Full-length cDNAs for two additional mammalian adenylyl cyclases have been described (*19, 20*). The type-III enzyme is abundant in olfactory tissue. We expressed this adenylyl cyclase in Sf9 cells (*17*); activity is stimulated synergistically by $G_{s\alpha}$ and forskolin (Fig. 4A). There was little or no effect of bovine brain $\beta\gamma$ on type-III adenylyl cyclase activity in the presence of $G_{s\alpha}$, forskolin, or a combination of the two activators (Fig. 4A). The type-IV enzyme is found in brain and several peripheral tissues. Its sequence most resembles that of type II, and its activity can be stimulated by a combination of $G_{s\alpha}$ and $\beta\gamma$ in much the same manner as type-II adenylyl cyclase can be (*20*). S49 cells express two other adenylyl cyclases, designated V and VI (*21*). Much of the initial work on the effects of G protein α and $\beta\gamma$ subunits on adenylyl cyclase activity was performed with S49 cell membranes (*1, 22*). In keeping with other results, we observed only weak potentiation of cyc^- ($G_{s\alpha}$-deficient) S49 cell adenylyl cyclase activity by $\beta\gamma$ in the presence of $G_{s\alpha}$ (Fig. 4B). Thus, it may be possible to classify adenylyl cyclases as being potentiated or inhibited by $\beta\gamma$ or as relatively immune to the subunits. It will be of interest to see if any non–calmodulin-sensitive form of the enzyme can be inhibited by $\beta\gamma$.

The major question is the physiological significance of these phenomena. An obvious prediction is that agents that interact with receptors that are coupled to G proteins other than G_s might alter the effects of G_s-linked receptors on adenosine 3′,5′-monophosphate (cAMP) accumulation (by releasing $\beta\gamma$) while having no primary effect on adenylyl cyclase themselves. G_o in particular could serve as a large pool of $\beta\gamma$ because this G protein represents 1 to 2% of brain membrane protein. Such a mechanism might explain the effects of combinations of neurotransmitters on cAMP concentrations in brain slices (*23*). Agents such as glutamate and α-adrenergic agonists, which do not stimulate adenylyl cyclase by themselves, potentiate the actions of compounds such as histamine, β-adrenergic agonists, and adenosine, which can interact with G_s-linked receptors. It will be of interest to discover if effects of this sort can be ascribed to regulation of the concentration of the free $\beta\gamma$ subunit complex.

