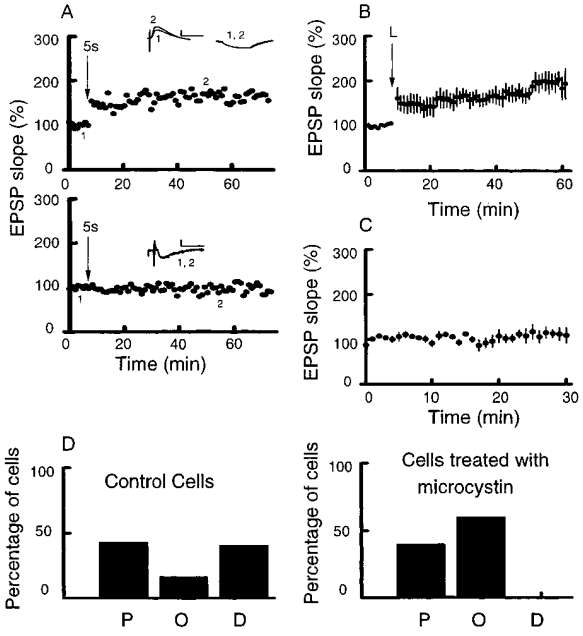

Figure 6. Photoactivation of Nitr-5 Is Unable to Depress Synaptic Transmission in Cells Perfused with Microcystin-LR

(A) A whole-cell recording was made from a surface cell, held at a membrane potential of −76 mV, and perfused with the standard internal solution containing 2.5 mM nitr-5, 1.25 mM Ca^{2+}, and 10 μM microcystin-LR, (ethanol, 0.25%). Photoactivation of nitr-5 induced LLP of EPSPs to 0.1 Hz stimulation of an afferent pathway.

(B) Averaged responses from four experiments in which photolysis of nitr-5 induced LLP in cells perfused with 10 μM microcystin-LR.

(C) Control experiment. Five cells were perfused with the same solution as for the cell described in (A). But in this case, no photoactivation of nitr-5 was performed. The EPSPs remained stable for 30 min.

(D) Histograms showing the percentage of cells presenting a potentiation (P), no effect (O), or a depression (D) after photoactivation of nitr-5 in the control population of 42 cells, or in a population in which ten cells have been perfused with microcystin-LR (10 or 20 μM).

nearly uniform. Since we select pyramidal cells at the surface of the slice and rapidly rising EPSPs that are electrically near the soma, we should be activating synapses near the surface of slices. For electrically proximal synapses that are almost certainly located within 150 μm of the soma, the 20 min we allow for perfusing cells should be adequate for the soma to reach equilibrium with the pipette and for the proximal dendrites to reach diffusional equilibrium for a compound with molecular weight less than 1 kDa. This is confirmed by the rapidity of filling pyramidal cells with fluorescent dyes.

It is important to recognize that even if there is a gradient in the $[Ca^{2+}]_i$ rise evoked by nitr-5 photolysis across the synapses from which we record, and even if $[Ca^{2+}]_i$ is elevated to different levels in different cells, if LTD had a lower induction threshold than LTP, it would still on the average require less photolysis of nitr-5 for its induction than LTP. As exposures of longer duration are used, those synapses closest to the surface, or those in cells that were more affected by nitr-5 photolysis, would indeed respond with a higher rise in $[Ca^{2+}]_i$. These synapses would be the first to be affected, and they would always show LTD. We did not observe this. The fact that both LLP and LLD occurred with equal frequency and with similar requirements for minimal light exposure suggest strongly that both processes have similar minimal $[Ca^{2+}]_i$ thresholds for their activation.

