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Temporal Limits on the Rise in Postsynaptic Calcium Required for the Induction of Long-Term Potentiation

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Summary

The induction of long-term potentiation (LTP) in hippocampal CA1 pyramidal cells requires a rise in postsynaptic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To determine the time for which Ca^{2+} must remain elevated to induce LTP, the photolabile Ca^{2+} buffer diazo-4 was used to limit the duration of the rise in postsynaptic $[Ca^{2+}]_i$ following a tetanus. The affinity of diazo-4 for Ca^{2+} increases approximately 1600-fold upon flash photolysis, permitting almost instantaneous buffering of $[Ca^{2+}]_i$ without disturbing resting $[Ca^{2+}]_i$ prior to the flash. Photolysis of diazo-4 1 s following the start of the tetanus blocked LTP, while delaying photolysis for more than 2 s had no discernible effect on LTP. Photolyzing diazo-4 at intermediate delays (1.5–2 s) or reducing photolysis of diazo-4 often resulted in short-term potentiation (STP). These results indicate that a tetanus-induced rise in postsynaptic $[Ca^{2+}]_i$ lasting at most 2–2.5 s is sufficient to generate LTP. Smaller increases or shorter duration rises in $[Ca^{2+}]_i$ may result in STP.

Introduction

The mechanism proposed for the induction of long-term potentiation (LTP) in the CA1 region of the hippocampus involves a rise in postysynaptic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This proposal is supported by several lines of experimental evidence. First, LTP depends upon the activation of the N-methyl-D-aspartate (NMDA) receptor ionophore (Collingridge et al., 1983), which is permeable to Ca^{2+} (Ascher and Nowak, 1988; Jahr and Stevens, 1987; Mayer and Westbrook, 1987). Experiments directly measuring Ca^{2+} also have demonstrated that NMDA receptor activation leads to increased Ca^{2+} levels in cultured neurons (MacDermott et al., 1986) and dendrites of CA1 pyramidal cells in slices (Regehr and Tank, 1990). Second, LTP can be blocked or reduced by manipulations that should reduce the rise in $[Ca^{2+}]_i$ during LTP induction, such as injection of Ca^{2+} chelators (Lynch et al., 1983; Malenka et al., 1988) or strong postsynaptic depolarization (Malenka et al., 1988; Perkel et al., 1991, Soc. Neurosci., abstract), which would decrease the driving force for Ca^{2+} entry. Finally, a rise in postsynaptic $[Ca^{2+}]_i$ by release from the photolabile Ca^{2+} chelator nitr-5 causes a long lasting potentiation of synaptic transmission (Malenka et al., 1988).

Although the crucial importance of Ca^{2+} for triggering LTP seems established, there is little quantitative information regarding the necessary $[Ca^{2+}]_i$ increase. Entry of Ca^{2+} through voltage-dependent channels alone may cause an enhancement of synaptic transmission (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Perkel et al., 1991, Soc. Neurosci., abstract), raising the question of whether the NMDA receptor ionophore is the only Ca^{2+} source for LTP induction. It has been suggested that the magnitude of the rise in postsynaptic $[Ca^{2+}]_i$ is a critical variable controlling the duration of NMDA receptor-dependent synaptic enhancement (Malenka, 1991), a hypothesis that requires further examination. Finally, the duration for which $[Ca^{2+}]_i$ must remain elevated to induce LTP is unknown. This is particularly interesting given that repetitive synaptic stimulation in hippocampal slices (Muller and Connor, 1991) or glutamate application in acutely dissociated CA1 neurons (Connor et al., 1988) can cause dendritic $[Ca^{2+}]_i$ increases that last for several minutes. It also has been suggested that Ca^{2+} influx for a period of minutes may be required for LTP (Izumi et al., 1987) even though synaptic enhancement is evident right after LTP induction.

The experiments described here manipulate the timing between the LTP-inducing tetanus and an instantaneous increase in Ca^{2+} buffering capacity produced by rapid, flash photolysis of the photosensitive buffer diazo-4 (Adams et al., 1989; Figure 1A). This compound retains many of the properties of the parent compound BAPTA, including a high selectivity for Ca^{2+} over Mg^{2+}, rapid Ca^{2+} binding, and low pH sensitivity (Adams et al., 1989). Upon complete photolysis the dissociation constant (K_d) of diazo-4 changes dramatically from 89 μM to 55 nM, with a time constant in the range of 100–400 μs. Thus, introduction of diazo-4 into a cell will not affect ambient Ca^{2+} levels (50–100 nM for most neurons) yet will buffer $[Ca^{2+}]_i$ rapidly upon photolysis. The efficacy of this manipulation in CA1 pyramidal cells has been established recently in experiments examining the Ca^{2+}-dependent K^+ current responsible for the slow afterhyperpolarization (Lancaster and Adams, 1986; Lancaster and Zucker, 1991, Soc. Neurosci., abstract). Photolysis of diazo-4 at the peak of the slow afterhyperpolarization caused an immediate reduction in the response and prevented its subsequent generation, indicating that photolyzed diazo-4 is capable of rapidly buffering increases in $[Ca^{2+}]_i$.