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11. Sf9 cells were maintained in suspension culture as described (*9*). Cell membranes were prepared from 1-liter cultures after infection with either B-rACI or B-rACII (one plaque-forming unit per cell) as described (*9*).
12. W.-J. Tang and A. G. Gilman, unpublished data.
13. The concentrations of $G_{o\alpha}$ required to reverse the effects of $\beta\gamma$ were less than anticipated, based on stoichiometric interaction with $\beta\gamma$. $G_{o\alpha}$ was quantified by nucleotide binding; $\beta\gamma$ was quantified by protein assay. Inactive $\beta\gamma$ in the preparation may explain this discrepancy. In addition, bovine brain $\beta\gamma$ contains several species of both β and γ polypeptides. The relative affinities of different $\beta\gamma$ complexes for $G_{o\alpha}$ and adenylyl cyclase are not known.
14. Nine different preparations of bovine brain $\beta\gamma$ were provided by P. Casey, E. Lee, L. Quarmby, and G. Berstein and were purified as described [P. J. Casey, M. P. Graziano, A. G. Gilman, *Biochemistry* **28**, 611 (1989)]. Transducin $\beta\gamma$ and fractions enriched in $\beta_{35}\gamma$ and $\beta_{36}\gamma$ were provided by P. Casey. These preparations were in three different detergents: cholate (0.05 to 0.1%), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (0.07%), or lubrol PX (0.025 to 0.05%). The type of the detergent had little effect on the results.
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17. For mutant II-M_2C_2, the Afl II fragments of plasmid p1-4, which contained bovine type-II adenylyl cyclase cDNA, were isolated and ligated with phosphorylated adapters (5′-GATCCACCATGGGC-3′ and 5′-TTAAGCCCATGGTG-3′) and pBluescript SK^- (Stratagene), which had been digested with Bam HI. For II-NM_1C_1, plasmid prII, which contained rat type-II adenylyl cyclase cDNA, was digested with Afl II and linked with phosphorylated adapters (5′-TTAAAC-TAGGATCCTAGT-3′). For type-III adenylyl cyclase, the Eco RI fragment that contained the full-length type-III cDNA was transferred from the pCMV vector (*19*) to pBluescript SK^-. The resulting DNAs were then transferred to the baculovirus expression vector pVL1392 (into the Bam HI site for II-M_2C_2 and into the Eco RI site for II-NM_1C_1 and type-III). These DNAs were cotransfected with linearized AcRP23-*lacZ* viral DNA by lipofectin-mediated transfection. Positive viral clones were selected by plaque assay, and production of protein was confirmed either by protein immunoblotting or by visualizing the [^{35}S]methionine-labeled products [R. D. Possee and S. C. Howard, *Nucleic Acids Res.* **15**, 10233 (1987); D. R. Groebe, A. E. Chung, C. Ho, *ibid.* **18**, 4033 (1990); P. Kitts, M. D. Ayres, R. D. Possee, *ibid.*, p. 5667]. II-NM_1C_1 terminates and II-M_2C_2 is initiated 55 amino acid residues before the beginning of the second set of transmembrane spans of type-II adenylyl cyclase. Infection of Sf9 cells and preparation of cell membranes were performed as described (*9*).
18. Solubilization of proteins from Sf9 cell membranes with lubrol PX and purification of type-I adenylyl cyclase by forskolin-Sepharose (Pharmacia) chromatography were performed as described (*9*). Similar procedures were followed for the type-II enzyme.
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27. We thank R. Reed for type-II and type-III adenylyl cyclase cDNA and B-rACII; M. Linder, H. Itoh, and P. Middleton for $rG_{s\alpha}$ and $rG_{i\alpha}$; T. Higashijima for bovine brain $G_{o\alpha}$; P. Kitts for AcRP23-*lacZ* viral stock; M. Stanzel and L. Ortlepp for technical assistance; and T. Nguyen for the II-M_2C_2 construct. Supported by USPHS grant GM34497, American Cancer Society grant BE-30M, the Raymond and Ellen Willie Chair of Molecular Neuropharmacology, the Lucille P. Markey Charitable Trust, and the Perot Family Foundation.

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A Requirement for the Intercellular Messenger Nitric Oxide in Long-Term Potentiation

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Long-term potentiation (LTP) of synaptic transmission is a widely studied model of neuronal plasticity. The induction of LTP is known to require processes in the postsynaptic neuron, while experimental evidence suggests that the expression of LTP may occur in the presynaptic terminal. This has led to speculation that a retrograde messenger travels from the post- to the presynaptic cell during induction of LTP. Extracellular application or postsynaptic injection of two inhibitors of nitric oxide synthase, *N*-nitro-L-arginine or N^G-methyl-L-arginine, blocks LTP. Extracellular application of hemoglobin, which binds nitric oxide, also attenuates LTP. These findings suggest that nitric oxide liberated from postsynaptic neurons may travel back to presynaptic terminals to cause LTP expression.

IN THE SCHAFFER COLLATERAL–CA1 synapses of the hippocampus, LTP is induced by a series of postsynaptic events including activation of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor channel (*1*) and the influx of Ca^{2+} (*2*). The site of the expression of LTP is controversial (*3–5*), but recent studies with quantal analysis (*4, 5*) have strengthened the evidence that, after postsynaptic induction, LTP is expressed by an increase in transmitter release from presynaptic terminals (*6, 7*). If the mechanisms underlying the induction and expression of LTP reside

on opposite sides of the synapse, there must be a signal that travels in a retrograde direction from the postsynaptic cell to the presynaptic terminal (*6, 8*).

