Nitric Oxide Signaling Contributes to Late-Phase LTP and CREB Phosphorylation in the Hippocampus

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Long-term potentiation (LTP) in the hippocampus has an early phase (E-LTP) that can be induced by one- or two-train tetanization, lasts ~1 hr, and is cAMP-dependent protein kinase (PKA) and protein synthesis independent and a late phase (L-LTP) that can be induced by three- or four-train tetanization, lasts >3 hr, and is reduced by inhibitors of PKA and of protein or RNA synthesis. Nitric oxide (NO) is thought to be involved in E-LTP, but until now there has been no information about the role of the NO-signaling pathway in L-LTP. We examined this question at the Schaffer collateral–CA1 synapses in slices of mouse hippocampus. An inhibitor of NO synthase blocked L-LTP induced by three-train tetanization and reduced L-LTP induced by four-train tetanization, whereas an inhibitor of PKA was more effective in blocking four-train L-LTP than three-train L-LTP. Three-train L-LTP was also blocked by inhibitors of guanylyl cyclase or cGMP-dependent protein kinase (PKG).

Conversely, either NO or cGMP analogs paired with one-train tetanization produced late-phase potentiation, and the cGMP-induced potentiation was blocked by inhibitors of protein or RNA synthesis and an inhibitor of PKG, but not by an inhibitor of PKA. To test a possible downstream target of PKG, we examined changes in phospho-CRE- binding protein (phospho-CREB) immunofluorescence in the CA1 cell body area and obtained results similar to those of the electrophysiology experiments. These results suggest that NO contributes to L-LTP by stimulating guanylyl cyclase and cGMP-dependent protein kinase, which acts in parallel with PKA to increase phosphorylation of the transcription factor CREB.

Key words: nitric oxide; guanylyl cyclase; cGMP-dependent protein kinase; long-term potentiation; CREB; hippocampus

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MATERIALS AND METHODS

Male C57BL6 mice aged 6–9 weeks were housed and killed in accordance with the guidelines of the Health Sciences Division of Columbia University. The brain was quickly removed and immersed in ice-cold...
artificial CSF (ACSF) bubbled with a gas mixture of 95% O2 and 5% CO2, the hippocampus was dissected, and 400 μm transverse slices were prepared. The slices were incubated in an interface recording chamber maintained at 28.5 ± 0.5°C for at least 1.5 hr before recording and were constantly superfused with gas-saturated ACSF at 1–1.5 ml/min. The composition of the ACSF was as follows: NaCl, 124 mM; KCl, 4.4 mM; CaCl2, 2.5 mM; MgSO4, 1.3 mM; Na2HPO4, 1 mM; NaHCO3, 26 mM; and glucose, 10 mM.

Electrophysiological experiments. To record the field EPSP, a glass micropipette filled with ACSF (1–5 MΩ resistance) was placed in the stratum radiatum of the CA1 region, and a bipolar tungsten-stimulating electrode was placed along the Schaffer collateral fibers. In two-pathway experiments, two stimulating electrodes were placed on opposite sides of the recording electrode, and stimulation from the two electrodes was delivered alternately. The intensity of the stimulation was adjusted to produce an EPSP with a slope that was ~35% of maximum. The test stimulation was delivered once per minute (0.017 Hz). For inducing LTP, either single or multiple trains of stimulation at 100 Hz for 1 sec were delivered at the same intensity as the test stimulation. In the experiments using picrotoxin, the CA3 region was surgically removed from the slice, and the ACSF was adjusted by increasing both CaCl2 and MgSO4 to 4 mM to reduce seizure activity.

NO solution was prepared as described previously (Zhuo et al., 1993). Briefly, NO gas was bubbled to saturation in helium-saturated distilled water and then diluted to 0.1–1.0 μM in ACSF containing 30 units/ml superoxide dismutase. The NO solution was prepared immediately before use and injected directly into the recording chamber.

The following drugs were used: 8-bromo-cGMP (8-Br-cGMP), 8-(4-chlorophenylthio)-cGMP (8-CPPT-cGMP), β-phenyl-1,2,4-oxadiazolo[4,3-α]quinolin-1-one (ODQ) from Alexis; and U0126 from Research Biochemicals. The drug solutions were prepared as stock solutions and further diluted in ACSF immediately before application. Rp-8-Bromo-PET-cGMPS, and Sp-adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS) from BIolog Life; KT5823 and KT5720 from Calbiochem (La Jolla, CA); aminosyn, actinomycin D, N-nitro-l-arginine, and picrotoxin from Sigma (St. Louis, MO); 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODO) from Alexis; and U0126 from Research Biochemicals. The drug solutions were prepared as stock solutions and further diluted in ACSF immediately before application. Rp-8-Bromo-PET-cGMPS, Sp-cAMPS, KT5823, KT5720, ODQ, and U0126 were prepared in DMSO, and actinomycin D was prepared in ethanol. The final concentration of the DMSO or ethanol was 0.1%.