Results

The standard protocol used in all experiments (Figures 1B and 1C) incorporated a number of control

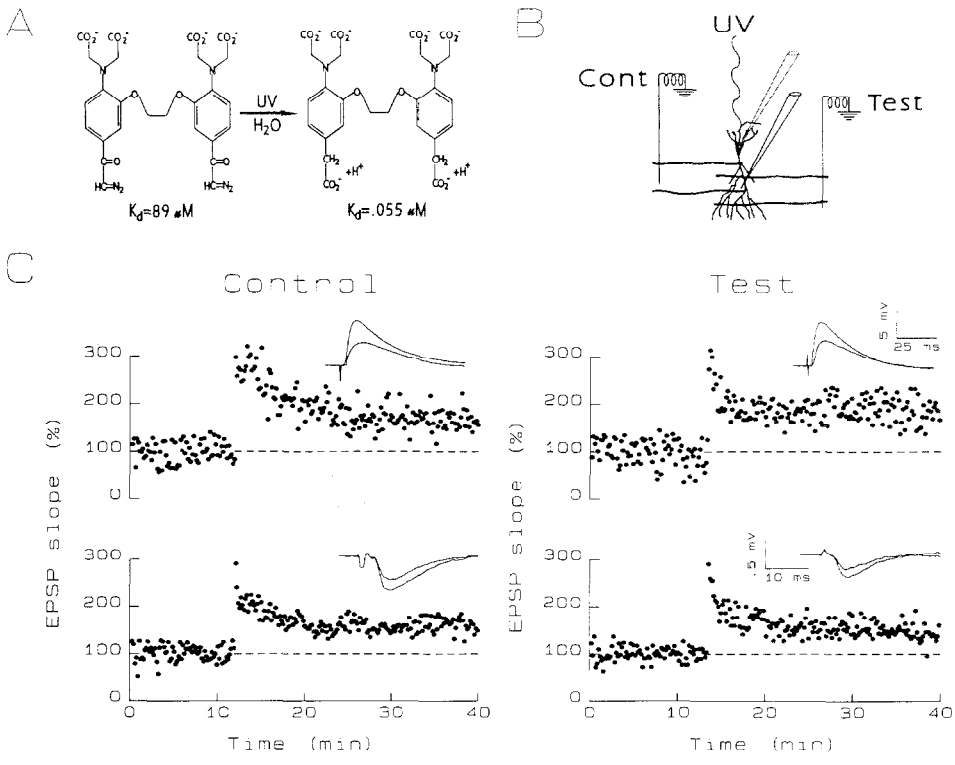

Figure 1. Experimental Protocol for Examining Effects of Diazo-4 on LTP Induction

(A) Structure of diazo-4 before and after photolysis. K_d decreases from 89 μM to 0.055 μM. (B) Arrangement of stimulating and recording electrodes in CA1 region. The control and test stimulating electrodes activate independent fiber bundles. Whole-cell recording electrode contains diazo-4. (C) Example from a control slice demonstrating that LTP can be generated in both the field and cell in response to a 1 s and 100 Hz tetanus applied first to the control pathway and then to the test pathway. In this and all following figures, the results obtained with whole-cell recording are shown in the top two panels, and those obtained with extracellular recording are displayed in the lower two panels. Insets in each panel show raw data traces taken before and 20–30 min following LTP induction.

experiments. To ensure that cells were capable of generating LTP, two independent afferent pathways were stimulated alternately; this provided a control for the test pathway upon which the effects of diazo-4 photolysis were examined. In most experiments (45 of 58) extracellular field potentials were also monitored simultaneously to ensure that LTP occurred in the population of cells not subject to the photosensitive manipulation. The test tetanus was applied within 1–2 min of the control tetanus to minimize any problems associated with internal perfusion ("washout") due to the whole-cell recording configuration. Figure 1C shows the results from one of five control cell–field combinations in which clear LTP was elicited in both pathways.

The ability of photolyzed diazo-4 to block LTP induction is shown in Figure 2. A tetanus to the control pathway was followed, 30 s later, by a single flash discharge (100 J) and another 30 s later by a tetanus to the test pathway. In the control pathway, LTP was observed in both the field and whole-cell recordings, while the test pathway produced LTP in the field recording but not in the cell. Similar results were obtained in six other cells (Figure 2B: 30 s flash delay, n = 3; 60 s flash delay, n = 3). These results indicate that diazo-4 photolysis blocks induction of LTP reliably and that buffering $[Ca^{2+}]_i$ 30 s or more following the tetanus does not prevent expression of LTP.