The diffusible molecule nitric oxide (NO) serves as an intercellular messenger in several biological systems, including the brain (*9–12*). Nitric oxide is formed from L-arginine in a nicotinamide adenine dinucleotide phosphate (NADPH)–dependent reaction catalyzed by NO synthase (*13*), a Ca^{2+}-calmodulin–dependent enzyme (*14*). Inhibitors of NO synthase have been used to identify the biological functions of NO, which include smooth muscle relaxation (*15*), gastrointestinal adaptive relaxations (*16*), and guanosine 3′,5′-monophosphate (cGMP) elevation in cerebellar neurons (*10, 11*). We have used inhibitors of NO synthase to probe the role of NO in the production of LTP (*17*).

Field excitatory postsynaptic potentials (EPSPs) were evoked in the stratum radiatum of hippocampal slices by test stimuli delivered to the Schaffer collaterals (*18*). To assess the contribution of NO to LTP, we bathed slices in artificial cerebrospinal fluid (ACSF) containing the NO synthase inhibitors N^G-methyl-L-arginine (L-Me-Arg) or *N*-nitro-L-arginine (L-No-Arg). In control ACSF, tetanic stimulation of the Schaffer collaterals resulted in a persistent potentiation of the EPSP [148.2 ± 8.9% (mean percent of baseline ± SEM) 1 hour after tetanus; n = 15] (Fig. 1A). In contrast, when slices were treated with 100 μM (*19*) L-Me-Arg before tetanus, high-frequency stimulation failed to produce a persistent enhancement of the EPSP (103.2 ± 6.5%; n = 16) (Fig. 1B). Application of 100 μM N^G-methyl-D-arginine (D-Me-Arg), an isomer of methyl arginine that does not inhibit NO synthase, did not prevent tetanus-induced LTP (158.6 ± 19.7%; n = 6) (Fig. 1C). In addition, slices exposed to L-No-Arg (100 μM) did not exhibit significant tetanus-induced LTP (109.4 ± 7.8; n = 8) (Fig. 1D). These results suggest that NO synthase activity and NO are necessary for the production of LTP.

L-Me-Arg inhibits NO synthase activity by competing with endogenous L-arginine for the substrate site on the enzyme (*11*). We attempted to reverse the L-Me-Arg block of LTP by the addition of excess L-arginine to the ACSF. Slices were initially incubated in ACSF containing L-Me-Arg. As above, tetanic stimulation failed to elicit LTP in the presence of the inhibitor (103.0 ± 1.5%; n = 4) (Fig. 2). L-arginine was then added to the bath in the continued presence of L-Me-Arg. Subsequent tetanic stimulation of the same presynaptic axons resulted in potentiation of the EPSP (138.5 ± 13.2%) (Fig. 2). Thus, the inhibition of LTP by L-Me-Arg can be reversed by excess arginine. These data indicate that the site of action of L-Me-Arg is the NO synthase.

To determine the location of the NO synthase activity critical for LTP induction, we delivered L-Me-Arg into postsynaptic CA1 pyramidal neurons through intracellular recording electrodes. LTP was induced by pairing constant postsynaptic depolarization with low frequency stimulation of presynaptic axons (*5, 20*). Under control condi-