Data are shown as mean (± SEM) percent of the baseline EPSP slope. Data were analyzed using either t tests to compare two conditions or ANOVA followed by planned comparisons of multiple conditions, and p < 0.05 was considered significant.

Immunocytochemical experiments. Hippocampal slices were prepared and treated with tetanic stimulation and/or drugs exactly as described in the electrophysiological experiments. Either 1 or 60 min after the treatment, the slices were rapidly immersed in ice-cold 4% paraformaldehyde in PBS, pH 7.4, and fixed for 60 min. The slices were then washed three times in PBS, permeabilized in 0.3% Triton X-100 in PBS for 60 min, and then washed three times in PBS again. The free aldehydes were quenched in 50 mM ammonium chloride in PBS for 20 min. Nonspecific antibody binding was blocked by incubation in 10% goat serum in PBS for 60 min at room temperature. The slices were then incubated with primary antibody, rabbit polyclonal anti-phospho-CREB (Upstate Biotechnology, Lake Placid, NY), diluted 1:100 in 10% goat serum in PBS at 4°C for 36 hr. This antibody is thought to be relatively selective for phospho-CREB, although it may have some cross-reactivity with the related molecules CRE modulator (CREM) and activating transcription factor 1 (ATF-1) (Ginty et al., 1993). The slices were then washed six times in PBS, for 2 hr each time. The slices were then washed in PBS six times, for 2 hr each time.

The slices were viewed using a Bio-Rad (Hercules, CA) MRC1000 laser confocal-scanning system coupled to a Zeiss Axiovert 100 inverted microscope. Images were taken using a 5×, 0.25 numerical aperture (NA) or a 40×, 0.75 NA water immersion objective. Kalman averages of five scans were collected for each image. The mean pixel intensity in the CA1 cell body area and in an apical dendritic area of CA3 that was relatively free of cell bodies was determined using Bio-Rad Comos software. The ratio of intensities in the two areas was determined in each slice to normalize for differences in background fluorescence. These values were in turn normalized to the values obtained from untreated control slices from the same animal. All data are presented as mean (± SEM) percent of control. The experimental data were analyzed by a two-way ANOVA (treatment and time) followed by planned comparisons of individual conditions. The specificity of the immunofluorescence was confirmed by omitting the primary antibody, which resulted in a significant reduction in fluorescence intensity.

RESULTS

NO signaling is involved in L-LTP

Previous studies have shown that four trains of tetanic stimulation can induce long-lasting LTP (L-LTP) that is dependent on PKA in the CA1 region of mouse hippocampus (Abel et al., 1997; Winder et al., 1998). We first replicated those studies and found that four trains of 100 Hz/1 sec stimulation induced stable long-lasting LTP (the EPSP slope was 227 ± 17% of baseline 3 hr after the end of tetanization; n = 5; Fig. 1A) that was almost completely blocked by KT5720 (1 μM), an inhibitor of PKA [117 ± 7% at 3 hr; n = 6; F(1,28) = 30.89; p < 0.01 compared with normal saline; Fig. 1B]. In agreement with previous studies, KT5720 also reduced an intermediate phase of LTP that is expressed within the first hour after multiple-train tetanization and is mechanistically distinct from both E-LTP and L-LTP (Blitzer et al., 1995, 1998; Winder et al., 1998). We then tested the effect of N′-nitro-l-arginine (NO-Arg), an inhibitor of nitric oxide synthase, NO-Arg (100 μM) applied at least 60 min before tetanic stimulation and throughout the experiment reduced L-LTP by four-train tetanization but did not completely block it (147 ± 10% at 3 hr; n = 6; Fig. 1A). These results are consistent with those of Zhuo et al. (1998), who found that although NO-Arg completely blocked E-LTP by one-train tetanization and significantly reduced E-LTP by two-train tetanization, it only partially reduced LTP 60 min after four-train tetanization.

We next tested whether PKA and the NO-signaling pathway might contribute differently to L-LTP induced with a weaker stimulation protocol. Three trains of 100 Hz/1 sec stimulation with 5 min between trains induced stable L-LTP that was slightly smaller than four-train L-LTP (193 ± 21% at 3 hr; n = 6; Fig. 1C). KT5720 reduced this L-LTP but did not completely block it (138 ± 17%; n = 5; Fig. 1D), suggesting that three-train L-LTP has a PKA-independent component. By contrast, NO-Arg almost completely blocked three-train L-LTP [116 ± 6% at 3 hr; n = 6; F(1,28) = 16.28; p < 0.01 compared with normal saline; Fig. 1C]. The results with KT5720 and NO-Arg with three-train tetanization were the reverse of those with four-train tetanization, as indicated by a significant drug × train number interaction in a two-way ANOVA [F(1,19) = 6.67; p < 0.05]. These results suggest that NO signaling and PKA contribute preferentially to somewhat different components of L-LTP, with NO contributing importantly to three-train L-LTP and to a lesser extent to four-train L-LTP.

