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Nitric Oxide and Carbon Monoxide Produce Activity-Dependent Long-Term Synaptic Enhancement in Hippocampus

Min Zhuo, Scott A. Small, Eric R. Kandel, Robert D. Hawkins*

Nitric oxide (NO) and carbon monoxide (CO) may act as retrograde messages for long-term potentiation (LTP) in the hippocampus. Zinc protoporphyrin IX, an inhibitor of the enzyme that produces CO, blocked induction of LTP in the CA1 region of hippocampal slices. Application of either NO or CO to slices produced a rapid and long-lasting increase in the size of evoked synaptic potentials if, and only if, the application occurred at the same time as weak tetanic stimulation. This long-term enhancement was spatially restricted to synapses from active presynaptic fibers and appeared to involve mechanisms utilized by LTP, occluding the subsequent induction of LTP by strong tetanic stimulation. The enhancement by NO and CO was not blocked by an *N*-methyl-D-aspartate (NMDA) receptor blocker, suggesting that NO and CO act downstream from the NMDA receptor. Also, CO produced long-term enhancement when paired with low-frequency stimulation. These results are consistent with the hypothesis that NO and CO, either alone or in combination, serve as retrograde messages that produce activity-dependent presynaptic enhancement during LTP.

Long-term potentiation is a type of synaptic plasticity that is thought to contribute to certain forms of learning in mammals (*1*). Whereas induction of LTP in the CA1 region of the hippocampus requires Ca^{2+} influx through postsynaptic NMDA receptor channels, maintenance of LTP apparently involves in part a presynaptic increase in transmitter release, implying that the postsynaptic cell must send one or more retrograde messages to the presynaptic terminals (*2–5*). Previous experiments with inhibitors of NO synthase have indicated that the membrane-permeant molecule NO may be such a message (*2–4*). Inhibitors of NO synthase also impair certain kinds of learning in vivo (*6*). To determine how NO contributes to LTP, we examined the effects of applying NO directly to hippocampal slices. In addition, we compared these actions with those produced by CO because it has actions similar to those of NO in other systems (*7, 8*).

A diffusible retrograde message could spread laterally and potentiate transmission at inactive presynaptic terminals, in contrast with the observed pathway specificity of LTP (*9*). A possible solution would be for the message to be effective only at recently active presynaptic fibers, as during activity-dependent presynaptic facilitation in *Aplysia* (*10*). We tested this possibility by applying NO to hippocampal slices either alone or paired with weak tetanic stimulation of the presynaptic fibers that by itself produced little or no LTP (*11*). Alone, NO (0.1 to

Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, New York State Psychiatric Institute, and Howard Hughes Medical Institute, 722 West 168th Street, New York, NY 10032.

*To whom correspondence should be addressed.

0.9 μM) had no consistent effect on the field excitatory postsynaptic potential (EPSP) in the CA1 region, and neither did weak tetanic stimulation (50 Hz for 0.5 s) (Fig. 1, A and B). However, when NO was applied at the same time as weak tetanic stimulation (paired training), the EPSP was immediately enhanced and remained enhanced for at least 1 hour (Fig. 1C). When applied 5 min after weak tetanic stimulation (unpaired training), NO produced no significant long-term effect (Fig. 1D), which demonstrates that the effects of NO and weak tetanic stimulation are synergistic and not simply additive during paired training.

Paired training with either the NO vehicle (n = 3) or a vehicle containing NO that had been prepared the previous day and allowed to oxidize (n = 4) produced no significant long-term effect. In an additional test of whether the long-term synaptic enhancement was attributable to NO or an oxidation product, weak tetanic stimulation during perfusion with 1 mM Arg, the precursor of NO, also produced long-term enhancement of the synaptic potential [n = 4, $\bar{x}$ = 192 ± 24%, analysis of variance (ANOVA) gives $F(1, 6) = 28.09$, $P < 0.01$]. Moreover, this enhancement was blocked when the slice was perfused with Arg and 20 μM hemoglobin, which binds extracellular NO [t test gives $t(6) = 5.38$, $P < 0.01$ comparing Arg and Arg plus hemoglobin].