To estimate the effectiveness of diazo-4 photolysis in buffering rises in $[Ca^{2+}]_i$ we have developed a model (see Experimental Procedures) that assumes a tetanus-induced peak $[Ca^{2+}]_i$ in the undisturbed dendritic spine of 5 μM, a value broadly consistent with published measurements (Muller and Connor, 1991) and estimates (Holmes and Levy, 1990; Zador et al., 1990). Whole-cell recording solution (200 μM EGTA plus 2.5 mM diazo-4) will reduce this value to 2.4 μM, which still generates LTP reliably in the control pathway. Following a 100 J flash, however, peak $[Ca^{2+}]_i$ can reach only 0.34 μM, and no LTP can be induced. It is unlikely that the prevention of LTP induction by diazo-4 photolysis can be attributed to nonspecific deleterious effects, since basal synaptic transmission was unaffected by the light flash. Furthermore, the ability to generate LTP in adjacent cells as recorded with the extracellular electrode indicates that exposure of cells to the light flash, in and of itself, does not contribute to the inhibition of LTP.

Diazo-4 was photolyzed at several shorter intervals following the LTP-inducing tetanus without a discern-

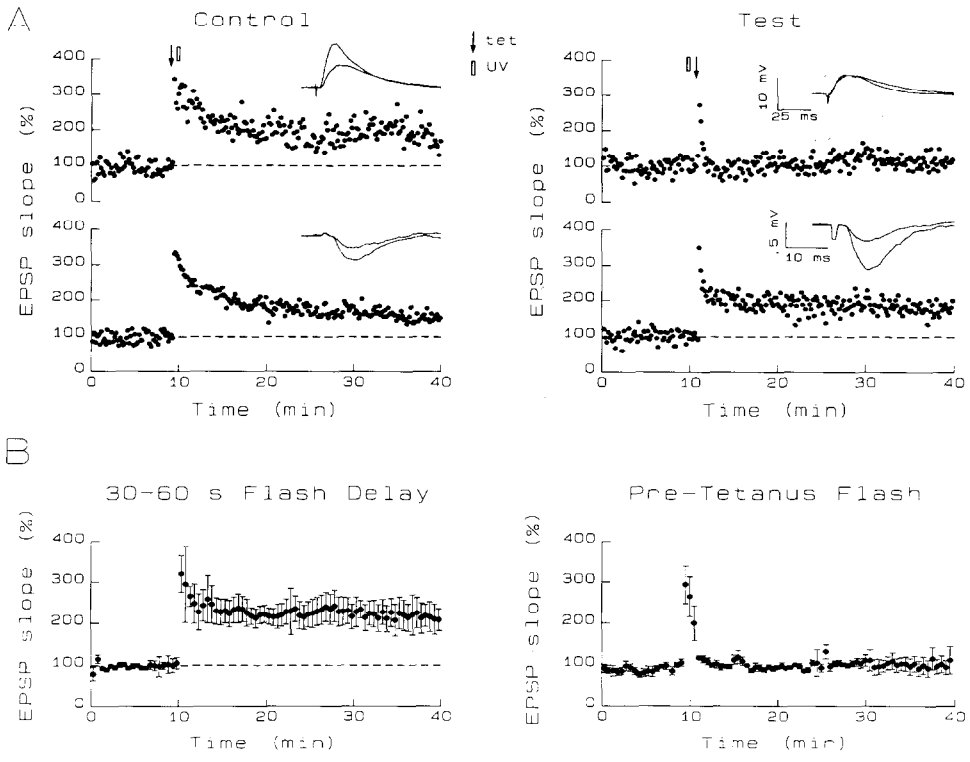

Figure 2. Photolysis of Diazo-4 Blocks the Induction of LTP

(A) Experiment in which diazo-4 photolysis took place 30 s after the control tetanus and 30 s before the test tetanus. The test tetanus generated LTP in the field EPSP but not in the cell. (B) Summary of experiments (n = 7) in which diazo-4 photolysis followed the control tetanus by 30–60 s and preceded the test tetanus.