Previous studies have shown that NO paired with a weak tetanus (50 Hz/0.5 sec) induced LTP that lasted for at least 60 min (Zhuo et al., 1993). To test whether NO also contributes to the induction of L-LTP, we used a modified protocol in which we paired NO with a single 100 Hz/1 sec train of stimulation. Stimulation alone (100 Hz/1 sec) produced early-phase LTP (130 ± 14% at 60 min; n = 5) but not L-LTP (114 ± 9% at 3 hr). However, when NO was paired with one-train tetanization, stable late-phase potentiation was obtained [163 ± 15% at 3 hr; n = 4; t(3) = 4.20; p < 0.05; t(7) = 3.21; p < 0.05 compared with one-train alone; Fig. 2A]. These results, together with the inhibitory effect of NO-Arg on L-LTP induced by multiple-train tet-
Inhibitors of NOS or PKA preferentially reduce L-LTP induced by different stimulation protocols. A, L-LTP induced by four trains of 100 Hz/1 sec tetanization (arrows) was reduced but not completely blocked by the NO synthase inhibitor NO-Arg (100 μM). B, Four-train L-LTP was blocked by the PKA inhibitor KT5720 (1 μM). C, Three trains of 100 Hz/1 sec tetanization with 5 min between trains induced stable L-LTP that was blocked by NO-Arg. NO-Arg was applied at least 1 hr before the tetanic stimulation and throughout the experiment. Insets, Representative field EPSPs before and 3 hr after tetanic stimulation are shown. Calibration: 5 msec, 1 mV. D, Three-train L-LTP was reduced but not completely blocked by KT5720.

These results show that guanylyl cyclase is involved in NO-induced late-phase potentiation.

Because NO-induced potentiation is thought to be produced in part by activation of guanylyl cyclase resulting in the production of cGMP, we predicted that exogenous application of cGMP analogs should produce the same effect as NO. We tested this idea by using 8-Br-cGMP, a membrane-permeable cGMP analog that is resistant to degradation by phosphodiesterases. We used a low concentration of 8-Br-cGMP (1 μM) that should specifically activate cGMP-dependent protein kinase (PKG) but not PKA because the $K_a$ of 8-Br-cGMP is 0.01–0.21 μM for PKG and 12 μM for PKA (Butt et al., 1992; Sekhar et al., 1992). Perfusion with 8-Br-cGMP for 10 min before a one-train tetanus produced robust late-phase potentiation [180 ± 18% at 3 hr; $n = 9$; $t(8) = 4.44$; $p < 0.01$; $t(12) = 2.62$; $p < 0.05$ compared with perfusion with normal saline; Fig. 3C]. Perfusion of the slice with 8-Br-cGMP alone had no effect on the baseline EPSP (92 ± 8% at 3 hr; $n = 3$). We also tested 8-pCPT-cGMP, another cGMP analog that has greater membrane permeability and is also more selective for stimulating PKG as opposed to other cGMP targets such as cGMP-stimulated phosphodiesterases (Geiger et al., 1992). The $K_a$ of 8-pCPT-cGMP is 0.04–0.44 μM for PKG and 7.0 μM for PKA (Butt et al., 1992; Sekhar et al., 1992). 8-pCPT-cGMP (1 μM) paired with a one-train tetanus produced robust late-phase potentiation [208 ± 13% at 3 hr; $n = 3$; $t(2) = 8.31$; $p < 0.05$], whereas 8-pCPT-cGMP alone had no effect on the baseline EPSP (86 ± 3%; $n = 3$; Fig. 3D).

The enhanced potentiation by cGMP analogs paired with one-train tetanus might simply result from increased depolarization of L-LTP.
the postsynaptic cells during the tetanus, perhaps because of disinhibition of GABAergic interneurons (Wexler et al., 1998). However, Son et al. (1998) found no effect of 8-Br-cGMP on the field EPSP during tetanic stimulation. As another way to examine this possibility, we tested the effect of 8-Br-cGMP during blockade of GABAergic inhibition. In the presence of the GABA antagonist picrotoxin (100 μM), 8-Br-cGMP paired with one-train tetanization still produced late-phase potentiation [176 ± 5% at 3 hr; n = 3; t(2) = 15.20; p < 0.01]. Picrotoxin itself had no detectable effect on potentiation induced by one-train tetanization (109 ± 8% at 3 hr; n = 3).