We next examined a possible role of CO in LTP. Zinc protoporphyrin IX (ZnPP), which inhibits heme oxygenase by binding to its active site, blocked the induction of LTP in a dose-dependent manner (*12*) (Fig. 2A). The ZnPP (20 μM) also blocked LTP in the presence of picrotoxin (100 μM), a γ-aminobutyric acid (GABA) receptor blocker, suggesting that ZnPP does not act by enhancing inhibition (n = 4, $\bar{x}$ = 107 ± 2%). In contrast with its effect on LTP, ZnPP did not greatly affect the decrementing potentiation in the first 10 min after the tetanus. Moreover, ZnPP had no significant effect on the base-line EPSP or the NMDA component of the EPSP measured in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which blocks the non-NMDA component (*13*). These results do not rule out other possible effects of ZnPP but suggest that the drug's effects are relatively specific. A similar block of LTP by ZnPP has been observed independently by Stevens and Wang (*14*).

As with NO, CO applied alone (0.1 to 1.0 μM) had no consistent effect on the field EPSP in the CA1 region of slices (*15*) (Fig. 2B). However, when CO was paired with weak tetanic stimulation, the EPSP was rapidly enhanced and remained so for at least 1 hour (Fig. 2C). When applied 5 min after weak tetanic stimulation (unpaired training), CO produced no significant long-term effect (Fig. 2D). We obtained similar results when we first tested either CO alone (n = 1) or weak tetanic stimulation alone (n = 3) and then tested, in the same slice, CO paired with weak tetanic stimulation [20 to 30 min after CO or weak tetanus alone, $\bar{x}$ = 96 ± 8%; after CO paired, $\bar{x}$ = 173 ± 22%, $t(3) = 3.76$, $P < 0.05$ comparing CO or weak tetanus alone and CO paired].

These results demonstrate that both NO and CO can produce long-term synaptic enhancement. If that enhancement has properties similar to LTP, it should exhibit pathway specificity (*9*). We placed stimulation electrodes on two different presynaptic pathways in the same slice. When NO or CO was paired with weak tetanic stimulation of one pathway, the EPSP in that pathway was rapidly enhanced and remained so for at least 1 hour (Fig. 3, A and B). By contrast, the EPSP in the control pathway, which received weak tetanic stimulation 5 min

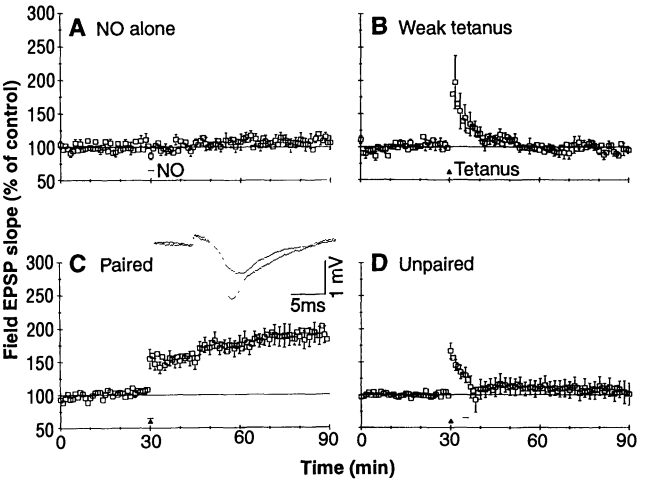

Fig. 1. Activity-dependent long-term enhancement of synaptic transmission in hippocampus by NO. (**A**) Average initial slope of the field EPSP in the CA1 region of the hippocampus, normalized to the average value before application of 0.1 μM NO (horizontal bar). Vertical bars, SEM (n = 5). (**B**) Weak tetanic stimulation delivered to the presynaptic fibers (filled triangle) (n = 5). (**C**) Paired NO and weak tetanus [n = 13, $\bar{x}$ = 190 ± 14% SEM, $F(1, 31) = 84.17$, $P <$ 0.01 comparing the potential 50 to 60 min after training to the average for 30 min before training]. (**Inset**) Representative records of the EPSP before and 50 to 60 min after NO application. (**D**) Unpaired NO application 5 min after weak tetanus (n = 6). Paired training produced significantly greater long-term enhancement of the synaptic potential than NO or weak tetanus alone, unpaired training, or vehicle paired with weak tetanus ($P < 0.01$ in each case, Duncan's multiple range test); no other groups were significantly different from each other. The average prevalues were (A) −0.32, (B) −0.20, (C) −0.30, and (D) −0.27 mV/ms, which were not significantly different from each other by one-way analysis of variance. Control was level of transmission before application to tetanus.