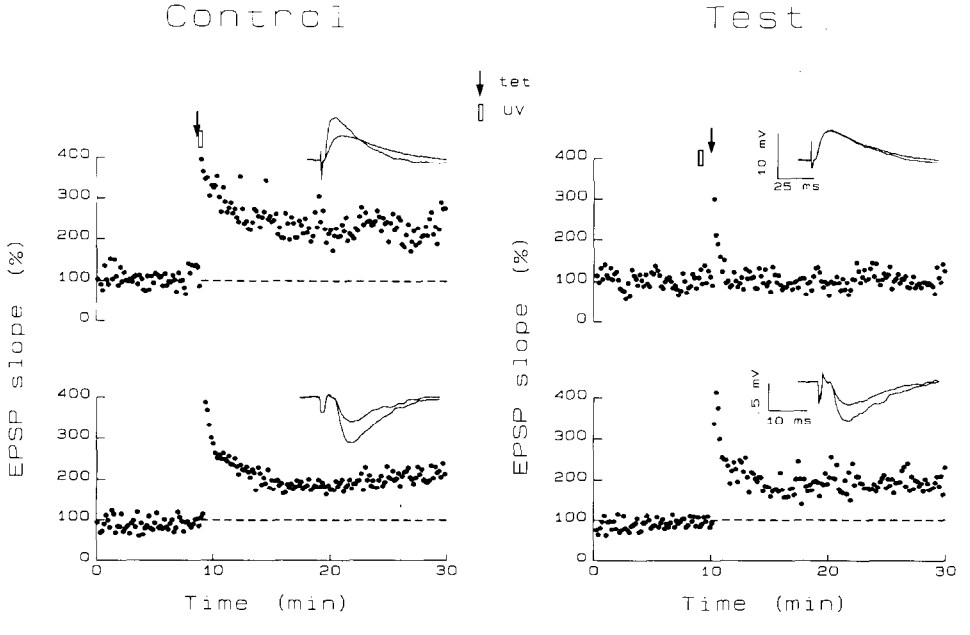

Figure 3. Delaying the Photolysis of Diazo-4 by 2.5 s Does Not Prevent LTP Induction

Discharging the flash lamp 2.5 s following the start of the control tetanus had no effect on LTP in the control pathway but blocked LTP in the cell in response to the test tetanus, which was given following diazo-4 photolysis. The test field EPSP graph demonstrates that the test tetanus did induce LTP in adjacent cells.

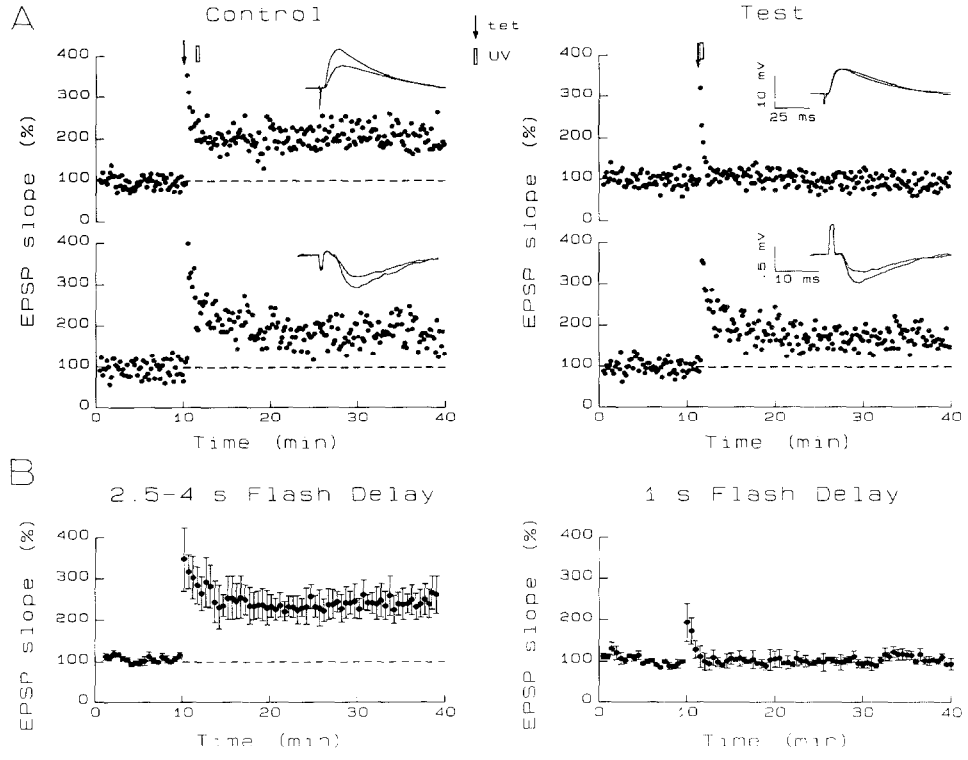

Figure 4. Photolysis of Diazo-4 1 s after the Start of the Tetanus Prevents LTP Induction

(A) Experiment in which LTP was first induced in the control pathway and then diazo-4 was photolyzed 1 s after the start of the test tetanus. The test tetanus induced LTP in the field EPSP but not in the cell. (B) Summary of experiments in which the photolysis of diazo-4 was delayed by 2.5–4 s (n = 8) or by 1 s (n = 5) following the start of the tetanus.

ible effect on the ability to generate LTP (10 s flash delay, n = 2; 4 s flash delay, n = 3; 2.5 s flash delay, n = 5). Figure 3 shows an experiment in which diazo-4 was photolyzed 2.5 s following the start of the initial control tetanus. The control pathway showed LTP even at this short delay, while subsequent inhibition of LTP induction in the test pathway testified to the effectiveness of the buffer.