If late-phase potentiation induced by cGMP analogs paired with one-train tetanization shares mechanisms with L-LTP induced by three-train tetanization, it was interesting to know whether cGMP-induced late-phase potentiation is also protein and RNA synthesis dependent. Replicating previous results (Huang and Kandel, 1994), we found that L-LTP induced by three-train tetanization was blocked by preincubation with the translational inhibitor anisomycin (30 μM) for 30 min [128 ± 5%; n = 4; t(8) = 2.47; p < 0.05 compared with normal saline]. Similarly, after perfusion with anisomycin, 8-Br-cGMP paired with one-train tetanization produced fairly normal early-phase potentiation (152 ± 10% at 1 hr) but reduced late-phase potentiation [123 ± 8% at 3 hr; n = 7; t(14) = 2.46; p < 0.05 compared with normal saline; Fig. 5A]. Anisomycin had no effect on the baseline EPSP (95 ± 12% at 3 hr; n = 2). Although anisomycin could have additional molecular actions, these results suggest that late-phase potentiation induced by cGMP is dependent on protein synthesis.

If protein synthesis is required for the expression of late-phase potentiation, the synthesis might occur in the soma of the neurons, but it could also occur at the synapses. For example, in Aplysia some of the new protein necessary for long-term facilitation was found to be synthesized at the synapses by local mRNA transduction.
(Martin et al., 1997a). In the hippocampus, a BDNF-induced enhancement of synaptic transmission was also shown to be dependent on local protein synthesis and was still present after removal of both the pre- and postsynaptic cell bodies (Kang and Schuman, 1996). Therefore, the inhibitory effect of anisomycin may be produced by blockade of the translational mechanism at the cell body and/or synapse. To distinguish between these possibilities and as an additional test of the involvement of macro-molecular synthesis, we examined the effect of the transcriptional inhibitor actinomycin D (40 μM) and found that it also reduced late-phase potentiation induced by 8-Br-cGMP paired with one-train tetanus [122 ± 14%; n = 6; t(13) = 2.19; p < 0.05 compared with normal saline; Fig. 5B]. These results indicate that like L-LTP produced by multiple-train tetanization, cGMP-induced potentiation is protein and RNA synthesis dependent.

**Relationship of the PKG- and PKA-signaling pathways in L-LTP**

A number of studies have demonstrated the role of PKA in L-LTP (Frey et al., 1993; Huang and Kandel, 1994; Abel et al.,...
In the present study we have shown that the PKG-signaling pathway also appears to be involved in L-LTP by using cGMP analogs (8-Br-cGMP and 8-pCPT-cGMP) and PKG inhibitors (KT5823 and Rp-8-Br-PET-cGMPS) at relatively low concentrations that were chosen to selectively affect PKG but not PKA. Therefore, it was interesting to investigate the relationship of PKA and PKG in the induction of L-LTP.

One possibility is that cGMP and PKG produce late-phase potentiation by stimulating PKA. Consistent with this possibility, the type 1 regulatory subunit of PKA (PKAR1) is a substrate for PKG (Geahlen and Krebs, 1980), and phosphorylation of PKAR1 by PKG results in loss of inhibition of the catalytic subunit of PKA \(_{\text{in vitro}}\), although it is not clear whether this also happens \(_{\text{in vivo}}\) (Geahlen et al., 1981). However, the finding that PKG and PKA are preferentially involved in three- and four-train L-LTP, respectively (Figs. 1, 4), argues against this possibility. As an additional test of this idea, we examined the effect of a PKA inhibitor on cGMP-induced potentiation. Preincubation with the PKA inhibitor KT5720 (1 µM) produced only a small reduction of late-phase potentiation by 8-Br-cGMP paired with one-train tetanus (158 ± 11%; \(n = 3\); Fig. 6A). This result suggests that cGMP does not act by stimulating PKA during the induction of L-LTP. As a positive control for the effectiveness of the drug, we found that the same concentration of KT5720 (1 µM) completely blocked the long-lasting potentiation of synaptic transmission produced by the PKA agonist Sp-cAMPS (100 µM) (Fig. 6B).

If PKG and PKA activate independent pathways initially but converge at some later step during the induction of L-LTP, activating one pathway should occlude potentiation induced by the other pathway. To test this possibility, we first applied the

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**Figure 4.** Effect of PKG inhibitors on L-LTP. **A,** L-LTP induced by three-train tetanization was blocked by the PKG inhibitors KT5823 (2 µM) or Rp-8-Br-PET-cGMPS (1 µM). The drugs were applied 30 min before until 20 min after the tetanus, as indicated by the horizontal bar. **B,** L-LTP induced by four-train tetanization was reduced but not completely blocked by Rp-8-Br-PET-cGMPS. **C,** Late-phase potentiation induced by 8-Br-cGMP (1 µM) paired with one-train tetanization was also blocked by KT5823 (2 µM).

