Chemically Induced, Activity-Independent LTD Elicited by Simultaneous Activation of PKG and Inhibition of PKA

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INTRODUCTION

Homosynaptic long-term depression (LTD) is an input-specific, long-lasting reduction in synaptic strength that can be generated by prolonged low-frequency stimulation in a number of cortical areas, including at Schaffer collateral/commissural-CA1 synapses in the hippocampus (for review, see Bear and Abraham 1996; Christie 1996). Although the precise, physiologically relevant functions of LTD are still unclear, most neural network models include both long-term increases and decreases in synaptic strength to accomplish memory storage, information processing, and mnemonic classifications (Bienenstock et al. 1982; Sejnowski 1977). It is a popular hypothesis that memories may have their cellular correlate in precise spatiotemporal patterns of synaptic strengths. Since the demonstration of homosynaptic, associative LTD (Stanton and Sejnowski 1989), much work has focused on the elucidation of cellular mechanisms underlying both the induction and expression of cortical LTD.

Cyclic nucleotide signal transduction cascades have been suggested to play a role in synaptic plasticity in a wide variety of systems. In mammalian hippocampus, it is now well established that cyclic AMP-activated pathways play necessary roles in the full expression of long-term potentiation (LTP) of synaptic strength (Abel et al. 1997; Blitzer et al. 1995; Frey et al. 1993; Hopkins and Johnston 1988; Matthies and Reymann 1993; Stanton and Surgey 1985a,b; Stanton et al. 1989). Although most of these studies have focused on a late-phase of LTP involving both activation of postsynaptic kinases and alterations in gene expression and protein synthesis, there is also evidence that supports a potential presynaptic role of cyclic AMP at mossy fiber terminals in field CA3 (Huang et al. 1994) and at Schaffer collateral/commissural terminals in field CA1 of rat hippocampal slices (Chavez-Noriega and Stevens 1994).

Recent work in our and other laboratories (Gage et al. 1997; Izumi and Zorumski 1993; Reyes-Harde et al. 1999a,b) has linked another cyclic nucleotide second-messenger cascade, the nitric oxide (NO)-cyclic GMP system, to the induction of LTD. As a first step toward identifying downstream effectors of cyclic GMP, recent evidence (Reyes-Harde et al. 1999a,b) implicates presynaptic cyclic GMP-dependent protein kinase (PKG) as a necessary component in the LTD induction pathway. Thus cyclic AMP and cyclic GMP appear to be involved reciprocally in long-term regulation of synaptic strength. However, it also has been reported that cyclic AMP-dependent protein kinase (PKA) activity is necessary to permit the induction of LTD (Brandon et al. 1995). Given that PKA and PKG have reciprocal actions in many cells, (Inoue et al. 1995; Polanowska-Grabowska and Gear 1994; Wexler et al. 1998), it seemed surprising to us that these two cyclic nucleotides would have opposing actions on LTD. We set out to test our hypothesis of a bidirectional regulation model in which these two cyclic nucleotides would have opposing actions on LTD. We show here that inhibition of PKA results in an enhancement of stimulus-evoked LTD via a PKG-dependent mechanism. In addition, we demonstrate that blockade of the hydrolysis of cyclic GMP, in conjunction with simultaneous inhibition of PKA, elicits a sustained, chemically induced, activity-independent LTD that appears to share mechanisms in common with stimulus-evoked LTD.

METHODS

Transverse hippocampal slices (400-μm thick) were obtained from Sprague-Dawley rats of either sex (14–21 days old). Briefly, animals were anesthetized and decapitated. The brain was removed rapidly, the hippocampus dissected and placed in 4°C artificial cerebrospinal fluid under aseptic conditions. The brain was placed in a cutting chamber and coronal slices were cut at 400 μm. The slices were transferred to a recording chamber and the temperature was increased to 34°C.

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RESULTS

PKA inhibitors do not block the induction of homosynaptic LTD

To test the necessity for PKA activation for the induction of LTD at Schaffer collateral-CA1 synapses, we employed two selective, cell-permeant PKA inhibitors, H-89 (a synthetic inhibitor) (Chijjiwa et al. 1990) and KT5720 (a secondary metabolite-derived inhibitor) (Kase et al. 1987). Figure 1A illustrates the induction of LTD by LFS (1 Hz/900 s; solid bar) in the presence of bath-applied H-89 (10 μM; open bar). LTD in these slices was indistinguishable from control LTD (−36.6 ± 2.9%; n = 16, data not shown) in either magnitude or duration (LTD in H-89 = −35.1 ± 6.0%; P > 0.20; Student’s t-test compared with controls; n = 9). Similarly, the second PKA inhibitor, KT5720 (1 μM; open bar; Fig. 1B), was also unable to block the induction of LTD by LFS (LTD in KT5720 = −35.2 ± 6.4%; n = 6). Taken together, these experiments lead to the conclusion that PKA activation is not a necessary step in the induction of homosynaptic LTD at Schaffer collateral-CA1 synapses.