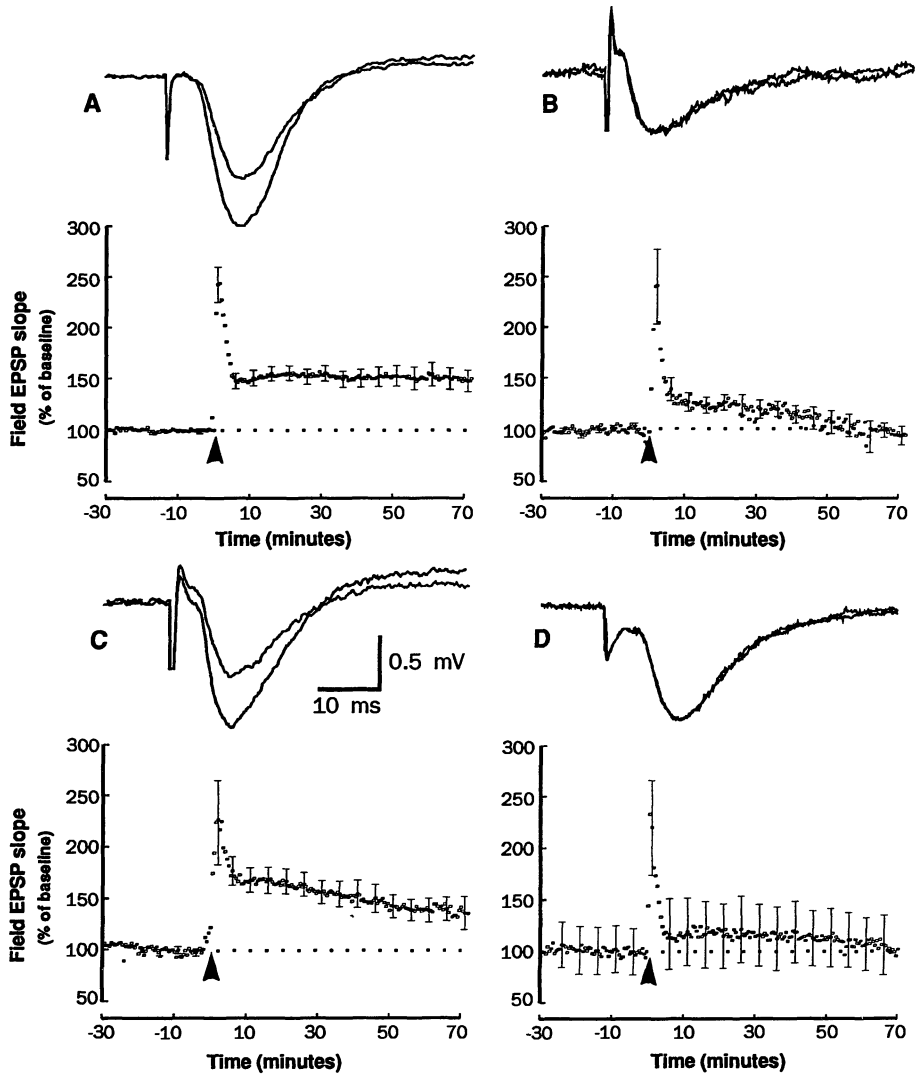

Fig. 1. Extracellular application of the NO synthase inhibitors L-Me-Arg and L-No-Arg prevents tetanus-induced LTP of the field EPSP. Tetanus is indicated by an arrowhead. (**A**) Control. (Top) Two representative EPSPs from a slice bathed in control ACSF, recorded 10 min before and 60 min after tetanic stimulation. (Bottom) Ensemble averages for all control experiments. Field EPSP slope before tetanus was 0.44 ± 0.05 mV/ms (mean ± SEM) and after tetanus was 0.63 ± 0.06, mV/ms (P < 0.001). (**B**) L-Me-Arg. (Top) Two representative EPSPs from a slice bathed in ACSF containing L-Me-Arg (100 μM), recorded 10 min before and 60 min after tetanic stimulation. (Bottom) Ensemble average for all L-Me-Arg experiments. Field EPSP slope before tetanus was 0.37 ± 0.04 mV/ms and after tetanus was 0.38 ± 0.04 mV/ms. (**C**) D-Me-Arg. (Top) Two representative EPSPs from a slice bathed in ACSF containing D-Me-Arg (100 μM), recorded 10 min before and 60 min after tetanic stimulation. (Bottom) Ensemble average for all D-Me-Arg experiments. Field EPSP slope before tetanus was 0.36 ± 0.06 mV/ms and after tetanus was 0.54 ± 0.09 mV/ms (P < 0.01). (**D**) L-No-Arg. (Top) Two representative EPSPs from a slice bathed in ACSF containing L-No-Arg (100 μM), recorded 10 min before and 60 min after tetanic stimulation. (Bottom) Ensemble average for all experiments with L-No-Arg. Field EPSP slope before tetanus was 0.47 ± 0.11 mV/ms and after tetanus was 0.50 ± 0.12 mV/ms. The amount of potentiation produced in L-Me-Arg and L-No-Arg experiments was not significantly different. However, the potentiation in the presence of these inhibitors was significantly less than that occurring in control (P < 0.01) and D-Me-Arg experiments (P < 0.01), which did not differ significantly from one another.