For LTP, it had previously been suggested that such a threshold lies in the range of several μM (Malenka et al., 1988). The present results make it likely that less than 1.0 μM $[Ca^{2+}]_i$ is sufficient to induce either LLD or LLP, though the actual $[Ca^{2+}]_i$ has not been measured. This level of $[Ca^{2+}]_i$ is similar to what is required to activate the native calcium/calmodulin-dependent kinase II (CaMKII) (Kuret and Schulman, 1984) and is consistent with the essential role of this protein kinase in the induction of LTP (Malenka et al., 1989; Malinow et al., 1989). It is also similar to levels of $[Ca^{2+}]_i$ needed to activate the Ca^{2+}-dependent phosphatase calcineurin (Armstrong, 1989) and is consistent with an essential role for this protein phosphatase in the induction of LTD (Mulkey et al., 1994).

In earlier experiments, elevation of $[Ca^{2+}]_i$ by photolysis of nitr-5 evoked only LLP of synaptic transmission in CA1 pyramidal cells (Malenka et al., 1988). However, those experiments were performed on adult rats, in which LTD is generally smaller and more difficult to obtain (Dudek and Bear, 1993). The present experiments appear to be the first to show that a rise in postsynaptic $[Ca^{2+}]_i$ is able to trigger LTD in cortical synapses.

Interpreting the Effects of DM-Nitrophen Photolysis

The amplitude and duration of Ca^{2+} signals were also varied in experiments using DM-nitrophen. This caged Ca^{2+} compound allows production of briefer (<1 s) rises in $[Ca^{2+}]_i$ than nitr-5, but to similar modest levels of about 1 μM (Zucker, 1994). Above a certain duration of light exposure, all remaining DM-nitrophen will be occupied by Ca^{2+}, and $[Ca^{2+}]_i$ will rise to much higher levels (probably exceeding 5 μM). This more elevated $[Ca^{2+}]_i$ should recover within a few seconds, due to removal of excess Ca^{2+}, buffered only by native cytoplasmic buffers, by extrusion mechanisms (Regehr and Tank, 1992; Sprusston et al., 1995; Yuste and Denk, 1995).

Surprisingly, only LLD was induced with DM-nitrophen, even when the resting Ca^{2+} was maintained at a physiological level. Since both brief, modest $[Ca^{2+}]_i$ elevations and longer, larger $[Ca^{2+}]_i$ elevations generated LLD, it is difficult to interpret these results in terms of differential effects of elevated $[Ca^{2+}]_i$ depending on either the amplitude or the duration of the stimulus. Since modest $[Ca^{2+}]_i$ elevations generated by nitr-5 photolysis often produced LLP, we worry that the DM-nitrophen results reflect some nonspecific or toxic effect, present even when the solution was adjusted to have a physiologically normal free $[Ca^{2+}]_i$ level and an antioxidant (glutathione) was used to counter effects of oxidizing photoproducts (Kaplan and Somlyo, 1989).

Perfusion of cells with DM-nitrophen leads to a transient elevation in $[Ca^{2+}]_i$ to about 1 μM during the loading process, as dilute Ca^{2+}-loaded DM-nitrophen confronts excess $[Mg^{2+}]_i$ in cytoplasm that drives Ca^{2+} off the DM-nitrophen until final equilibrium levels of DM-nitrophen are reached (Neher and Zucker, 1993). It might be that this transient $[Ca^{2+}]_i$ elevation somehow prepotentiated cells so that further potentiation was impossible and only depression could be expressed to subsequent Ca^{2+} signals. Our results with nitr−5 make it unlikely that the loading transient would preferentially potentiate, rather than depress, synapses, but it remains possible that the more prolonged duration of the loading transient preferentially prepotentiated synapses.

It is also possible that the photolysis-induced $[Ca^{2+}]_i$ elevations using DM-nitrophen were always too brief to induce potentiation, since both short and long light exposures would be expected to lead to briefer $[Ca^{2+}]_i$ elevations than those caused by nitr-5 photolysis, in which extrusion is slowed by the extra buffer capacity of nitr-5 and its photoproduct. This is an example of a hypothesis in which only LTD is induced by brief $[Ca^{2+}]_i$ elevation, while more prolonged $[Ca^{2+}]_i$ elevations can induce either LTP or LTD. Since LTD is normally induced by prolonged 1 Hz stimulation, and LTP by short tetani, this interpretation of the DM-nitrophen results seems rather unlikely.