Homosynaptic LTD is enhanced by inhibiting PKA

The preceding observations, and previous studies showing that PKA activity is necessary for the full expression of LTP (Frey et al. 1993; Matthes and Reymann 1993; Stanton and Surve 1985a,b; Stanton et al. 1989), raise the question of whether preventing activation of PKA in fact might enhance or unmask the induction of a reciprocal modification of synaptic strength, LTD. To test this hypothesis, we used a shorter, submaximal LFS stimulation, 1 Hz for 400 s, which induced only a small amount of LTD in control slices (Fig. 1C; −11.5 ± 2.9%; n = 11). In a second group of slices (Fig. 1D; n = 14), we bath-applied H-89 (10 μM; open bar) 45 min before submaximal LFS of Schaffer collateral/commissural axons (1 Hz/400 s; solid bar). In contrast to control slices, submaximal LFS elicited virtually maximal LTD when PKA was inhibited (−35.4 ± 4.1%, P < 0.05; Student’s t-test compared with controls 60 min post-LFS). This intriguing result prompted further investigation into the synaptic locus of action of H-89 as well as studies aimed at testing the possibility of a co-requirement for an NO-cyclic GMP-PKG pathway (Gage et al. 1997) in this “unmasked” LTD.

Postsynaptic PKA inhibition does not enhance LTD

In a recent study (Gage et al. 1997), we presented evidence that at least one form of homosynaptic LTD requires activation of a presynaptic cyclic GMP-mediated cascade triggered by the gaseous intercellular messenger NO. To test the possibility that PKA counteracts this cascade directly, it was necessary to determine whether H-89 acts pre- and/or postsynaptically. To accomplish this, we used sharp intracellular microelectrodes to fill single CA1 pyramidal neurons with H-89. Intracellular electrodes were backfilled with a solution containing a 50-fold higher concentration of H-89 (500 μM) than that which was effective in enhancing LTD when applied extracellularly. Figure 2A illustrates the effect of submaximal Schaffer collateral LFS (1 Hz/400 s; solid bar) on intracellularly recorded EPSPs in control, untreated CA1 pyramidal neurons (n = 7). The left inset shows examples of single EPSPs before and after submaximal LFS in a control cell. The right inset illustrates burst-induced afterhyperpolarization (AHPs; +0.5–1.0 mV/100 ms injected current) before and after bath application of the adenylyl cyclase stimulant forskolin (100 μM), which elicited a well-characterized PKA-mediated block of the late AHP (Madison and Nicoll 1986).
Figure 2B illustrates the effects of identical submaximal LFS on CA1 pyramidal neurons filled with H-89 to selectively inhibit postsynaptic PKA. CA1 pyramidal neurons were impaled with microelectrodes filled with 500 μM H-89 (in 2 M KCl, open bar). At least 45 min were allowed for leakage of H-89 into neurons (open bar), after which submaximal LFS (1 Hz/400 s; solid bar) was applied to one Schaffer collateral input, while a second input served as control (not shown). In contrast to extracellular application of H-89, selective inhibition of postsynaptic PKA did not statistically enhance the magnitude of LTD evoked by submaximal LFS (LTD in control (1) and 40 min after (2) LFS (calibration bars: 2 mV/5 ms; each point is the normalized mean ± SE)).

NO synthase is necessary for the form of LTD unmasked by inhibiting PKA

We have shown recently that full LFS-evoked homosynaptic LTD consists of both NO-guanylyl cyclase-dependent and -independent components (Gage et al. 1997). However, it was not clear which component(s) contribute to the LTD unmasked by inhibiting PKA in our current experiment. Therefore, we used the cell-permeant NO synthase inhibitor N\textsubscript{o}-nitro-L-arginine (L-NA) to determine whether NO production is, in fact, a necessary component of LTD evoked under either condition. Figure 3A (n = 6) illustrates that, in agreement with previous reports (Izumi and Zorumski 1993; Otani and Connor 1995), bath application of L-NA (100 μM; open bar) partially reduced the magnitude of LTD produced by LFS (1 Hz/900 s; solid bar;
The observed H-89 effect also may be NO-independent, perhaps acting through a postsynaptic cascade involving protein phosphatases (Mulkey et al. 1993).