In contrast, photolysis inducing an increase in buffer capacity immediately at the end of the 1 s tetanus routinely prevented induction of LTP (n = 5; Figure 4A). Figure 4B shows a summary comparison of the experiments in which the flash was delayed 2.5–4 s or 1 s following the start of the tetanus. Photolysis of diazo-4 at the termination of the tetanus had the same effect as photolysis of diazo-4 before applying a tetanus (see Figure 2B), while increasing buffer capacity at intervals of 2.5 s or greater following the start of an LTP-inducing tetanus had no discernible effect on LTP. These results suggest that to generate LTP, an elevated $[Ca^{2+}]_i$ is required for longer than the duration of the tetanus (1 s) but not longer than 2.5 s from the start of the tetanus.

To probe this transition period further, the effects of diazo-4 photolysis at two additional shorter delays were examined. Both delays gave mixed results. Photolysis of diazo-4 1.5 s following the start of the tetanus resulted in posttetanic potentiation alone (2/8), a decremental synaptic enhancement like short-term potentiation (Malenka, 1991) (STP; 4/8; Figure 5A), or stable LTP (2/8). At a 2 s delay, photolysis of diazo-4 resulted in either STP (2/5) or LTP (3/5). Thus at least in some cases in which LTP was prevented, STP could be observed in isolation (Figure 5A). A summary of these experiments (Figure 5B) demonstrates that shortening the duration of the Ca^{2+} increase may represent a transition point at which activation of the requisite Ca^{2+}-dependent processes is partially, but not completely, inhibited.

A second method of partial activation of the Ca^{2+}-dependent processes involved in synaptic enhancement would be to damp the rise in $[Ca^{2+}]_i$, that is, to suppress the amplitude rather than the duration of the postsynaptic Ca^{2+} change. To accomplish this, a lower concentration of diazo-4 (1.0 rather than 2.5 mM) and a lower intensity light flash (30 J rather than 100 J) were used. Using the assumption for a tetanus-induced peak $[Ca^{2+}]_i$ of 5 μM, the pipette buffers (without photolysis) will reduce this to 3 μM. Following a 30 J flash, however, the peak $[Ca^{2+}]_i$ induced by a second tetanus will be reduced by 40% to 1.8 μM. Such a manipulation (n = 7) caused the LTP-inducing tetanus to elicit either STP (Figure 6A) or small magnitude LTP (Figure 6B, summary graph).

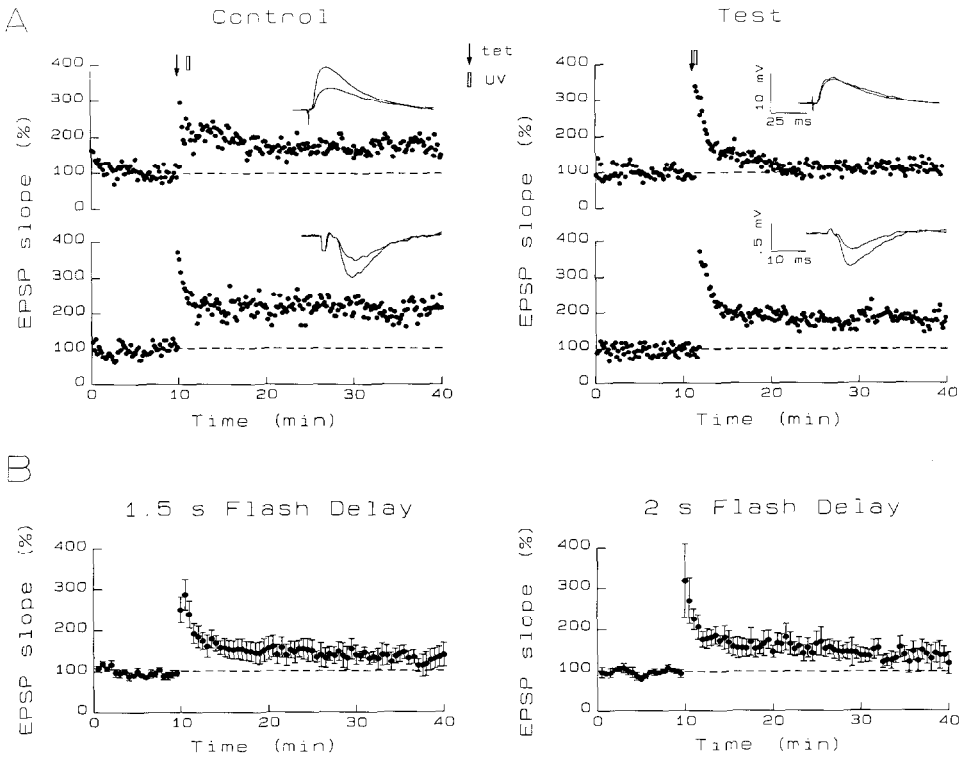

Figure 5. STP Is Often Generated when the Photolysis of Diazo-4 Is Delayed by 1.5 or 2 s