Recent results using the photosensitive caged chelator diazo-4 (Malenka et al., 1992) bear on the question of the effect of varying the amplitude and duration of the Ca^{2+} stimulus. It was found that a tetanus-induced rise in $[Ca^{2+}]_i$ lasting at most 2–2.5 s is sufficient to induce LTP. A shorter or a smaller rise in $[Ca^{2+}]_i$ induced only a decrementing or short-term potentiation (STP) that decayed within 30 min. This is consistent with earlier results that manipulations that should reduce the entry of postsynaptic $[Ca^{2+}]_i$ during a tetanus can lead to the production of only STP (Malenka, 1991; cf. Mulkey and Malenka, 1992). Thus, it is unlikely that LTD induction requires a brief $[Ca^{2+}]_i$ elevation. The most likely explanation of the failure of DM-nitrophen photolysis to induce LLP is that it is a consequence of the loading transient or a nonspecific effect of this photolabile chelator.

Relationship of Synaptic Modifications Induced by Nitr-5 Photolysis to Conventional LTP and LTD

The modifications in synaptic efficiency generated by nitr-5 photolysis appear to be equivalent to LTP and LTD. LLP and LLD were occluded either by electrically induced LTP or LTD or by inhibiting the biochemical steps involved in the induction of LTP or LTD, i.e., calmodulin-dependent enzymes and protein phosphatase, respectively. The electrical occlusions were performed in experiments using two independent pathways, in which one pathway received either several tetani at 100 Hz for 1–2 s to induce saturated LTP, or 1 Hz for 10 min to induce saturated LTD (Mulkey and Malenka, 1992). Then, the cell was perfused with nitr-5 and the results of photolysis observed in both test and control pathways.

We had to establish LTP or LTD before patching onto a cell because otherwise wash-out of the recorded cell might occur during the time it takes to generate synaptic plasticity and confirm its persistence. This would interfere with the ability to subsequently elicit synaptic plasticity. Furthermore, unphotolyzed nitr-5 is a strong Ca^{2+} buffer that would prevent the initial induction of LTP or LTD by afferent stimulation. Similarly, occlusion experiments in which nitr-5 is first photolyzed to generate LLP or LLD, followed by tests of the effects of afferent stimulation, would be confounded by the continued buffering action of nitr-5 photoproducts. These might interfere with the ability to subsequently alter synaptic strength. Waiting long enough to confirm that nitr-5 photolysis had induced a long-term plasticity would risk wash-out of constituents needed for subsequent induction of plasticity by afferent stimulation. Our occlusion protocol avoids these pitfalls by performing the conditioning treatment under conditions that avoid wash-out and interference by exogenous Ca^{2+} buffering.

A rise in $[Ca^{2+}]_i$ upon photolysis of nitr-5 was never able to induce further LLP or LLD in a test pathway that exhibited either LTP or LTD, respectively. In a few cases, it was even possible to record LLD on a prepotentiated test pathway and LLP on the control pathway, and vice versa. These results confirm that a single uniform rise in $[Ca^{2+}]_i$ can simultaneously trigger LLP and LLD in

different pathways onto that cell and suggest that LLP and LLD share common mechanisms with LTP and LTD, respectively.

This idea was strengthened by the experiments using antagonists of calmodulin and protein phosphatases that have been shown to block LTP and LTD, respectively (Malenka et al., 1989; Mulkey et al., 1993). When protein phosphatases 1A and 2A were inhibited by microcystin-LR, LLD was never observed. Nitr-5 photolysis induced LLP or no change in synaptic transmission. When calmodulin, which is necessary to activate CaMKII, was blocked by calmidazolium, LLP was never observed. Nitr-5 photolysis induced LLD or no change in synaptic transmission.