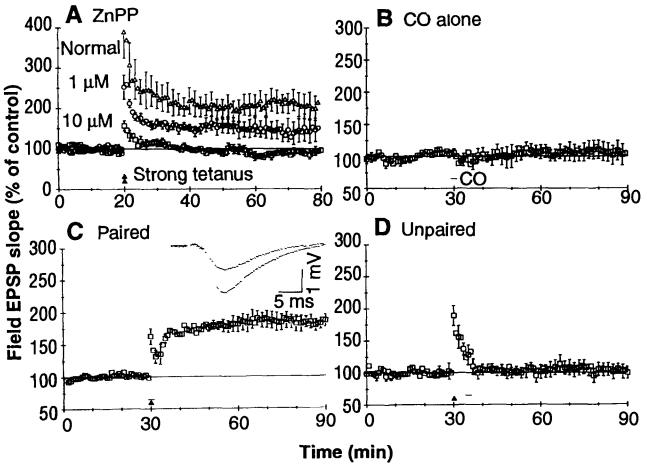

Fig. 2. Inhibition of long-term potentiation by the heme oxygenase inhibitor ZnPP, and activity-dependent long-term enhancement by CO. (**A**) Average potentiation of the field EPSP by a strong tetanus (twice at 100 Hz for 1.0 s each, separated by 20 s) in normal ACSF and in ACSF containing 1 or 10 μM ZnPP. Perfusion with ZnPP started at least 30 min before the tetanus (normal LTP: n = 5, $\bar{x}$ = 208 ± 30%; 1 μM ZnPP: n = 5, $\bar{x}$ = 144 ± 29%; 10 μM ZnPP: n = 6, $\bar{x}$ = 90 ± 9%; overall: $F(2, 13) = 6.73$, $P < 0.01$). In the presence of 10 μM ZnPP, LTP was significantly reduced compared with control LTP ($P < 0.01$, Duncan's multiple range test). The average prevalues were (normal) −0.23, (1 μM) −0.33, and (10 μM) −0.27 mV/ms. (**B**) Application of 0.1 μM CO (horizontal bar) (n = 5). (**C**) Paired CO and weak tetanus [n = 5, $\bar{x}$ = 182 ± 9%, $F(1, 11) = 50.76$, $P < 0.01$]. (**D**) Unpaired CO 5 min after weak tetanus (n = 4). Paired training produced significantly greater enhancement of the synaptic potential than either CO alone or unpaired training ($P < 0.01$ in each case, Duncan's multiple range test). The average prevalues were (B) −0.24, (C) −0.27, and (D) −0.23 mV/ms.

before application of the NO or CO, showed no significant long-term effect. Therefore, long-term enhancement by NO or CO is spatially restricted to synapses from active presynaptic pathways. Also, enhancement does not involve a generalized postsynaptic effect but rather must involve either presynaptic enhancement or a localized postsynaptic effect.

If long-term enhancement by either NO or CO contributes to normal LTP, then that enhancement should occlude LTP. We tested this possibility with strong tetanic stimulation at the end of eight of the experiments in Fig. 1C and five of the experiments in Fig. 2C. After long-term enhancement by either NO or CO, the strong tetanic stimulation produced decrementing potentiation but no significant long-term effect (Fig. 3, C and D). We obtained similar results in the two-pathway experiments in Fig. 3, A and B: Strong tetanic stimulation produced no significant long-term effect in the pathway that had received paired training (NO: $\bar{x}$ = 112 ± 9%; CO: $\bar{x}$ = 111 ± 8%) and significantly greater long-term potentiation in the pathway that had received unpaired training [NO: $\bar{x}$ = 167 ± 18%, $t(6) = 2.68$, $P < 0.05$; CO: $\bar{x}$ = 168 ± 12%, $t(6) = 4.00$, $P < 0.01$].