Cyclic GMP accumulation alone is not sufficient to induce LTD

One physiological function of presynaptic nitric oxide is to bind to the heme moiety of soluble guanylyl cyclase (sGC), causing the synthesis of cGMP. However, it was unclear whether an elevation in [cGMP] alone could be sufficient to elicit LTD. Isozyme-specific phosphodiesterase (PDE) inhibitors are useful pharmacological tools for examining the physiological roles of cyclic nucleotides in a variety of systems (Beavo 1995). In particular, it is the type V PDE that is primarily responsible for the selective breakdown of cyclic GMP. Therefore, a selective PDE V inhibitor, such as zaprinast, markedly and selectively elevates intracellular [cGMP] (Gillespie and Beavo 1989).
Figure 4A shows that bath application of zaprinast (5 μM; open bar; n = 6) caused a transient depression of synaptic EPSPs that fully reversed within 30 min of drug washout. Thus raising [cGMP] by this method, in the absence of any stimulus train, produced only a transient, rapidly reversible short-term depression, consistent with previous reports (Boulton et al. 1994). Similarly, Fig. 4B demonstrates that zaprinast (open bar; n = 6), added along with coincident submaximal LFS (1 Hz/400 s; solid bar), produced a depression that, though much larger in magnitude, was still fully reversible on drug washout. Using a higher concentration of zaprinast (20 μM), Fig. 4C (n = 8) shows that, even at this concentration, zaprinast-induced depression was still rapidly and completely reversible on drug washout. Thus, the reversibility of zaprinast’s effect is probably not attributable to some threshold level in the [cGMP] achieved. Zaprinast at this concentration (20 μM), paired with submaximal stimulation, also produced only a reversible depression (n = 2, data not shown). Taken together, these experiments indicate that simply elevating [cGMP] alone is insufficient to elicit fully saturated, stable LTD of synaptic strength.

**Simultaneously raising [cyclic GMP] while inhibiting PKA induces LTD**

Studies in our and other laboratories (Boulton et al. 1994; Gage et al. 1997) support a role for cGMP in synaptic depression at CA1 synapses in rat hippocampal slices. Because raising cGMP alone is insufficient to produce a long-lasting depression, but we do find a marked enhancement in the magnitude of LTD when another signaling system (PKA) is inhibited, we hypothesized that these two pharmacological manipulations in concert might supply the factors needed to produce a long-lasting depression of synaptic strength.

In contrast to the effect of zaprinast alone, Fig. 4D illustrates the induction of an apparently saturated LTD when zaprinast (20 μM; solid bar; n = 8) and H-89 (10 μM; open bar) were coapplied. This “chemical LTD” remained stable for ≥2 h following washout of both drugs (−41.8 ± 3.8%, P < 0.05, Student’s t-test, 60 min postapplication compared with preapplication baselines). These data support our hypothesis that these two cyclic nucleotide second messengers mediate opposing biological signals and that simultaneous elevation of [cGMP] and inhibition of PKA is, in fact, **sufficient** to elicit LTD at Schaffer collateral-CA1 synapses.

**Chemical LTD is not associated with alteration in paired-pulse facilitation**

Our intracellular data with postsynaptic infusion of H-89 (Fig. 2B) suggests that the synaptic locus of this effect may be presynaptic. A common, but not universal, feature of a presynaptic site of action is an alteration in paired pulse facilitation (PPF) (Zucker 1989). PPF is an enhancement in the response magnitude to a second stimulus when preceded at a short interstimulus interval (10–100 ms) by a conditioning stimulus. PPF is believed, though not without debate, to result from residual-free Ca2+ in the presynaptic terminal, which is retained near sites of transmitter release for a period of time after the conditioning pulse (Katz and Miledi 1968; but see also Bertram et al. 1996).