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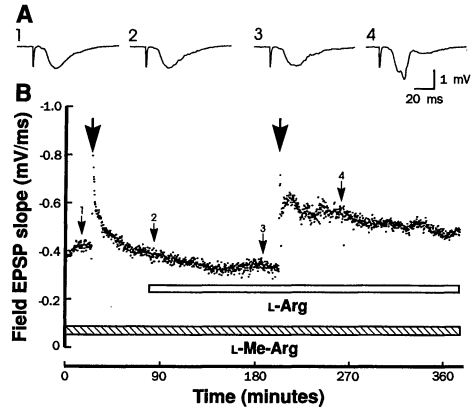

Fig. 2. L-Arginine reverses the L-Me-Arg blockade of LTP. Tetanic stimulation was delivered twice in this experiment (large arrows), once in the presence of L-Me-Arg (100 μM) (hatched bar) and again after the addition of L-arginine (1 mM) (open bar). (**A**) Field EPSPs recorded in stratum radiatum. EPSPs were collected 15 min before (1) and 60 min after (2) the first tetanic stimulation (in L-Me-Arg); 15 min before (3) and 60 min after (4) the second tetanic stimulation (after addition of L-arginine). (**B**) The slope of recorded field EPSPs throughout the experiment. Each point represents the rising slope of a single, nonaveraged EPSP. EPSPs were evoked at a rate of two per minute; data points for all EPSPs collected during the experiment are shown. Numerals over arrows indicate the time at which the records in (A) were collected. After L-Me-Arg treatment, field EPSP slope before tetanic stimulation was 0.43 ± 0.07 mV/ms and after tetanus was 0.45 ± 0.08 mV/ms (not significantly different). After the addition of L-arginine, field EPSP slope before tetanus was 0.35 ± 0.06 mV/ms and after tetanus was 0.48 ± 0.07 ($P < 0.05$). The amount of potentiation in the presence of L-Me-Arg plus L-arginine was significantly greater than that in the presence of L-Me-Arg alone ($P < 0.05$).

tions, this protocol resulted in a robust and long-lasting potentiation of the intracellular EPSP (190.3 ± 24.3%; $n = 7$) (Fig. 3A). However, when postsynaptic neurons were filled with L-Me-Arg, the pairing protocol did not elicit persistent potentiation of synaptic potentials (109.5 ± 4.5%; $n = 15$) (Fig. 3B). Cells filled with the inactive isomer D-Me-Arg exhibited LTP similar to control (172.2 ± 16.7%; $n = 6$) (Fig. 3C). These data demonstrate that NO synthase activity in the postsynaptic cell is critical for the production of LTP.

A postsynaptically generated retrograde messenger would likely pass outside the postsynaptic cell on the way to its presynaptic target. To address this possibility, we tested whether extracellular application of hemoglobin attenuates LTP. Hemoglobin binds and inactivates NO but itself does not readily cross cell membranes (*21*). As before, when slices were bathed in normal ACSF, tetanic stimulation resulted in LTP (159 ± 10.7%; $n = 10$) (Fig. 4A). However, after incubation in ACSF containing 100 μM hemoglobin, tetanized synapses failed to exhibit significant LTP (115.8 ± 6.2%; $n = 10$) (Fig. 4B). The simplest interpretation of this finding is that hemoglobin, present outside the cell, scavenges extracellular NO and prevents it from reaching its ultimate site of action, perhaps the presynaptic terminal.