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**Figure 5.** cGMP analog-induced late-phase potentiation is dependent on macromolecular synthesis. **A,** Late-phase potentiation induced by 8-Br-cGMP (1 µM) paired with one-train tetanization was blocked by the protein synthesis inhibitor anisomycin (30 µM), whereas the baseline EPSP was not affected by anisomycin. Anisomycin was applied from 30 min before until 25 min after the tetanus. **B,** Late-phase potentiation induced by 8-Br-cGMP (1 µM) paired with one-train tetanization was also blocked by the transcriptional inhibitor actinomycin D (ATD; 40 µM). Actinomycin D was applied from 30 min before until 25 min after the tetanus.
PKA activator Sp-cAMPS (100 μM) for 15 min. In agreement with previous studies (Frey et al., 1993; Nguyen et al., 1994; Winder et al., 1998), Sp-cAMPS produced inhibition during the application, followed by long-lasting potentiation after washout of the drug [135 ± 5% 90 min after application of Sp-cAMPS; n = 3; t(2) = 7.00; p < .05; Fig. 6C]. The test stimulation intensity was then reduced to obtain a new baseline similar to that before Sp-cAMPS application. Under this condition, 8-Br-cGMP paired with one-train tetanization induced early-phase potentiation but no late-phase potentiation [106 ± 3% 3 hr after the tetanus; n = 3; t(10) = 2.57; p < 0.05 compared with normal 8-Br-cGMP-induced potentiation]. This result suggests that PKG and PKA share some common downstream mechanism during the induction of L-LTP.

Together with the finding that a PKA inhibitor did not block cGMP-induced potentiation, these results suggest that PKG and PKA activate independent pathways initially, but their signaling pathways merge at some later step. The evidence that both PKG- and PKA-induced late-phase potentiation are blocked by inhibitors of protein and RNA synthesis (Fig. 5) (Frey et al., 1993) also suggests that they might share some of the same mechanisms.

cGMP-induced late-phase potentiation is accompanied by CREB phosphorylation

The late, protein synthesis-dependent phase of LTP is thought to involve induction of immediate early genes via phosphorylation of CREB, mediated in part via PKA (Bouritchoaldze et al., 1994; Impy et al., 1996, 1998; Matthies et al., 1997; Gass et al., 1998). Previous studies in other systems have shown that NO is also involved in gene expression associated with activation of CREB (Peunova and Enlkolopov, 1993; Ohki et al., 1995; Ding et al., 1997) and that cGMP and PKG can trigger gene induction via CREB phosphorylation (Haby et al., 1994; Gudi et al., 1996, 1997). We therefore investigated whether PKG might also cause CREB phosphorylation during the induction of late-phase potentiation by examining phospho-CREB immunofluorescence in hippocampal slices that had received the same treatments described in the electrophysiological experiments. The slices were fixed either 1 or 60 min after the treatments, stained with an antibody for CREB phosphorylated at Ser-133, and viewed on a confocal microscope.

We first examined phospho-CREB immunofluorescence after either four- or three-train tetanization (Fig. 7). One minute after the end of four-train tetanization, the intensity of immunofluorescence in the CA1 cell body area was significantly increased compared with that in untreated control slices from the same animals [187 ± 12% of control; n = 7; F(1,71) = 93.63; p < 0.01]. The increase in phospho-CREB immunofluorescence was almost completely blocked by an inhibitor of PKA, KT5720 [112 ± 7%; n = 7; F(1,71) = 35.15; p < 0.01 compared with no inhibitor], and was also significantly reduced but not completely blocked by an inhibitor of PKG, KT5823 [132 ± 8%; n = 7; F(1,71) = 12.33; p < 0.01 compared with control; F(1,71) = 19.00; p < 0.01 compared with no inhibitor]. One minute after the end of three-train tetanization, there was also a significant increase in phospho-CREB immunofluorescence that was somewhat smaller than that after four-train tetanization [148 ± 8%; n = 8; F(1,71) = 32.96; p < 0.01 compared with control; F(1,71) = 9.89; p < 0.01 compared with four-train]. However, unlike the increase in immunofluorescence after four-train tetanization, the increase after three-train tetanization was not reduced by an inhibitor of PKA, KT5720 (156 ± 5%; n = 6), but it was almost completely blocked by an inhibitor of PKG, KT5823 [112 ± 5%; n = 6; F(1,71) = 8.16; p < 0.01 compared with no inhibitor]. Thus, like L-LTP by three- and four-train tetanization (Figs. 1, 4), the increases in immunofluorescence after three- and four-train tetanization were affected differently by inhibitors of PKA and PKG [drug × train number interaction, F(1,22) = 18.78; p < 0.01]. These results suggest that

Figure 6. Relationship between PKG and PKA in the induction of late-phase potentiation. A, Late-phase potentiation induced by 8-Br-cGMP paired with one-train tetanization was not significantly reduced by the PKA inhibitor KT5720 (1 μM). KT5720 was applied from 30 min before until 20 min after the tetanus. B, KT5720 (1 μM) blocked enhancement of synaptic transmission by the PKA activator Sp-cAMPS (100 μM). C, Potentiation induced by Sp-cAMPS (100 μM) occluded late-phase potentiation by 8-Br-cGMP paired with one-train tetanus. Ninety minutes after Sp-cAMPS-induced potentiation, the stimulation intensity was reduced to obtain a new baseline similar to that at the beginning of the experiment, and then 8-Br-cGMP was applied paired with one-train tetanus.
PKA and PKG contribute preferentially to somewhat different components of CREB phosphorylation, with PKA contributing more importantly than PKG with four-train tetanization and PKG contributing more importantly than PKA with three-train tetanization.