(A) Experiment in which LTP was first induced in the control pathway and diazo-4 was photolyzed 1.5 s after the start of the test tetanus. The test tetanus induced STP in the cell but LTP in the field EPSP. (B) Summary of experiments in which the photolysis of diazo-4 was delayed by 1.5 s (n = 8) or by 2 s (n = 5).

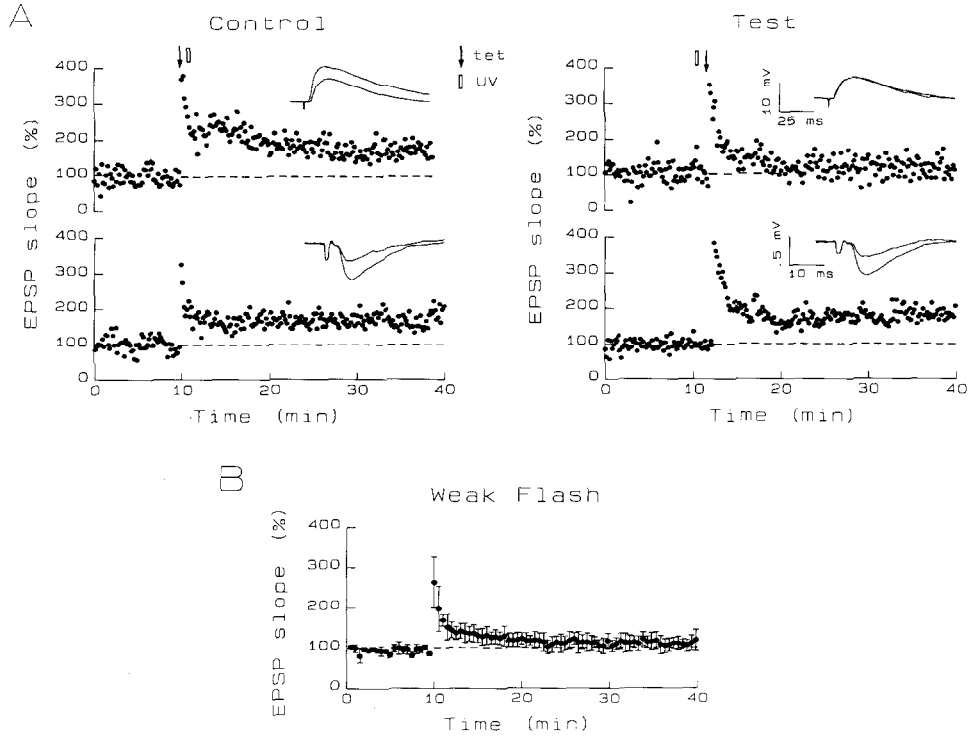

Figure 6. Partial Diazo-4 Photolysis Results in the Generation of STP

(A) Experiment in which the whole-cell recording electrode was filled with 1.0 mM diazo-4 and a 30 J flash was used for photolysis. LTP was first induced in the control pathway, and following partial diazo-4 photolysis the test tetanus induced STP in the cell and LTP in the field EPSP. (B) Summary of experiments (n = 7) in which diazo-4 (1.0 mM) was photolyzed with a 30 J flash prior to application of the test tetanus.

Discussion

The rapid flash photolysis of diazo-4 described in these experiments represents a method for raising instantaneously the Ca^{2+} buffering capacity within a cell. By changing the timing between photolysis of diazo-4 and a tetanus that reliably induced LTP, the minimum duration of raised postsynaptic $[Ca^{2+}]_i$ necessary to generate LTP was determined. Because ambient $[Ca^{2+}]_i$ levels should not be affected significantly by unphotolyzed diazo-4 the inhibition of LTP by photolyzed diazo-4 can be assumed to be due to the buffering of the tetanus-induced rise in $[Ca^{2+}]_i$ and not to a lowering of resting $[Ca^{2+}]_i$ (Malenka and Nicoll, 1990). If a flash followed the tetanus without delay, induction of LTP was prevented, despite having been demonstrated in the control pathway for that cell within the preceding 2 min. Assuming that $[Ca^{2+}]_i$ started to rise at the beginning of the tetanus, this result indicates that postsynaptic $[Ca^{2+}]_i$ rising for 1 s was insufficient to produce any long-term synaptic enhancement (only posttetanic potentiation, a presynaptic phenomenon, was observed).