If NO or CO acts as a retrograde message, its effects should not require NMDA receptor activation because supplying a retrograde message exogenously would presumably bypass the NMDA receptor step in the induction of LTP. We therefore repeated these experiments in the presence of 2-amino-5-phosphonovaleric acid (APV), which blocks NMDA receptors and induction of LTP by tetanic stimulation (*16*). APV blocks long-term enhancement by arachidonic acid with a similar paired protocol, suggesting that arachidonic acid may not act as a retrograde message (*3*). Paired training with NO still produced significant long-term enhancement of the synaptic potential in the presence of APV (Fig. 4A), as did paired training with CO in two-pathway experiments similar to those in Fig. 3B [n = 5; 45 min after paired training, $\bar{x}$ = 183 ± 28%; after unpaired training, $\bar{x}$ = 129 ± 21%, $t(4) = 5.71$, $P < 0.01$ comparing paired and unpaired training].

A form of LTP can be induced in the presence of APV by very strong tetanic stimulation or bath application of the K^+-channel blocker tetraethylammonium (TEA) (*17*). These NMDA-independent forms of LTP are blocked by the voltage-dependent Ca^{2+}-channel blocker nifedipine, suggesting that sufficient Ca^{2+} influx through those channels can substitute for Ca^{2+} influx through the NMDA receptor channels in the induction of LTP. However, in the presence of both APV and nifedipine, paired training with either NO or CO still produced enhancement (*18*) [NO: n = 5, $\bar{x}$ = 148 ± 4%, $F(1, 12) = 12.64$, $P < 0.01$; CO: see Fig. 4B]. Therefore, NO and CO do not simply act by enhancing postsynaptic Ca^{2+} influx through either the NMDA receptor channels or the L-type voltage-dependent Ca^{2+} channels during the weak tetanic stimulation. In addition, the long-term enhancement was not blocked in the presence of both APV and 100 μM picrotoxin, suggesting that it does not importantly involve postsynaptic disinhibition [NO: n = 4; $\bar{x}$ = 147 ± 11%, $F(1, 12) = 9.62$, P