If NO participates solely in the induction of LTP, then it should be produced for only a short time after tetanic stimulation. On the other hand, NO production could persist after tetanic stimulation and thereby participate in maintaining potentiation. Our data demonstrating that the reversal of L-Me-Arg inhibition of LTP by L-arginine occurs only after a second tetanus (Fig. 2) suggest that NO acts only as an initial messenger in LTP induction. If tetanic stimulation resulted in the persistent activation of NO synthase, then the addition of L-arginine would have resulted in the production of NO and the immediate expression of LTP, independent of a second tetanus.

Our data provide evidence that the postsynaptic pyramidal neuron is the site of NO production. Very few cell bodies in the CA1 cell region stain positive for NO synthase (*13, 22*). However, stained fibers are apparent in stratum radiatum (*22*), where the Schaffer collaterals synapse onto CA1 dendrites. In addition, a different isoform (*23*) of NO synthase, which is not recog-

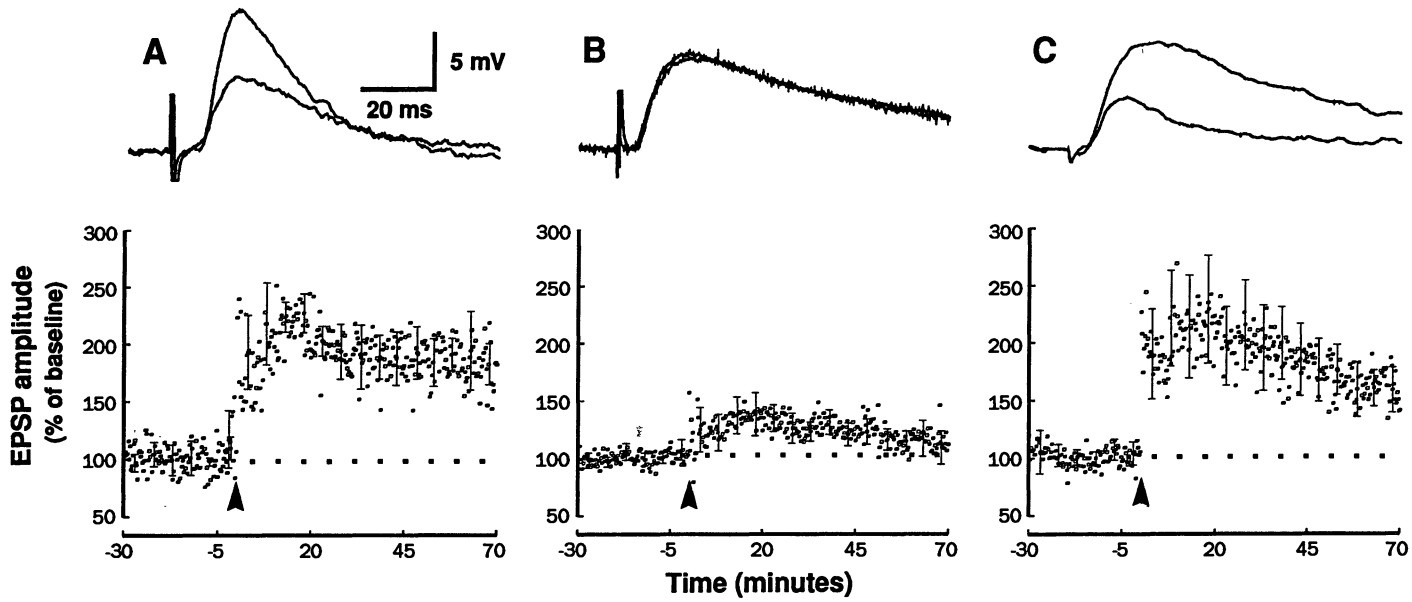

Fig. 3. Injection of L-Me-Arg into postsynaptic cells blocks LTP. Pairing indicated by arrowheads. (**A**) Control. (Top) Representative intracellular EPSPs recorded from the same control cell 15 min before and 60 min after pairing. (Bottom) Ensemble average for all control experiments. The intracellular EPSP amplitude before pairing was 3.76 ± 0.42 mV and after pairing was 6.72 ± 0.49 mV ($P < 0.001$). (**B**) L-Me-Arg. (Top) Representative intracellular EPSPs recorded from a cell injected with L-Me-Arg. Shown are two superimposed EPSPs taken 15 min before and 60 min after pairing. (Bottom) Ensemble average for all L-Me-Arg–injected neurons. The intracellular EPSP amplitude before pairing was 3.38 ± 0.25 