In contrast, when photolysis of diazo-4 occurred at intervals 2.5 s or greater after the start of the tetanus, LTP could be induced without fail and presumably was no longer dependent on an increased $[Ca^{2+}]_i$ in the postsynaptic cell. These results indicate that a sustained increase in postsynaptic $[Ca^{2+}]_i$ is not required to generate stable LTP and that an increase lasting at most 2.5 s (1 s during the tetanus plus 1.5 s thereafter) is sufficient for LTP induction. This value does not preclude a role for Ca^{2+} release from intracellular stores in LTP (Obenaus et al., 1989; Harvey and Collingridge, 1992), but does rule out a requirement for any long lasting oscillations in $[Ca^{2+}]_i$ that may occur as a result of this release (Tsien and Tsien, 1990).

Decreasing the magnitude of the tetanus-induced rise in $[Ca^{2+}]_i$ by reducing diazo-4 photolysis often resulted in a transient synaptic enhancement similar to STP. Although, like LTP, STP requires activation of postsynaptic NMDA receptors (Anwyl et al., 1989; Malenka, 1991), its precise relationship to LTP is ambiguous. It may reflect partial activation or "weak induction" of the processes that lead to LTP (Gustafsson and Wigstrom, 1988). Alternatively, there is some evidence to suggest that it may be mediated by mechanisms distinct from those involved in maintaining stable LTP (Larson et al., 1986; Davies et al., 1989). The present results are consistent with the hypothesis that to activate the processes necessary to generate stable LTP requires a higher level of postsynaptic $[Ca^{2+}]_i$ and that smaller rises in $[Ca^{2+}]_i$ result in STP (Malenka, 1991). STP was also sometimes elicited by shortening the duration of the normal tetanus-induced rise in $[Ca^{2+}]_i$. This finding makes it unlikely that the higher level of $[Ca^{2+}]_i$ required for LTP activates processes not activated by lower levels of $[Ca^{2+}]_i$ but instead is consistent with the hypothesis that STP results from incomplete activation of the requisite LTP processes. However these experiments do not rule out the possibility that a rise in $[Ca^{2+}]_i$ sequentially activates several distinct biochemical processes and that interrupting this sequential activation results in STP.

Little is known of how Ca^{2+} interacts with its target or targets to trigger LTP, but two possibilities may be imagined: Ca^{2+} may act rapidly, perhaps by binding quickly and irreversibly to its target; or Ca^{2+} may bind to a site that initiates a process that must continue for a minimum time to trigger LTP successfully. Under the first possibility, once Ca^{2+} reaches a threshold for activating its target(s), LTP would be initiated, and subsequent Ca^{2+} concentration would be irrelevant. Our results rule out this hypothesis because we have shown that Ca^{2+} must remain high for more than 0.5–1 s after the tetanus and after the time at which $[Ca^{2+}]_i$ has presumably reached its peak.

One candidate mechanism involved in the generation of LTP is Ca^{2+}/calmodulin-dependent protein kinase II, which is concentrated in postsynaptic densities (Kennedy et al., 1983; Kelly et al., 1984). Endogenous postsynaptic calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II both appear to be required for LTP induction (Malenka et al., 1989; Malinow et al., 1989). A similar process is present in smooth muscle where neural stimulation leads to raised $[Ca^{2+}]_i$, activation of a calmodulin-dependent kinase, phosphorylation of the myosin light chain, and then contraction (Miller-Hance et al., 1988). In this system, substrate (myosin light chain) phosphorylation is maximal within 2 s from the beginning of neural stimulation and maximal at approximately 1 μM intracellular Ca^{2+} (Taylor et al., 1989). This time period is similar to that described here for LTP induction, indicating that the time course of activation of the Ca^{2+}-dependent processes required for LTP is consistent with an analogous system in smooth muscle.

Experimental Procedures

Standard procedures were used to prepare hippocampal slices from Sprague–Dawley rats (2–5 weeks) (Malenka, 1991). Following a recovery period (1–6 hr), one slice was transferred to the recording chamber where it was submerged beneath a continuously superfusing medium that had been saturated with 95% O_2, 5% CO_2. The medium was composed of the following: 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. The temperature of the medium was maintained between 28°C and 30°C.