We compared PPF ratios before and after the induction of chemical LTD (Fig. 5). The pooled chemical LTD that was elicited is illustrated in Fig. 5A (n = 13) and was similar to previous experiments. Associated PPF was measured at two interstimulus intervals, 50 ms (Fig. 5B; n = 8) and 15 ms (Fig. 5C; n = 5). PPF at either paired-pulse interval was not consistently altered during chemical LTD.
Chemical LTD requires PKG activity

While cGMP can exert its biological effects through a number of effector cascades, it is unclear which of these downstream pathways are required to induce LTD. A primary action of cGMP is the stimulation of PKG, which in many systems is considered the primary intracellular receptor protein for cGMP. Recent data from our laboratory (Reyes and Stanton 1997) demonstrate that PKG activation is necessary for the induction of LTD. This result, together with our present data involving PKA inhibition, led us to hypothesize a role for reciprocal regulation of long-term synaptic plasticity by the two cyclic nucleotide-activated protein kinases. To determine if the LTD observed in H-89 also requires the coincident activation of PKG, we used the selective, cell-permeant PKG inhibitor KT5823. Figure 6A illustrates that, when H-89 and KT5823 (10 and 1 μM respectively; open bar) were co-bath applied 45 min before submaximal LFS (1 Hz/400 s; solid bar), the H-89 unmasking of LTD was blocked completely (−4.3 ± 1.9%; n = 10). This shows that the unmasking of LTD by inhibiting PKA does require the unopposed activity of PKG. Just as significantly, this result also ensured that the LTD-enhancing effect seen with H-89 was, in fact, due exclusively to PKA inhibition and not attributable to cross-inhibition of PKG.

Because LTD in H-89 was prevented by simultaneous addition of KT5823, we investigated whether PKG activity also is needed for chemically induced LTD. Figure 6B illustrates that blockade of PKG could, indeed, prevent the expression of chemical LTD produced by raising [cGMP] while inhibiting PKA (60 min postwash; −6.1 ± 9.7%; n = 4). These data lend strong support to the contention that PKG is the primary physiological target for cyclic GMP generated in response to zaprinast and that PKG activity unopposed by PKA is what is required to yield stable, long-lasting LTD.

Both PKG and PKA function presynaptically to regulate chemical LTD

Before considering possible substrates for PKG, it was first necessary to determine the synaptic locus of PKG activity required for LTD. To address this, we dissolved KT5823 (50 μM) at 50 times the extracellularly effective concentration in 2 M potassium acetate plus 0.5% DMSO and backfilled microelectrodes for intracellular perfusion. Intracellular evoked EPSPs in single CA1 pyramidal neurons were recorded for a baseline period of 45 min to allow KT5823 to leak into the postsynaptic neuron. H-89 (10 μM) then was bath applied alone for 10 min, followed by 30-min bath application of H-89 plus zaprinast (20 μM). Figure 6C illustrates the results of these experiments in which postsynaptic infusion of KT5823 was unable to block the expression of chemical LTD (−43.1 ± 1.2%; n = 6). This result supports the hypothesis that presynaptic, not postsynaptic, PKG is necessary for chemical LTD. Our previous experiment (Fig. 2) suggests a presynaptic locus for PKA inhibition in the enhancement of LTD; however, the site of action of H-89 in the expression of chemical LTD could be different. To answer this question, we backfilled electrodes with H-89 (500 μM) for intracellular perfusion of CA1 pyramidal neurons. After 45 min of infusion and baseline recording, we bath applied zaprinast (20 μM) for 30 min, followed by drug washout. Figure 6D shows that chemical LTD could no longer be elicited when H-89 was applied postsynaptically (−10.5 ± 8.5%; n = 6), indicating that the negative role for PKA important to the induction of LTD is probably also presynaptic.
Chemical LTD is reversed by HFS and occluded by stimulus-evoked LTD, but is activity-independent.

Associated with studies of LTD is an ongoing concern that depression of synaptic potentials may reflect irreversible synaptic damage rather than a reversible physiological phenomenon. Figure 7A demonstrates that, similar to stimulus-induced LTD, chemical LTD can be reversed by the induction of LTP. After inducing stable chemical LTD (−33.3 ± 0.76%), high-frequency stimulation (HFS; theta burst stimulation; 4 trains of 100 Hz/5 pulse burst times 10, interburst interval 200 ms; see Methods) was applied 45 min after drug washout (TBS indicated by arrows), which evoked robust LTP (+208% change from chemically depressed baseline). fEPSPs remained stably potentiated for 1 h, at which time a second TBS was given which resulted in a slight additional potentiation. A third TBS given 45 min later elicited no further potentiation, indicating that LTP was saturated at this level. This demonstration of reversibility argues against irreversible damage underlying chemical LTD.