mV and after pairing was 3.77 ± 0.31 mV (not significantly different). (**C**) D-Me-Arg. (Top) Representative intracellular EPSPs recorded from a D-Me-Arg–injected cell. Shown are two superimposed EPSPs taken 15 min before and 60 min after pairing. (Bottom) Ensemble average for all D-Me-Arg–injected neurons. The intracellular EPSP amplitude before pairing was 3.29 ± 0.42 mV and after pairing was 5.61 ± 0.73 mV ($P < 0.01$). The amount of potentiation in L-Me-Arg–injected cells was significantly less than that observed in controls ($P < 0.001$) or D-Me-Arg–injected cells ($P < 0.001$), which did not differ significantly from one another.

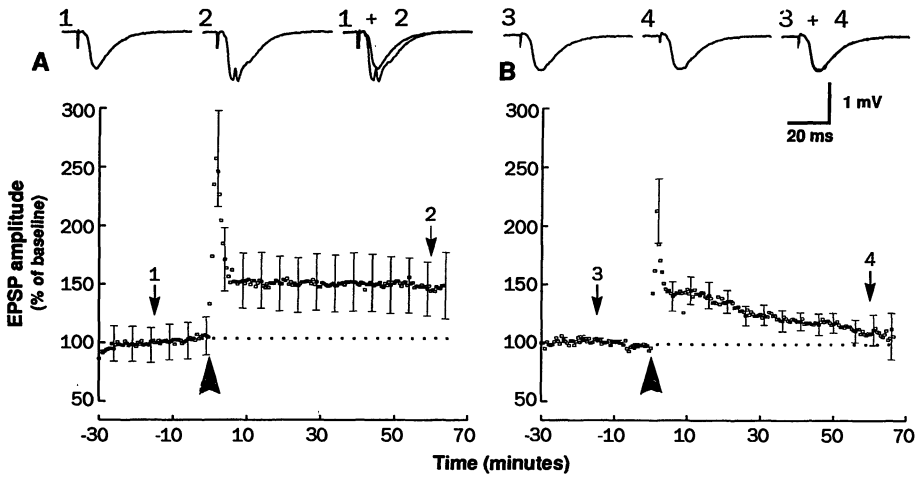

Fig. 4. Extracellular hemoglobin attenuates LTP. All records in this figure were obtained from the same slices. EPSPs were evoked by stimulation of two independent groups of Schaffer collateral fibers, one tetanized before and the other after addition of hemoglobin. Tetanus is indicated by arrowheads. (**A**) Control. (Top) Field EPSPs from a slice bathed in control ACSF collected 15 min before (1) and 60 min after (2) tetanic stimulation. (Bottom) Ensemble averages for all control experiments. The field EPSP before tetanic stimulation was 0.63 ± 0.05 mV/ms (15 min before tetanus) and after tetanus was 1.04 ± 0.14 mV/ms ($P < 0.005$). (**B**) Hemoglobin. (Top) Field EPSPs from a slice bathed in ACSF containing hemoglobin (100 μM) obtained 15 min before (3) and 60 min after (4) tetanic stimulation. (Bottom) Ensemble averages for all hemoglobin experiments. The field EPSP before tetanus was 0.58 ± 0.08 mV/ms and after tetanus was 0.68 ± 0.12 mV/ms (not significantly different). The amount of LTP observed in control pathways was significantly greater than that observed in hemoglobin-treated pathways ($P < 0.01$).