Whole-cell patch-clamp recordings were made using standard techniques (Blanton et al., 1989; Coleman and Miller, 1989). Electrodes (3–7 MΩ) were filled with a solution containing the following: 117.5 mM cesium gluconate, 17.5 mM CsCl, 8 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 2–3 mM Mg-ATP, and 0.2 mM GTP (pH 7.2). By blocking K^+ channels, internal Cs^+ increases cells' input resistance, and we have found this results in more stable recordings and more reliable induction of LTP. For the diazo-4 photolysis experiments, the tips of electrodes (2–3 mm) were filled with this same solution, to which 2.5 mM or 1.0 mM diazo-4 had been added. Photolysis of diazo-4 results in the release of H^+ (2 mol of H^+ per mol of diazo-4) (Adams et al., 1989). To compensate further for proton release, some experiments (n = 24) were performed with a higher concentration of HEPES (20 mM). When this pipette solution was challenged with 2 mM H^+ it sustained a change of only 0.1 pH unit. The estimated proton release

in these experiments was 1.24 mM (100 J flash, 2.5 mM diazo-4) or 0.19 mM (30 J flash, 1.0 mM diazo-4; see below). Results with 10 or 20 mM HEPES in the pipette solution were indistinguishable.

Extracellular field potentials were recorded in stratum radiatum using electrodes (3–6 MΩ) filled with 3 M NaCl. During synaptic stimulation cells were held between −70 and −85 mV under current clamp. Independent fiber bundles were alternately activated at 0.1 Hz by placing bipolar stainless steel electrodes in stratum radiatum on opposite sides of the recording electrodes. Data were collected and analyzed on line (5–10 kHz sampling rate) using an Intel 80-386–based computer programed with the AXOBASIC system (Axon Instruments). Initial slopes of field and whole-cell excitatory postsynaptic potentials (EPSPs) were calculated using a least-squares regression.

The standard experimental protocol for all experiments began by obtaining a stable, 10 min baseline, at which point a tetanus (1 s at 100–200 Hz) was applied to the control pathway. Cells were included in the data analysis only if following this tetanization an increase in the control (but not test) pathway EPSP slope was recorded and lasted at least 15–20 min. A second tetanus was given to the test pathway always within 120 s (and routinely within 60–90 s) of the initial tetanus. Summary graphs were obtained by normalizing each experiment according to the average value of all points on the 10 min baseline prior to the tetanus, aligning the points with respect to the time of the tetanus, and dividing each experiment into 30 s bins and averaging these across experiments (Malenka et al., 1989). Each point shows the mean ± SEM.

Photolysis of diazo-4 was accomplished with a xenon arc lamp of flash duration 1.6 ms. A 100 J flash represents the discharge of that amount of electrical energy from the power supply (Chadwick Helmuth). Prior to the experiment, the lamp was focused onto a marked area of the recording chamber so that the light flash would photolyze diazo-4 in the cell and in the tip of the recording electrode. The area of the slice from which the field EPSP was recorded was also completely exposed to the light flash. The "flash delay" is defined as the time following the start of the tetanus at which the flash was given.

Diazo-4 has two photochemically sensitive diazoacetyl groups (Figure 1A; Adams et al., 1989) and a low affinity for Ca^{2+} of 89 μM. Photolysis of both diazoacetyl groups gives a Ca^{2+} affinity of 55 nM, while single photolysis gives a Ca^{2+} affinity of 2.2 μM. A flash produces a mixture of these species and several other "frozen" or mixed conformations, in which one or both photolyzable sites is converted to a form with no effect on Ca^{2+} affinity but loss of sensitivity to UV light (Adams et al., 1989). To estimate changes in buffering capacity of diazo-4 after photolysis, a model was developed that considered the six species of diazo-4 (unphotolyzed, singly photolyzed, doubly photolyzed, singly frozen, doubly frozen, and singly photolyzed and frozen) and the nine possible transition probabilities between species on exposure to repeated flashes of UV light. The model was confirmed, and our flash lamp was calibrated by measuring flash-induced Ca^{2+} concentration changes expressed as absorbance changes in microcuvettes filled with mixtures of diazo-4 and the Ca^{2+}-sensitive dye arsenazo III (M. W. Fryer and R. S. Zucker, unpublished data). Allowing for tissue absorbance (assuming a 100 μM depth), we calculated that a 100 J flash of 2.5 mM diazo-4 produces 0.92 mM of singly photolyzed or equivalent frozen products with a Ca^{2+} affinity of 2.2 μM and 0.16 mM of doubly photolyzed product with 55 nM affinity. A 30 J flash of 1 mM diazo-4 should produce 0.15 mM of medium affinity products and 0.02 mM of high affinity product. It was assumed that the diazo-4 in the electrode tip had equilibrated within the dendritic processes by the time of the tetanus. When combined with an estimate of the tetanus-induced rise in $[Ca^{2+}]_i$ in the presence of only a native cytoplasmic buffer, with an assumed concentration of 0.4 mM and a Ca^{2+} affinity of 2.2 μM, we arrive at the estimates of the effect of photolysis of diazo-4 on the peak Ca^{2+} concentration reached in a tetanus given in Results. Effects of photolysis of diazo-4 qualitatively similar to those presented in Results are obtained when the peak rise in $[Ca^{2+}]_i$ in the absence of diazo-4 is assumed to be 1 or 20 μM rather than 5 μM.

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