These data also hint that stimulus-induced and chemical LTD may share some common mechanisms because both can be reversed by the same HFS. We set out to test the hypothesis that these may not be separate and distinct forms of LTD by determining whether saturation of stimulus-induced LTD at one set of synapses could selectively occlude chemical LTD. As shown in Fig. 7B, we alternately stimulated each of two independent Schaffer collateral pathways once every 30 s (S1 and S2) and recorded the evoked fEPSPs in stratum radiatum of field CA1. After the baseline period, the S1 input (solid circles) received repeated LFS trains (4–5 trains, 1 Hz/600 s), spaced 10 min apart, until saturated, homosynaptic LTD was induced. Ten minutes after the last train, we bath applied H-89 (10 μM; open bar) for 30 min, followed by coapplication of zaprinast (20 μM; closed bar). Zaprinast caused equal short-term depression of synaptic potentials, but inhibition of PKG completely blocked chemically induced LTD (each point is the normalized mean ± SE). In contrast, the naive input (S2, open circles) demonstrated chemical LTD that was indistinguishable...
in magnitude from stimulus-induced LTD (−42.0 ± 6.4%, P > 0.20, Student’s t-test comparing S1 to S2). The occlusion of chemical LTD by stimulus-evoked LTD further supports the hypothesis that they share at least some common expression mechanisms.

To determine whether chemical LTD requires any synaptic activity at all for its generation, we performed experiments where all synaptic stimulation was suspended during the period of drug application and not resumed until 30 min postwashout. As shown in Fig. 7C, virtually identical chemical LTD was elicited in the absence of electrical stimulation (−37.1 ± 3.1%, n = 5). To completely silence both evoked and spontaneous synaptic activity, we also performed the same experiment in the presence of the Na⁺ channel blocker TTX (0.5 μM). As Fig. 7D shows, chemical LTD was induced in the presence of TTX, after which all three drugs were washed out. Although considerable time was needed for TTX to completely wash out of these slices (~80 min), once fEPSPs stabilized, the magnitude of LTD was the same as in controls (−36.2 ± 4.3%; n = 4). Thus, chemically induced LTD does appear to converge on some of the same mechanisms as stimulus-induced LTD while bypassing the need for any synaptic stimulation.

**Induction of chemical LTD does not require either NMDA or GABA receptor activation**

An initial trigger for one form of stimulus-induced LTD is activation of the N-methyl-D-aspartate (NMDA) class of glutamate receptors (Dudek and Bear 1992). One possible physiological cascade for raising presynaptic [cGMP] begins with the activation of postsynaptic NMDA receptors, Ca²⁺ influx, and activation of the Ca²⁺/calmodulin-dependent enzyme NO synthase (NOS). NO then diffuses readily across membranes, allowing it to act on neighboring cells and/or presynaptic terminals (Boulton et al. 1994). One of the targets activated by NO is soluble guanylate cyclase (sGC), elevating [cGMP]. It seems likely that chemical LTD, by directly elevating [cGMP], bypasses the need for NMDA receptor activation. To test whether NMDA receptor activation is required for chemical LTD, we attempted to elicit chemical LTD in the presence of the NMDA receptor blocker D-AP5. Figure 8A shows that chemical LTD still could be evoked despite NMDA receptor blockade (50 μM AP5; hatched bar; n = 8). These results demonstrate that effectively raising [cGMP] in response to zaprinast is enough to overcome the need for NMDA receptor-only. 