The amount of LLD in calmidazolium was not statistically different from the control population of cells perfused with only nitr-5. Lisman (1989) and Mulkey et al. (1994) proposed a biochemical scheme for LTD that involves calcineurin in a phosphatase cascade that induces LTD. Calcineurin is activated by Ca^{2+} by both calmodulin-dependent and calmodulin-independent mechanisms (Merat et al., 1985), so that calmidazolium would be expected to reduce but not abolish the ability to generate LLD, as previously reported by Mulkey et al. (1993). The present results suggest that substantial LLD can be established by calmodulin-independent processes.

We may inquire whether a rise in $[Ca^{2+}]_i$ evokes a mixture of LLP and LLD, with one process tending to dominate, or whether only a single process is induced at a time. The occlusion experiments show that tetanically induced LTP occludes LLP and electrically induced LTD occludes LLD to a rise in $[Ca^{2+}]_i$ released by nitr-5 photolysis. However, these experiments do not show clearly whether the blocked process is replaced by the other process or by nothing. The pharmacological experiments, on the other hand, definitely suggest that when one process is blocked, pathways that would have exhibited that process do not show the opposite effect, but rather remain unchanged (Figures 5D and 6D).

To see this, we compare the distribution of results in experiments with calmidazolium with two models: in the first, synapses that would normally be potentiated (18 of 42 or 43%) are now nonresponsive (added to 7 of 42 for a total of 60%), leaving 17 of 42 or 40% subject to depression. Our results are fully consistent with this model ($p = 0.8$). In the second model, synapses that would normally be potentiated would show depression (83%), leaving 17% nonresponsive. The results differ significantly from this prediction ($p < 0.01$). Similarly, the effects of microcystin-LR are fully consistent with the predicted results if synapses that would show depression are nonresponsive ($p = 0.98$), but differ significantly from the predicted results if synapses that would show depression are potentiated ($p < 0.01$). Therefore, it appears that synapses begin with a bias toward responding to a rise in $[Ca^{2+}]_i$, with either LLP or LLD. Blocking the response that would occur leaves those synapses nonresponsive.

What Determines Whether a $[Ca^{2+}]_i$ Rise Induces LTP or LTD?

We were unable to distinguish separate amplitude or duration thresholds to a rise in $[Ca^{2+}]_i$ for inducing LLP and LLD. These results, taken together with our occlusion and pharmacological results, do not support the frequent suggestion that different $[Ca^{2+}]_i$ thresholds are involved in the induction of LTP and LTD (Lisman, 1989, 1994; Mulkey and Malenka, 1992; Artola and Singer, 1993; Schulman, 1994; Bear and Malenka, 1994; Linden and Connor, 1995; Larkman and Jack, 1995). These models require that when $[Ca^{2+}]_i$ exceeds the first low threshold, a mechanism is activated that leads to LTD. A second higher threshold must be exceeded before another mechanism is triggered leading to LTP. However, the present study shows that modest rises in $[Ca^{2+}]_i$ do not preferably induce LLD, nor are higher rises in $[Ca^{2+}]_i$ necessary to induce LLP. Similarly, the results of Malenka et al. (1992) show that reducing the amplitude or duration of the Ca^{2+} rise triggering LTP does not result in LTD, but rather only STP. These results also appear to conflict with predictions of the "sliding threshold model" (Bear, 1995), in which the $[Ca^{2+}]_i$ level separating LTP from LTD induction is a function of prior activity; even in this model, LTD always has a lower induction threshold than LTP. The choice between induction of LLP and LLD does not seem to be based on whether the Ca^{2+} signal exceeds different minimum threshold amplitudes or durations.

Our results bear on one other hypothesis for what determines whether a stimulus generates LTD or LTP. It may be that the Ca^{2+} target for producing LTD is located closer to the site of critical Ca^{2+} entry (presumably NMDA receptor channels) than the Ca^{2+} target for LTP induction. Then, weaker stimuli would activate LTD while only stronger stimuli would admit enough Ca^{2+} to reach the more distant LTP induction target, even if the two targets have similar $[Ca^{2+}]_i$ thresholds. However, this model requires that for stimuli that admit sufficient Ca^{2+} to activate both targets, LTP always wins out. Our results are not consistent with this implication.