The similarity between the results on phospho-CREB immunofluorescence and L-LTP supports the idea that CREB phosphorylation is a key step in the induction of the potentiation. However, there was one case in which the two sets of results were not parallel: with three-train tetanization, an inhibitor of PKA significantly reduced L-LTP (Fig. 1D) but had no effect on the increase in phospho-CREB immunofluorescence. This result suggests that PKA may play some role in L-LTP in addition to CREB phosphorylation, perhaps by acting on another transcription factor or a coactivator necessary for transcription.

Sixty minutes after these treatments CREB phosphorylation was very similar to that seen 1 min after the treatments, with one exception: the increase in immunofluorescence after four-train tetanization was significantly less at 60 min than at 1 min \( [F(1,71) = 15.29; p < 0.01] \) in a two-way ANOVA (group and time). In subsequent planned comparisons, \( *p < 0.05 \) compared with control slices; \( #p < 0.05 \) compared with slices with no inhibitor.

although it was still significantly greater than control \( [F(1,71) = 41.37; p < 0.01] \). There was no such decline in phospho-CREB immunofluorescence after three-train tetanization, suggesting that these two different training protocols may also produce temporally different patterns of CREB phosphorylation.

We next examined phospho-CREB immunofluorescence after treatments with cyclic nucleotide analogs (Fig. 8). There was a significant increase in immunofluorescence 1 min after treatment with 8-Br-cGMP paired with 1 train tetanization \( [169 \pm 14%; n = 7; F_{(1,115)} = 44.76; p < 0.01] \). By contrast, there was no significant change in immunofluorescence after treatment with one-train tetanization alone \( (97 \pm 8%; n = 6) \) or 8-Br-cGMP alone \( (109 \pm 6%; n = 9) \). The increase in immunofluorescence by 8-Br-cGMP paired with one-train tetanization was significantly reduced by an inhibitor of PKG, KT5823 \( [104 \pm 8%; n = 7; F_{(1,115)} = 19.77; p < 0.01 \) compared with no inhibitor], but not by an inhibitor of PKA, KT5720 \( (179 \pm 11%; n = 8) \). There was also a significant increase in immunofluorescence 1 min after treatment with the cAMP analog Sp-cAMPS \( [213 \pm 25%; n = 6; F_{(1,115)} = 101.03; p < 0.01] \) that was significantly reduced by the
PKA inhibitor KT5720 [124 ± 10%; n = 6; \( F_{(1,115)} = 31.34; p < 0.01 \) compared with no inhibitor]. In all of these cases the results on CREB phosphorylation were similar to the electrophysiological results on late-phase potentiation (Figs. 4C, 6A, B) and support the idea that PKG and PKA act in parallel to phosphorylate CREB during the induction of L-LTP.

Recent evidence indicates that MAP kinase also plays an important role in late-phase potentiation and CREB phosphorylation (English and Sweatt, 1997; Atkins et al., 1998; Impey et al., 1998) and that cAMP and PKA may act in part by activating MAP kinase (Martin et al., 1997b; Impey et al., 1998; Michael et al., 1998). Consistent with that idea, the increase in phospho-CREB immunofluorescence by Sp-cAMPS was almost completely blocked by an inhibitor of MAP kinase, U0126 [104 ± 2%; \( n = 9; F_{(1,115)} = 56.47; p < 0.01 \) compared with no inhibitor], as well as by the PKA inhibitor KT5720. We therefore investigated whether cGMP-induced CREB phosphorylation also involves MAP kinase. The increase in immunofluorescence by 8-Br-cGMP paired with one-train tetanization was significantly reduced but not completely blocked by the MAP kinase inhibitor U0126 [138 ± 8%; \( n = 8; F_{(1,115)} = 15.67; p < 0.01 \) compared with control; \( F_{(1,115)} = 4.76; p < 0.05 \) compared with no inhibitor]. These results suggest that cGMP may cause CREB phosphorylation in part via MAP kinase but that it can also cause CREB phosphorylation independent of both MAP kinase and PKA.