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**FIG. 7.** Chemically induced LTD is reversed by high-frequency stimulation (HFS), occluded by stimulus-induced LTD, but is activity-independent. A: representative experiment in which repeated trains of high-frequency theta burst stimulation (HFS, see METHODS) were given at 3 time points after drug washout (45, 105, and 150 min postdrug application). HFS reversed chemically induced LTD. Schaffer collateral-evoked EPSPs remained stably potentiated until recording was terminated. B: 2 independent Schaffer collateral pathways (S1 and S2; see inset) were stimulated alternately each 30 s (n = 5). After 30 min of baseline recording, 1 input (S1, closed circles) received repeated trains of LFS (4–5 trains; 1 Hz/600 s) at 10-min intertrain intervals until saturated LTD was evoked. Ten minutes after the last LFS train, H-89 (10 μM; open bar) was bath applied for 30 min; after which time zaprinast (20 μM; solid bar) was coapplied for an additional 30 min. Significant chemical LTD was evoked only at the naive synapses, but not those where stimulus-induced LTD had been saturated (each point is the normalized mean ± SE). C: in a separate group of 5 slices, all electrical stimulation was terminated immediately before drug application (10 μM H-89; 20 μM zaprinast, open and solid bars respectively) and resumed 30 min after drug washout. Expression of chemical LTD was indistinguishable from stimulated slices (each point is the normalized mean ± SE). D: TTX (0.5 μM) was coapplied throughout the period of H-89 addition (10 μM, open bar; n = 4) to block all action potential generation, and electrical stimulation suspended during drug application and the first 30 min of drug washout. Under these conditions, chemical LTD was still elicited (each point is the normalized mean ± SE).
Chemically induced LTD does not require N-methyl-D-aspartate (NMDA) receptor activation or intact GABAergic inhibition. A: NMDA receptor antagonist d-2-amino-5-phosphonopentanoic acid (AP5; 50 μM, hatched bar; n = 7) was bath applied 15 min before H-89. H-89 and zaprinast (open and solid bars respectively) then were applied as previously, in the continued presence of AP5. Chemical LTD still was elicited, despite NMDA receptor activation or intact GABAergic inhibition. A receptor antagonist D-(+)methyl- D-aspartate (AP5; 50 μM) and the GABA B receptor blocker CGP 35348 (1 mM, hatched bar; n = 8) were bath applied 30 min before H-89. These experiments were performed in 4 mM Mg 2+ to reduce hyperexcitability, and area CA1 was isolated by cutting Schaffer collateral axons in stratum radiatum, separating them from CA3 neurons. Chemical LTD still was expressed during GABA receptor blockade. Inset: iEPSPs at the onset of picrotoxin plus CGP 35348 application (1), 20 min in H-89 application (2), and 40 min after washout of all compounds (3) (calibration bars: 2 mV/5 ms; each point is the normalized mean ± SE).

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** Chemically induced LTD does not require intact GABAergic modulation, supporting a direct effect of chemical LTD on monosynaptic excitatory transmission.

**DISCUSSION**

While there has been some progress in elucidating cellular mechanisms underlying LTD, we are far from a complete understanding of either the molecular processes or cognitive function(s) of this form of plasticity. Interestingly, many of the same second-messenger cascades appear to be involved in both LTP and LTD, leading one to question where the critical points of divergence in the two phenomena may be. Here, we supply the first evidence of reciprocal regulation of LTD by cyclic GMP-(LTD enhancing) and cyclic AMP-(LTD suppressing) dependent protein kinases in presynaptic nerve terminals.

The fact that PKA plays an important role in the persistence of LTP is well established (Frey et al. 1993; Hopkins and Johnston 1988; Stanton and Sarvey 1985a,b; Stanton et al. 1989). If PKA-mediated phosphorylation of protein substrates favors the expression of LTP, we predicted that inhibition of PKA should shift phosphorylation states in favor of LTD. In this study, we demonstrate a marked enhancement of the magnitude of LTD when PKA is inhibited. This result is in contrast to the observations of Brandon et al. (1995), who reported a complete block of LTD in control slices by PKA inhibition. The reasons for these differences are unclear. One explanation could be that effects of PKA blockade may be age and/or genus dependent. In our study, we used juvenile rats (14–21 days), whereas older mice (4–6 wk) were used by Brandon et al. (1995).

Intracellular infusion of H-89 into postsynaptic pyramidal neurons produced far less enhancement of LTD than bath application, suggesting that the effect involves, at least in part, a presynaptic site of action. However, because the delivery of substances from sharp microelectrodes is difficult to ensure, we used a 50-fold higher concentration of H-89 than for bath application and allowed ample diffusion time (≈45 min) before stimulation. We previously have found these methods to be effective in achieving intracellular activity (Reyes and Stanton 1996; Stanton and Gage 1996), but we further verified the presence of inhibition directly by testing the effect of H-89 infusion on a well-characterized postsynaptic PKA-dependent response, suppression of the AHP (Madison and Nicoll 1986). In control cells, PKA-dependent inhibition of the AHP by the adenylate cyclase stimulant forskolin was complete. Forskolin’s block was reduced >80%, but not completely prevented, in cells impaled with electrodes containing 500 μM H-89.