nized by the available antiserum or oligonucleotide probes, may be present in CA1 neurons. At least two possible molecular targets of NO have been identified. In smooth muscle (*24*) and cerebellar granule cells (*10, 11*), NO activates guanylate cyclase, resulting in elevation of cGMP. However, application of membrane-permeant cGMP analogs [8-bromo or dibutyrl-cGMP ($n = 9$)] has no effect on synaptic transmission (*25*). In platelets, a cytosolic adenosine diphosphate ribosyltransferase, which may ribosylate G proteins, is activated by sodium nitroprusside, an agent that generates NO (*26*). This result is particularly relevant because a presynaptic G protein may be necessary for the production of LTP (*27*).

A retrograde messenger involved in the production of LTP must conform to certain criteria. First, it must be generated in postsynaptic neurons. Second, it must pass from postsynaptic to presynaptic neurons. Third, it must exert its effect over a short distance to preserve the synapse specificity of LTP. Only tetanized (or paired) synapses undergo LTP, while nearby quiescent synapses on the same postsynaptic cell do not potentiate (*28*). Our data, and that of others (*29*), establish that NO production is necessary for LTP. In addition, our data suggest that NO is produced by the postsynaptic cell and must pass outside that cell on the way to its target. Furthermore, the very short half-life of NO (4 to 6 s) is consistent with the preservation of synapse specificity. These properties make NO an excellent candidate for a retrograde messenger in LTP.

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18. Hippocampal slices were prepared as described [D. V. Madison and R. A. Nicoll, *J. Physiol. (London)* **398**, 123 (1988)]. Field or intracellular EPSPs were measured with standard techniques in stratum radiatum or in CA1 pyramidal cells and were evoked by stimulation of the Schaffer collateral–commissural afferents (2 or 4 per min). L-Me-Arg, D-Me-Arg, L-No-Arg, and hemoglobin (100 μM) were applied to slices for at least 1.5 to 2 hours prior to tetanic stimulation or pairing. None of these agents affected normal synaptic transmission. In field potential studies, all control experiments were conducted in the same slices that were later exposed to ACSF containing L-Me-Arg, D-Me-Arg, or hemoglobin. Tetanic stimulation was delivered at the test intensity in 1-s trains at 100 Hz, with four to five trains 15 or 30 s apart. Pairing was accomplished by sustained depolarization of the pyramidal neuron by dc current injection in conjunction with low frequency (1 Hz) stimulation of the test pathway for 30 to 45 s. Extracellular recording electrodes were filled with 3 M NaCl; intracellular recording electrodes were filled with 2 M cesium acetate. In intracellular injection experiments L-Me-Arg and D-Me-Arg (100 mM) were dissolved in cesium acetate. Hippocampal slices were submerged in a stream of ACSF (119 mM NaCl; 2.5 mM KCl; 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$; 1.0 mM NaH_2PO_4; 26.2 mM $NaHCO_3$; 11.0 mM glucose), maintained at 22°C and gassed with 95% O_2, 5% CO_2. Data were collected and analyzed with software written by us for this purpose under the Axobasic programming environment (Axon Instruments Inc., Foster City, CA). Ensemble average plots represent group means of each EPSP, for all experiments, aligned with respect to the time after tetanic stimulation. Statistical comparisons were made with a *t* test, performed on non-normalized data.
19. At 1 μM, L-Me-Arg slightly attenuated LTP (135.8 ± 11.1; $n = 4$), and 10 to 20 μM L-Me-Arg produced an even greater attenuation of LTP (116.1 ± 11.2; $n = 7$). We used 100 μM L-Me-Arg for most of our studies because it produced the most reliable block of LTP.
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