Our results do not exclude other hypotheses based on the nature of the Ca^{2+} signal. For example, it is possible that a very large rise in $[Ca^{2+}]_i$, greater than what we could achieve with our methods, would always induce LLP, and that very prolonged elevation in $[Ca^{2+}]_i$, longer than what we could achieve, would always induce LLD. It is also possible that special spatial patterns of Ca^{2+} within cells are required for the induction of LTP and LTD. These are quite different hypotheses from the usual differential threshold hypothesis that we have addressed, and they remain to be tested experimentally.

Probably the most straightforward interpretation of our results is that some other as yet undetermined factor or factors influence the response of a cell to a rise in $[Ca^{2+}]_i$. Variability in these factors would explain why a modest $[Ca^{2+}]_i$ rise sometimes triggers LLD, and sometimes LLP. This result is obtained even in the total absence of afferent stimulation (Neveu and Zucker, 1996). This explains why when LTP or LTD is blocked pharmacologically, a rise in $[Ca^{2+}]_i$ more often has no effect. Apparently, blocking one process did not "flip the switch" that determines which process will respond to a rise in $[Ca^{2+}]_i$. This suggests that normally some other consequence of the pattern of previous pre- or postsynaptic activity determines how synapses respond to the rise in $[Ca^{2+}]_i$ that triggers both LTP and LTD. This suggestion is reminiscent of various conditions that appear

to influence the ability of a stimulus to induce LTP or LTD, including prior synaptic activity, prior activation of metabotropic glutamate receptors, or the state of CaMKII (Kauer et al., 1988; Huang et al., 1992; Kullmann et al., 1992; Bolshakov and Siegelbaum, 1994; Bortolotto et al., 1994; Bear, 1995).

Experimental Procedures

Preparation of Slices

Hippocampal slices were obtained from young (11–22 days) Sprague–Dawley rats. Animals were anesthetized with halothane and decapitated. The cerebral hemispheres were quickly removed and placed in a cold (4°C) solution containing: 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose that was saturated with 95% O_2 and 5% CO_2. Hippocampi were removed, placed on an agar block, and transferred to a slicing chamber containing the same cold solution described above. Transverse hippocampal slices (400–600 μm thick) were cut with a vibratome tissue slicer. Slices were then transferred into a humidified holding chamber and allowed to recover for at least 1 hr.

Electrophysiological Recordings

After a recovery period (1–8 hr), one slice was transferred to a recording chamber (Nicoll and Alger, 1981) and held between two nylon nets, where it was submerged beneath a continuously superfusing medium identical to that used for the preparation of the slices. The flow rate of the solution was ~2.5 ml/min. All the experiments were performed at room temperature (24°C–26°C).

Usually, afferent fibers (Schaffer collateral–commissural afferents) were stimulated at 0.1 Hz using bipolar, stainless steel electrodes. For some experiments, independent fiber bundles were alternatively activated at 0.1 Hz by placing bipolar stainless steel electrodes in stratum radiatum on opposite sides of the recording electrodes.

Extracellular field potentials were recorded in stratum radiatum using electrodes (thick wall glass, WPI Incorporated, 3–6 MΩ resistance, filled with 3 M NaCl). Whole-cell patch–clamp recordings were made from CA1 pyramidal cell bodies located on the top surfaces of slices, using the techniques of Blanton et al. (1989). For control experiments, electrodes (thin wall glass with filament, WPI Incorporated, 4–7 MΩ resistance), were filled with the following standard solution: 117.5 mM cesium gluconate, 17.5 mM CsCl, 8 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM Na_2-ATP, AND 0.2 mM GTP (pH 7.2 adjusted with CsOH, osmolarity: 290–300 mosM). For the caged Ca^{2+} experiments, the tips of the electrodes (2–3 mm) were filled with the standard solution to which 2.5–5 mM of the photosensitive compound, loaded 40%–80% with Ca^{2+}, had been added. Then the electrodes were back-filled with the standard internal solution. We used Cs^+ to block Ca^{2+}-activated K^+ currents that might be activated by the Ca^{2+} rise following photolysis of the caged Ca^{2+} compound. During the course of the experiment, cells were held under current clamp (using an Axoclamp 2B, Axon Instruments), at a membrane potential ranging from -70 mV to -80 mV. Bridge balance was continuously maintained by monitoring a response of the cell to a 50 ms hyperpolarizing current step (0.03 nA). When the input resistance of the cell varied more than 20%, the cell was discarded. Access resistance ranged between 6 and 55 MΩ (mean ± SD, 28 ± 13, n = 95), and the membrane potentials recorded at the soma were −59 ± 5 mV (n = 95). Membrane potentials were corrected by adding a measured junction potential of −6 mV to recorded values. Manipulations to induce long-lasting changes in synaptic transmission were performed within 20 min of breaking into a cell with a patch pipette, to minimize washout (Kato et al., 1993).