Although postsynaptic inhibition of H89 as well as KT5823 support a presynaptic site for LTD, our PPF data does not. PKP is believed to reflect enhanced transmitter release due to residual presynaptic [Ca2+]i, fostering the idea that manipulations that change release probability should alter PPF. It is more questionable whether PPF alterations are a sensitive measure of presynaptic plasticity. Early field potential studies in field CA1 failed to show changes in PPF after induction of LTP (Muller and Lynch 1989), whereas some later studies have reported significant decreases in average PPF ratio (Kleschevnikov et al. 1997). It has been suggested that intracellular EPSPs may be more accurate in assessing PPF because they are less contamin...
inated by polysynaptic events or population action potentials. Unfortunately, intracellular results have proven just as equivocal; some showing changes during LTP (Voronin and Kuhnt 1990) and LTD (Bolshakov and Sieglebaum 1994), others do not (Hjelmstad et al. 1997; Manabe et al. 1993). Interestingly, Bertram et al. (1996) recently supplied support for an alternative model of PPF that, by depending on Ca<sup>2+</sup> binding domains directly on release apparatus proteins, could allow for LTP and LTD in the presynaptic terminal that would not alter PPF. Our data may point to presynaptic targets insensitive to PPF, or damage the presynaptic hypothesis. Other methods of evaluating transmitter release will be needed to resolve this question.

The actions of zantrapin appear somewhat different from those of the NO donor, S-nitroso-N-acetyl penicillamine (SNAP). We previously have observed that combining submaximal LFS with SNAP elicits a robust, stable LTD (Gage et al. 1997; Reyes-Harde et al. 1999b). In the present study, zantrapin plus submaximal LFS produced only reversible depression, implying that SNAP, and, hence, NO, possess additional properties that zantrapin does not. Although nonspecific actions of SNAP are possible, the concentration we used (100 μM) is below those reported to act directly on sulfhydryl moieties. An alternative explanation could involve differences in subcellular localization of PDE, PKA, and PKG. De Vente et al. (1996) have shown that both the magnitude and localization of NO-mediated [cGMP] accumulation in hippocampus is influenced by isozyme-specific PDE inhibition. Kinase activity can be restricted spatially, at least for PKA (for review, see Coghlan et al. 1993) and also possibly for PKG (Vo et al. 1998). Shakur et al. (1993, 1995) have shown membrane compartmentalization of a specific PDE4 isofrom, while Whalin et al. (1988a,b) have shown the same for cyclic GMP-stimulated PDE2. In pyramidal cells, it is possible that inhibiting PDE V might cause local increases in [cGMP] that activate only a subset of the substrates necessary for LTD. In contrast, NO-activated GC may either activate a different pool of cyclic GMP or PKG-dependent events and/or stimulate PDE2-mediated hydrolysis of cAMP.

Perhaps of greatest interest, our investigations have culminated in the discovery of conditions that are both necessary and sufficient to elicit sustained depression of synaptic efficacy that completely bypasses the requirement for electrical stimulation, NMDA, or GABA receptor activation. Interestingly, in the dentate gyrus, Wu et al. (1998) recently have reported a long-lasting depression of synaptic strength produced by zantrapin alone, which was, however, activity-dependent and required metabotropic glutamate receptor (mGluR) activation. Group II mGluRs are coupled negatively to adenylate cyclase, providing a potential means of lowering [cAMP]. It is tempting to speculate that group II mGluRs might effectively serve the same purpose for LTD in the dentate as H-89 does in our chemical LTD in CA1.

The demonstration that saturating stimulus-induced LTD occludes chemically induced LTD indicates that these two forms of synaptic depression either act via the same mechanisms or, at some level, converge on shared pathways. Previous studies in our laboratory have shown that stimulus-induced LTD requires PKG activity, and the demonstration here that the PKG inhibitor KT5823 blocked chemically induced LTD provides additional support for a convergence. In earlier studies, we showed that presynaptic ryanodine-sensitive Ca<sup>2+</sup> stores (Reyes and Stanton 1996) and activation of presynaptic Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (Stanton and Gage 1996) are both needed to induce LTD. We recently have found that antagonists of the cGMP-stimulated messenger cyclic ADP ribose, which releases calcium from stores in sea urchin eggs (Galione et al. 1991), also can block the induction of LTD (Reyes-Harde et al. 1999a,b). The role of this cascade in chemical LTD, and how it modulates glutamate release, remain to be determined.