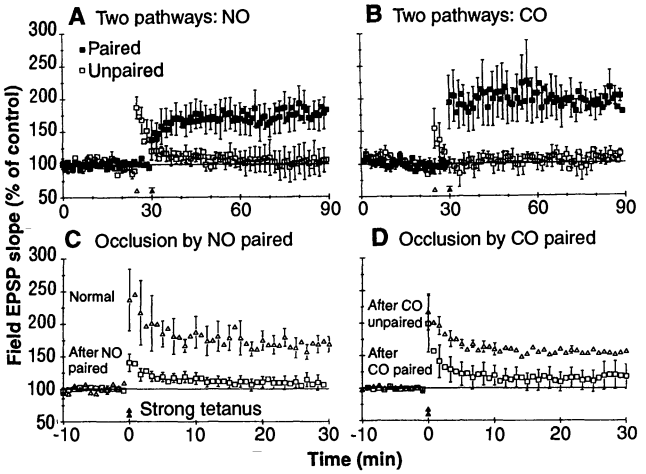

Fig. 3. Activity-dependent long-term enhancement by NO or CO is pathway-specific and occludes normal LTP. (**A**) One pathway (filled squares) received paired NO and weak tetanus, while a second pathway in the same slice (open squares) received unpaired NO application 5 min after a weak tetanus (open triangle). Long-term enhancement in the paired pathway [n = 4, $\bar{x}$ = 178 ± 23%, $F(1, 3)$ = 5765, $P < 0.01$] was significantly greater than enhancement in the unpaired pathway in the same slice [$t(3) = 52.66$, $P < 0.01$]. The average prevalues were (paired) −0.24 and (unpaired) −0.20 mV/ms. (**B**) Similar to (A), with application of CO instead of NO. Long-term enhancement in the paired pathway [n = 4, $\bar{x}$ = 185 ± 17%, $F(1, 3) = 24.34$, $P < 0.05$] was significantly greater than enhancement in the unpaired pathway in the same slice [$t(3) = 3.29$, $P < 0.05$]. The average prevalues were (paired) −0.20 and (unpaired) −0.18 mV/ms. (**C**) Decrementing potentiation produced by strong tetanic stimulation (twice at 100 Hz for 1.0 s) after long-term enhancement by NO paired with weak tetanus (squares) (n = 8, $\bar{x}$ = 109 ± 10% comparing the EPSP 20 to 30 min after training to the average for 10 min before training). In three of the experiments, the test stimulation was decreased to return the field EPSP slope to approximately its original value before the strong tetanus; in the remaining five, it was not. The results were similar in the two cases and have been pooled. The same strong tetanic stimulation produced significantly greater long-term potentiation ("normal") when it was not preceded by NO paired training (triangles) [n = 5, $\bar{x}$ = 169 ± 14%, $t(11) = 3.48$, $P < 0.01$]. (**D**) Similar to (C) after long-term enhancement by CO paired with weak tetanus (squares) (n = 5, $\bar{x}$ = 114 ± 16%). In two of the experiments, the test stimulation was decreased before the strong tetanus; in the remaining three, it was not. The results were similar in the two cases and have been pooled. The strong tetanic stimulation produced significantly greater long-term potentiation when it was preceded by unpaired CO (n = 3) or CO alone (n = 2); the results were also similar and have been pooled (triangles) [$\bar{x}$ = 156 ± 3%, $t(8) = 2.50$, $P < 0.05$].

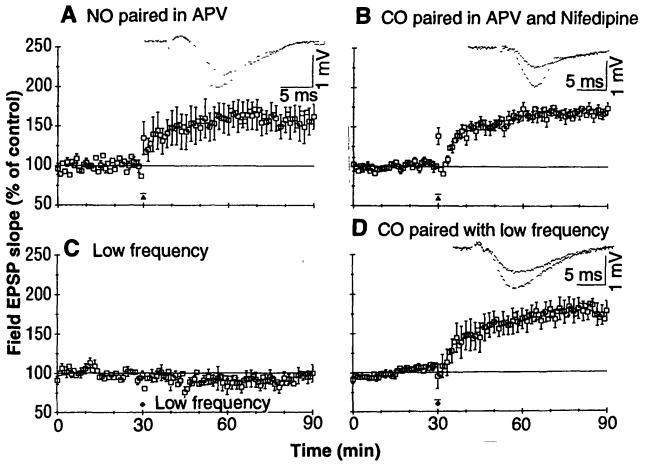

Fig. 4. Long-term enhancement by NO or CO paired with weak tetanus in the presence of APV and by CO paired with low-frequency stimulation. (**A**) NO paired with weak tetanus; 50 μM APV present throughout experiment [n = 6, $\bar{x}$ = **158 ± 17%**, $F(1, 12) = 21.91$, $P < 0.01$]. (**B**) Carbon monoxide paired with weak tetanus; 50 μM APV and 10 μM nifedipine (in 0.05% ethyl alcohol) present throughout experiment [n = 4, $\bar{x}$ = **170 ± 5%**, $F(1, 6) = 116.37$, $P < 0.01$]. The average prevalues were (A) −0.31 and (B) −0.24 mV/ms. (**C**) Low-frequency stimulation delivered to the presynaptic fibers (filled diamond) (n = 5). (**D**) Long-term enhancement by CO paired with low-frequency stimulation [n = 5, $\bar{x}$ = **177 ± 11%**, $F(1, 8) = 71.22$, $P < 0.01$] was significantly greater than enhancement by low-frequency stimulation alone [$t(8) = 6.40$, $P < 0.01$]. The average prevalues were (C) −0.24 and (D) −0.25 mV/ms.

< 0.01; CO: $n = 4$, $\bar{x} = 164 \pm 8\%$, $F(1, 6) = 97.20$, $P < 0.01$]. These results strengthen the possibility that NO and CO have a presynaptic locus of action.