Data were collected and analyzed on line (5–10 kHz sampling rate) using a Pentium-based personal computer programmed with the AXOBASIC system (Axon Instruments). To minimize the contribution of voltage-dependent conductances, maximal rising slopes of EPSPs were calculated using a least-squares regression and cubic spline curves. Summary graphs were obtained by normalizing each experiment according to the average value of all points of the 5–10 min baseline prior to the light exposure, aligning the points with respect to the light exposure, and dividing each experiment into 60 s bins and averaging these across experiments (Malenka et al., 1989). Each point shows the mean ± SEM. Student's t tests and χ^2 tests were used to evaluate significance in differences between means and distributions, respectively.

Caged Ca^{2+} Compounds

[Ca^{2+}]$_i$ was elevated by release from nitr-5 (Calbiochem) photolyzed by ultraviolet light, as previously described (Landò and Zucker, 1989). We used a collimated light beam from a continuously run xenon lamp (150 W Cermax, ILC Technology, Sunnyvale, CA) fitted with a Uniblitz shutter (Vincent Associates, Rochester, NY) that had been found to photolyze 12% of a dilute solution of fully Ca^{2+}-loaded nitr-5 in 1 s at the same source-to-target distance as we used in our experiments. The expected average rise in free [Ca^{2+}]$_i$ upon exposure to light was calculated as described by Landò and Zucker (1989) and Marrion et al. (1991). For calculations in cell processes assumed to lie 50 μm below the surface of a slice, we reduced the light intensity by 25% to account for the absorbance of slices at 360 nm, which we measured as 2.5 mm^{-1}.

In some experiments, [Ca^{2+}]$_i$ was elevated by release from DM-nitrophen (a gift from Drs. J. Kaplan and G. Ellis-Davies) photolyzed using the same Xe lamp and shutter. The light source was calibrated following procedures described by Delaney and Zucker (1990). The photolysis rate was 0.67 s^{-1} for the Ca^{2+}-loaded form. The free [Ca^{2+}] of solutions of DM-nitrophen loaded with Ca^{2+} and Mg^{2+} was measured with a Ca^{2+}-selective electrode (Microelectrodes Incorporated, Londonderry, NH). These measurements coincided with estimates made with a program that calculates equilibrium levels of ions in the presence of multiple buffers.

The purity and K_D of each batch of nitr-5 and DM-nitrophen were checked using procedures previously described (Zucker, 1994).

Solutions

Calmidazolium (Sigma) was dissolved in pure DMSO to reach a final concentration of 10 mM. Microcystin-LR (Calbiochem) was dissolved in pure ethanol to reach a final concentration of 4 mM. Aliquots (5 μl) of these stock solutions were kept frozen (-20°C). Before each experiment, an aliquot was thawed and diluted in standard internal solution including nitr-5 (2.5 mM) loaded with Ca^{2+} (1.25 mM) to get the final concentration: 2.5 mM for calmidazolium and 10 μM or 20 μM for microcystin-LR.

Control experiments were carried out to ensure that light exposure had no effect on the EPSP recorded in cells perfused with DMSO or ethanol.

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