In contrast to chemical LTD, the initial reversible depression induced by zantrapin was not blocked by KT5823. Boulton et al. (1994) reported this action of zantrapin to be presynaptic, suggesting there must be a cyclic GMP-mediated mechanism...
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for depressing transmitter release that is PKG-independent. Based on the literature, we propose the following. Elevation of [cGMP] activates cyclic GMP-stimulated (type II) PDE, which hydrolyzes cyclic AMP and reduces a tonic cyclic AMP-mediated enhancement of glutamate release. Studies supporting this include demonstrations by Doerner and Alger (1988) that cyclic GMP can depress hippocampal Ca\(^{2+}\) currents through a PKG-independent mechanism and by Broome et al. (1994), who found that activation of presynaptic A1 adenosine receptors, which tonically reduce [cyclic AMP] and suppress glutamate release, is necessary for zanadin’s actions. Another possible target is a cyclic nucleotide-gated cation channel in hippocampus whose tonic activation requires cyclic AMP (Bradley et al. 1997). Whether reversible cyclic GMP-mediated depression is distinct from LTD, or PKG just adds phosphorylation events that increase the duration of this existing mechanism, is unknown.

There are multiple mechanisms whereby PKG and PKA might affect glutamate release (see Fig. 9). Both kinases can phosphorylate presynaptic channels known to alter transmitter release. PKA can enhance glutamate release by phosphorylating either presynaptic Ca\(^{2+}\) channels (Helli et al. 1995) or presynaptic kainate receptors (Glur6) (Chittajallu et al. 1996). Conversely, PKC can directly phosphorylate and open K\(^{+}\) channels in hippocampal neurons (Furukawa et al. 1996) and indirectly activate protein phosphatase 2A, which opens K\(^{+}\) channels in pituitary tumor cells (White et al. 1993). Simultaneously reducing Ca\(^{2+}\) conductance through PKA inhibition and activating K\(^{+}\) channels via PKG could elicit marked reductions in transmitter release.

Phosphorylation of synaptic vesicle proteins provide another way kinases might influence transmitter release. An attractive, but untested, hypothesis is that PKG may phosphorylate and downregulate a protein vital to synaptic vesicle mobilization (Sistiaga et al. 1997). Wang and Robinson (1995) have shown the existence of >40 PKG substrate proteins that remain uncharacterized and of unknown function. Several proteins involved in vesicle transport have been identified as potential presynaptic targets for PKA phosphorylation in vitro. These include synapsin1, \(\infty\)-SNAP (\(\infty\) soluble NSF attachment protein), and raphelin 3A. Injection of dephosphorylated synapsin1 into squid axons inhibits transmitter release (Llinas et al. 1985), but phosphorylated synapsin has no converse effect, and synapsin1 knockout mice show no deficits in LTP (Spillane et al. 1995). Phosphorylation of raphelin 3A is required for interaction with another protein, rab3A, allowing its association with the fusion apparatus and recruitment of vesicles for exocytosis (Fykse et al. 1995). Interestingly, in rab3A knockout mice all electrophysiological parameters are normal in area CA1 except for an increase in synaptic depression evoked by short stimuli (Geppert et al. 1994). In vivo phosphorylation of these proteins has yet to be shown, leaving the physiological relevance of in vitro studies uncertain.

Recent studies (Kameyama et al. 1998; Lee et al. 1998) describe another chemical means for inducing LTD that used a brief (3 min) bath application of NMDA to hippocampal slices. It was suggested that this form of LTD is critically dependent on selective dephosphorylation of postsynaptic GluR1 AMPA receptor subunits at a PKA-sensitive site and shares mechanisms with stimulus-induced LTD. It is becoming clear that there are multiple mechanistically independent forms of LTD (Bolshakov and Siegelbaum 1994; Gage et al. 1997; Oliet et al. 1997). Our chemical LTD is activity-independent, bypasses the need for NMDA-receptor activation, and appears to depend on presynaptic PKG activation and PKA inhibition. Therefore, we hypothesize that our chemical LTD activates a presynaptic, cyclic-GMP- and PKG-dependent form of LTD, whereas that described by Lee et al. (1998) is a postsynaptic, dephosphorylation-mediated form.

In summary, our data support the hypothesis that in the induction of homosynaptic LTD, cyclic GMP, acting via PKG, is involved in promoting cellular events that are antagonistic to those mediated by activating PKA. The notion that these two cyclic nucleotide second-messenger systems mediate opposing biological signals has been demonstrated in many other tissues. The idea that a specific form of synaptic plasticity depends on the bidirectional control of kinase activity presents an attractive “push-pull” model for the amplification of signal transduction cascades. In this regard, chemically induced LTD should prove useful for the study of the biochemical cascades underlying stimulus-induced LTD.


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