Either strong tetanic stimulation of the presynaptic fibers or low-frequency stimulation paired with strong postsynaptic depolarization can induce LTP (*19*). If NO or CO acts as a retrograde message for both types of LTP, then pairing the gas with low-frequency stimulation should also produce long-lasting synaptic enhancement. When CO was paired with low-frequency stimulation (0.25 Hz for 100 s), the EPSP was enhanced for at least 1 hour (Fig. 4D). Low-frequency stimulation alone produced no significant long-term effect and no posttetanic or decrementing potentiation (Fig. 4C), making it unlikely that CO acts by enhancing the effect of the low-frequency stimulation. By contrast, NO (0.1 μM) paired with low-frequency stimulation did not produce enhancement but rather produced long-lasting depression (*20*). We have not yet tested other concentrations of NO or CO paired with low-frequency stimulation. However, the opposite effects of CO and NO suggest that CO is preferentially involved in potentiation with low-frequency stimulation or that NO is involved in depression as well as potentiation with the balance depending on the frequency of the paired activity.

The results of these and previous experiments (*2–4*) are consistent with the hypothesis that NO or CO, or both, acts as retrograde messages during LTP. However, there is as yet no clear histochemical evidence for NO synthase in CA1 pyramidal cells, where it should be if NO acts as a retrograde message (*21*), whereas heme oxygenase is clearly present in the pyramidal cells (*8*). Furthermore, inhibitors of NO synthase did not block LTP produced by strong tetanic stimulation in the presence of picrotoxin (*22*), whereas heme oxygenase inhibitors did. Finally, NO paired with low-frequency stimulation did not produce long-lasting potentiation, whereas CO did. On the other hand, tetanic stimulation and NMDA are known to activate NO synthase in the hippocampus (*23*), but such data are currently lacking for heme oxygenase. Thus, the evidence for both NO and CO as retrograde messages is still incomplete, and they might play other roles either during the induction of LTP or as constitutively required substances. Because LTP is blocked by inhibitors of both NO synthase and heme oxygenase that are thought to be specific (*3, 8, 24*), NO and CO may both play essential roles in LTP. One possibility is that CO provides a widespread, basal level of stimulation, whereas NO provides local, phasic stimulation during the induction of LTP. This possibility would be consistent with the fact that NO has a very short half-life, whereas CO is more stable. Alternatively, NO and CO might both have similar roles, with their relative contributions depending on the experimental circumstances. This possibility would be consistent with the finding that inhibitors of NO synthase do not block LTP completely under some circumstances (*22, 25, 26*), suggesting that the residual potentiation might be mediated by another substance.

Long-term enhancement by either NO or CO requires coincident stimulation of the presynaptic fibers, consistent with the idea that activity makes the presynaptic terminals responsive to potentiation by these putative retrograde messages (*27*). According to this idea, activity-dependent long-term enhancement by NO or CO would be similar to activity-dependent presynaptic facilitation contributing to classical conditioning in *Aplysia* (*10*). However, whereas the facilitating substance in *Aplysia* is released from widely projecting modulatory neurons, in the hippocampus it is presumably released from the postsynaptic cells themselves in response to NMDA receptor activation. This arrangement would allow for the use of a diffusible retrograde message in Hebbian potentiation at synapses onto active postsynaptic cells and also in non-Hebbian potentiation at synapses onto neighboring postsynaptic cells that are not active (*28*). Both of these types of potentiation occur in the CA1 region of hippocampus (*19, 29*), where they could be used to perform computations somewhat different from the strictly Hebbian rule (*30*). Alternatively, activity might have postsynaptic effects, such as stimulation of non-NMDA glutamate receptors or receptors for other transmitters. Regardless of the specific mechanism, however, activity-dependence of the effects of NO and CO in the hippocampus provides new instances of the more general phenomenon of activity-dependent neuromodulation, which contributes to learning-related plasticity involving a variety of transmitters and messages (*10, 31*).