Sixty minutes after these treatments CREB phosphorylation was very similar to that seen 1 min after the treatments, with one exception: the increase in phospho-CREB immunofluorescence after treatment with Sp-cAMPS was significantly less at 60 min than at 1 min [141 ± 11%; \( n = 7; F_{(1,115)} = 22.01; p < 0.01 \) compared with 1 min], although it was still significantly greater than control \( F_{(1,115)} = 15.61; p < 0.01 \). There was no such decline in phospho-CREB immunofluorescence after 8-Br-cGMP paired with one-train tetanization \( [\text{drug} \times \text{time interaction}, F_{(1,28)} = 4.48; p < 0.05] \). These results suggest that the cGMP-dependent component of late-phase potentiation involves phosphorylation of CREB that is stable for at least 1 hr after treatment, whereas the cAMP-dependent component involves more transient phosphorylation of CREB. We obtained a similar pattern of results with three- and four-train tetanization, repre-
tively (Fig. 7), consistent with the idea that four-train tetanization recruits the cAMP-dependent component relatively more strongly.

**DISCUSSION**

**The NO–cGMP–PKG-signaling pathway and PKA both contribute to L-LTP**

NO signaling is thought to be involved in E-LTP that is induced by one or two trains of tetanic stimulation and lasts ~1 hr (for review, see Hawkins et al., 1998). By contrast, PKA is not thought to be involved in E-LTP induced by one-train tetanic stimulation (Huang and Kandel, 1994; Huang et al., 1996), although recent evidence indicates that PKA does make a contribution to a novel, intermediate phase of LTP that can be produced by two tetani and does not depend on protein synthesis (Blitzer et al., 1995, 1998; Winder et al., 1998) as well as to L-LTP that is induced by three or four tetani and is protein synthesis dependent (Frey et al., 1993; Huang and Kandel, 1994; Huang et al., 1996; Abel et al., 1997; Winder et al., 1998). In the present study we found that NO and PKG also contribute to L-LTP induced by three-train tetanization and to a lesser extent to L-LTP induced by four-train tetanization, whereas PKA contributes more to four-train L-LTP than to three-train L-LTP. Taken together, these results indicate that PKG and PKA are both involved in L-LTP induced by multiple trains of tetanic stimulation. However, the contribution of PKG evidently declines as that of PKA grows with increasing numbers of tetani, suggesting that PKG and PKA play somewhat complementary roles in LTP.

The idea that different molecular mechanisms make different contributions to LTP depending on the protocol may help to explain some of the conflicting results on the roles of NO, cGMP, and PKG in E-LTP. A number of studies have supported the involvement of the NO–cGMP–PKG-signaling pathway in E-LTP, but other studies have found either that those molecules are not involved or that they are involved with some protocols but not others (for review, see Hawkins et al., 1998). For example, one recent study found no effect on E-LTP of a double knock-out of two isoforms of PKG (Kleppisch et al., 1999). However, that same study also reported no effect of an inhibitor of soluble guanylyl cyclase, ODQ, whereas previous studies had found that ODQ produced a clear reduction of E-LTP (Boulton et al., 1995; Son et al., 1998). A plausible explanation for these discrepant results is that the NO–cGMP–PKG pathway contributes to LTP but that other, independent mechanisms such as PKA signaling also contribute, and their relative contributions (and thus the experimental results) depend on the experimental protocol. For example, a number of studies have found that NO makes a relatively larger contribution to E-LTP when it is induced by weaker tetanic stimulation (Chetkovich et al., 1993; Haley et al., 1993; O’Dell et al., 1994; Malen and Chapman, 1997; Zhuo et al., 1998; but see Gribkoff and Lum-Ragan, 1992), and we have now extended that finding to L-LTP. Similarly, Son et al. (1998) recently identified experimental variables that affect the contribution of cGMP to E-LTP. Thus, differences in experimental procedures can account for some of the discrepant results on the roles of NO, cGMP, and PKG in LTP.

**cGMP and PKG increase CREB phosphorylation in parallel with PKA and MAP kinase**

Our results on phospho-CREB immunofluorescence are consistent with the results of previous studies that have shown that the induction of L-LTP in hippocampal slices is accompanied by an increase in CREB phosphorylation (Impey et al., 1996; Matthies et al., 1997) as well as CRE-mediated gene expression that depends on PKA (Impey et al., 1996). In addition, we have found that cGMP and PKG can also contribute to CREB phosphorylation and that this signaling pathway plays a relatively larger role after three-train tetanization than after four-train tetanization. The increase in CREB phosphorylation by 8-Br-cGMP paired with one-train tetanization was not blocked by an inhibitor of PKA, indicating that cGMP and PKG do not act by stimulating PKA. The increase in phospho-CREB immunofluorescence was also only partially reduced by an inhibitor of MAP kinase, indicating that cGMP and PKG cause CREB phosphorylation, at least in part, via some other pathway.