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11. Adult male guinea pigs were housed and killed in accordance with the guidelines of the Health Sciences Division of Columbia University. Transverse slices of hippocampus 400 μm thick were rapidly prepared and maintained in an interface chamber at 30°C, where they were subfused with saline (ACSF) consisting of 124 mM NaCl, 4.4 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.0 mM Na_2HPO_4, and 10 mM glucose and bubbled with 95% O_2 and 5% CO_2. In experiments with picrotoxin, the concentrations of $CaCl_2$ and $MgSO_4$ were 4.0 mM and the CA3 region was removed to prevent epileptiform activity. A bipolar tungsten stimulating electrode was placed in the stratum radiatum of the CA1 region and extracellular field potentials were recorded with a glass microelectrode (3 to 11 megohm, filled with ACSF or 2 M NaCl) also in the stratum radiatum. The stimulation intensity was adjusted to give field EPSP amplitudes of about 1.0 mV so that the weak tetanus would produce decrementing potentiation ($\bar{x}$ = 1.17, 0.88, 1.04, and 0.99 mV in Fig. 1, A through D, respectively, not significantly different by one-way ANOVA), and test responses were elicited at 0.02 Hz. If the EPSP was stable (±25%) for at least 30 min, training stimulation was delivered and data were collected for another 60 min. Different types of training stimulation (paired NO and one or more controls) were usually delivered to different slices from the same animal. In most cases, strong tetanic stimulation was delivered at the end of the experiment, and the data were included only if the slice was shown to be capable of long-lasting potentiation by some stimulation. We prepared NO stock solution by bubbling the gas until saturation in helium-saturated distilled water [I. MacIntyre *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2936 (1991)] and immediately diluted it to the desired concentration in helium-saturated ACSF supplemented with 30 units of superoxide dismutase per milliliter. This solution was injected directly into the slice recording chamber with a gas-tight syringe for about 2 min before washout began.
12. The ZnPP (Porphyrin Products, Logan, UT) was dissolved in dimethyl sulfoxide (DMSO) immediately before the experiment and diluted to the desired concentration in ACSF by sonication (final concentration of DMSO was ≤0.05%). The solution was protected from exposure to bright light for the rest of the experiment.
13. Perfusion of slices with 1 to 100 μM ZnPP for 10 to 15 min produced on average a 2 ± 9% increase in the EPSP (n = 5, not significant). In separate experiments, perfusion with 10 μM CNQX for 20 to 25 min produced a 94 ± 2% reduction. The stimulation intensity was then increased, and a new stable base line was established before the addition of ZnPP. Perfusion with 10 μM ZnPP for 25 to 30 min in the continued presence of CNQX produced a 5 ± 10% reduction of the EPSP (n = 6, not significant). Subsequent perfusion with 100 μM APV for 10 to 15 min in the presence of CNQX and ZnPP produced a 72 ± 13% reduction (t = 5.69, $P < 0.01$).
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15. The CO solution was prepared and applied in the same manner as the NO solution (*11*) without the helium saturation or superoxide dismutase.

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18. Interestingly, the enhancement by NO or CO in the presence of APV has a somewhat slow onset, developing most rapidly during the 10 min after the training. This time period corresponds to the duration of the decrementing potentiation produced either by weak presynaptic stimulation alone (Fig. 1B) or by strong tetanic stimulation in the presence of 10 μM ZnPP (Fig. 2A) or after paired NO or CO training (Fig. 3, C and D). These results suggest that the decrementing potentiation, which is blocked by APV [R. Malenka, *Neuron* **6**, 53 (1991)], and the long-lasting potentiation by NO or CO are independent and may add to produce the time course of potentiation by paired NO or CO in normal saline (Figs. 1C and 2C). Similarly, the (presumably) larger decrementing potentiation produced by strong tetanic stimulation may add with the long-lasting potentiation by NO or CO to produce the time course of normal LTP (Figs. 2A and 3C).
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27. It is not clear why previous studies of potentiation by NO have not required paired activity (*2, 3*). Potentiation of evoked EPSPs by sodium nitroprusside in hippocampal slices (*2*) may have involved another substance released by the drug or a frequency of test stimulation that itself provided sufficient activity. Potentiation of spontaneous release by NO in culture (*3*) may have involved better access of NO to the cells or cells in a different state of development or with a different resting metabolic state.
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Binding of the Ras Activator Son of Sevenless to Insulin Receptor Substrate–1 Signaling Complexes

Kurt Baltensperger, Lynn M. Kozma, Andrew D. Cherniack, Jes K. Klarlund, Anil Chawla, Utpal Banerjee, Michael P. Czech