One possibility is that PKG may directly phosphorylate CREB at the same site as PKA. PKG can phosphorylate CREB directly in vitro (Colbran et al., 1992) and is thought to act directly in transfected kidney cells (Gudi et al., 1996), but it is not known whether this occurs in neurons. Another possibility is that PKG may phosphorylate CREB indirectly via some other kinase such Ca2+/calmodulin-dependent kinase (CamK), perhaps by triggering Ca2+ release from intracellular Ca2+ stores. PKG activates ADP ribosyl cyclase leading to the production of cyclic ADP ribose, which in turn acts synergistically with Ca2+ to stimulate ryanodine receptors and cause release of Ca2+ from intracellular stores (Galione et al., 1993; Lee, 1993). Ca2+ release from intracellular stores has been implicated in the induction of LTP in hippocampal slices (Harvey and Collingridge, 1992; Wang et al., 1996), and CamK is important for CREB phosphorylation during LTP in cultured hippocampal neurons (Bito et al., 1996; Deisseroth et al., 1996, 1998). In agreement with this possibility, in preliminary experiments we have found that both late-phase potentiation and the increase in CREB phosphorylation by 8-Br-cGMP paired with one-train tetanization are blocked by prolonged exposure to ryanodine, which decreases Ca2+ release from ryanodine-sensitive intracellular Ca2+ stores (Y.-F. Lu and R. D. Hawkins, unpublished observations).

**Roles of NO, cGMP, and PKG in E-LTP and L-LTP**

The finding that NO, cGMP, and PKG are involved in L-LTP as well as E-LTP raises the question of what (if anything) is the relationship between the roles of these molecules in the different phases of LTP. During E-LTP, NO is thought to act as a retrograde messenger that stimulates cGMP and PKG in the presynaptic terminals. Results from a number of experiments on hippocampal slices are consistent with this hypothesis (for review, see Hawkins et al., 1998), and experiments on dissociated cultures of hippocampal neurons strongly support it (Arancio et al., 1995, 1996) (Arancio, Antonova, Gambaryan, Lohmann, Wood, Lawrence, and Hawkins, unpublished observations). Our results indicate that NO, cGMP, and PKG are also involved in L-LTP that requires protein and RNA synthesis. Although there could be local protein synthesis at the synapses (Kang and Schuman, 1996; Martin et al., 1997a), the RNA synthesis critical for late-phase potentiation probably occurs in the cell bodies. Moreover, it most likely occurs in the postsynaptic neurons because L-LTP is blocked when the dendrites are surgically separated from the postsynaptic cell bodies (Frey et al., 1989), whereas both L-LTP by tetanic stimulation and late-phase potentiation by NO or cGMP analogs are normal in slices from which the postsynaptic cell bodies have been surgically removed (our picrotoxin experiments; Lu and Hawkins, unpublished observations). Furthermore, we have found that late-phase potentiation is accompanied
by an increase in phospho-CREB immunofluorescence in the postsynaptic (CA1) cell bodies. Thus, NO, cGMP, and PKG may have different sites of action for the different phases of potentiation.

One possibility is that NO generated in the postsynaptic neurons may diffuse not only to the presynaptic terminals to induce E-LTP but also to postsynaptic dendrites or cell bodies to induce protein- and RNA synthesis-dependent L-LTP. An interesting implication of this idea is that NO might also diffuse to neighboring neurons and produce late-phase potentiation in them as well, much as it is thought to for early-phase potentiation (Bonhoeffer et al., 1989; Schuman and Madison, 1994). However, because NO or 8-Br-cGMP must be paired with one-train tetanization to produce either late-phase potentiation or CREB phosphorylation, presumably only neighboring neurons with some minimum level of synaptic activity would undergo the potentiation. This idea is analogous to the idea that NO and activity act synergistically to preserve the pathway specificity of early-phase potentiation by a freely diffusible retrograde messenger molecule (Hawkins et al., 1993).

These arguments suggest that NO may act to produce early- and late-phase potentiation by two completely independent mechanisms in different cellular locations. However, the finding that the same signaling pathway (NO–cGMP–PKG) appears to be involved in both phases of potentiation suggests that they may be coordinated in some way. One way that early- and late-phase potentiation might interact is via synaptic “tagging.” Evidence from both hippocampus and Aplysia suggests that local events that occur during early-phase LTP or facilitation somehow “tag” the active synapses, so that those synapses can use the newly synthesized proteins when they arrive from the cell body for the late phase (Frey and Morris, 1997; Martin et al., 1997a). The finding that NO paired with a single tetanus to one input pathway produces late-phase potentiation in that pathway but not in a control pathway in the same slice (Fig. 2B) suggests that late-phase potentiation by NO also involves synaptic tagging. Because Frey and Morris (1997) and we (data not shown) have found that a single tetanus by itself is not sufficient to tag synapses for late-phase potentiation, these results also suggest that the NO-signaling pathway may play some role in local synaptic tagging that could serve to link its other roles in L-LTP and E-LTP.

**REFERENCES**


N0 Signaling, L-LTP, and CREB Phosphorylation

Lu et al. • J. Neurosci., December 1, 1999, 19(23):10250–10261 10261