Signal transmission by insulin involves tyrosine phosphorylation of a major insulin receptor substrate (IRS-1) and exchange of Ras-bound guanosine diphosphate for guanosine triphosphate. Proteins containing Src homology 2 and 3 (SH2 and SH3) domains, such as the p85 regulatory subunit of phosphatidylinositol-3 kinase and growth factor receptor–bound protein 2 (GRB2), bind tyrosine phosphate sites on IRS-1 through their SH2 regions. Such complexes in COS cells were found to contain the heterologously expressed putative guanine nucleotide exchange factor encoded by the *Drosophila son of sevenless* gene (dSos). Thus, GRB2, p85, or other proteins with SH2-SH3 adapter sequences may link Sos proteins to IRS-1 signaling complexes as part of the mechanism by which insulin activates Ras.

Insulin receptors as well as other tyrosine kinases can elicit biological actions by increasing the steady-state level of guanosine triphosphate (GTP)–bound Ras proteins (*1–4*). The mechanism of Ras activation by insulin appears to involve the stimulation of guanine nucleotide exchange activity, causing release of bound guanosine diphosphate (GDP) followed by GTP binding to Ras (*2*). Genetic studies of *Drosophila melanogaster* have identified a protein, Son of sevenless (dSos), as a putative activator of Ras. Its function is required downstream of the sevenless tyrosine kinase and upstream of Ras in a signaling pathway necessary for eye development (*5, 6*). The dSos protein and two mouse homologs denoted as mSos1 and mSos2 (*7*) each contain a region with amino acid sequence similarity to the catalytic domain of the yeast Ras exchange factor CDC25. The mSos1 and mSos2 proteins are widely distributed in mouse tissues, in contrast to brain-specific Ras exchange factors (*8*). Thus, the Sos proteins are excellent candidates for mediating the effects of the insulin receptor on GTP binding to Ras.

In our studies here the dSos complementary DNA (cDNA) was modified to encode a hemagglutinin (HA) epitope extension at its COOH-terminus to allow its immunoprecipitation and immunoblotting (*9*). Cotransfection of this dSosHA cDNA with that encoding human H-Ras increased the amount of GTP-bound Ras in ^{32}P-labeled COS-1 cells to 10 times that found in cells transfected with human H-Ras alone (Fig. 1). Co-expression of human insulin receptors with human H-Ras in COS-1 cells led to a threefold increase in GTP-bound Ras, even in the absence of added insulin. This is a result of the large amounts of the receptor expressed under these conditions, although

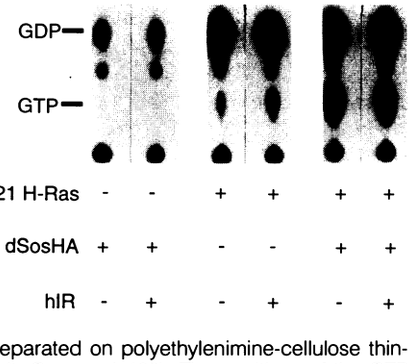

Fig. 1. Stimulation of Ras GTP-GDP exchange in intact cells by expression of recombinant dSosHA. COS-1 cells were transiently transfected (*25*) with the cDNAs encoding H-Ras (*26*), dSosHA (*9*), or the human insulin receptor (hIR) (*24*) subcloned into the mammalian expression vector pCMV (*27*) as indicated. Two days after transfection, the cells were labeled with carrier-free [^{32}P]orthophosphate (1.0 mCi/ml) for 4 hours in phosphate-free medium. The cells were lysed, and Ras was immunoprecipitated (*4*) with mAb to Ras [cell supernatant of the hybridoma cell line Y13-259 (American Type Culture Collection)]. Guanine nucleotides bound to Ras were dissociated and subsequently separated on polyethylenimine-cellulose thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) (*28*). The positions of the GTP and GDP standards on the same TLC plate are indicated.

K. Baltensperger, L. M. Kozma, A. D. Cherniack, J. K. Klarlund, A. Chawla, M. P. Czech, Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, MA 01605.
U. Banerjee, Department of Biology, University of California at Los Angeles, Los Angeles, CA 90024.