Acta Medica et Biologica
Vol. 46, No. 1, 17-24, 1998

Long-term Potentiation Suppressed by Carbon Monoxide in Layer V of the Rat Auditory Cortex

Hidemitsu WAKATSUKI[1,2], Masaharu KUDOH[1], Kota TAKAHASHI[2], Masayuki TAKEDA[2] and Katsuei SHIBUKI[1]

[1]Department of Neurophysiology, Brain Research Institute, Niigata University, [2]Department of Urology, Niigata University School of Medicine, Niigata, Japan

Received December 26 1997; accepted January 19 1998

Summary. **Carbon monoxide (CO) is a candidate for neural messengers involved in synaptic plasticity. Heme oxygenase (HO), which produces carbon monoxide (CO) by oxygenation of heme, is present in neocortical pyramidal cells. In this study, we investigated the role of CO in neocortical long-term potentiation (LTP) using slices prepared from the rat auditory cortex. Tetanic stimulation of layer IV pruduced LTP of field potentials in layer II/III ($LTP_{II/III}$) and in layer V (LTP_V). CO (8 nM-5 μM) showed no significant effect on $LTP_{II/III}$, while CO significantly suppressed LTP_V at concentrations of 40 nM and 200 nM. N^G-nitro-L-arginine (NA, 10 μM), a NO synthase inhibitor, blocked LTP_V. This blockade of LTP_V by 10 μM NA was reversed by 8-bromo-cGMP (Br-cGMP, 1 mM), a membrane-permeable cyclic GMP analog. CO (40 nM) did not suppress LTP_V in the presence of 10 μM NA and 1 mM Br-cGMP. δ-Aminolevulinic acid (ALA, 30 μM), which is a substrate for heme synthesis and is believed to facilitate endogenous CO production, significantly suppressed LTP_V. ALA showed no effect on $LTP_{II/III}$. These results strongly suggest that LTP_V but not $LTP_{II/III}$ is suppressed by exogenous and endogenous CO probably via suppression of NO-induced cGMP formation.**

Key words—carbon monoxide, long-term potentiation, cGMP, nitric oxide, auditory cortex, δ-aminolevulinic acid.

INTRODUCTION

Cyclic GMP formation is stimulated by nitric oxide (NO).[1] NO/cGMP signaling facilitates the induction of Long-term potentiation (LTP) in the hippocampal area CA1[2-5] and the induction of long-term depression (LTD) in cerebellar Purkinje cells.[6-10] Certain types of cerebellar motor learning are also dependent on NO/cGMP signaling.[11-13] In the visual cortex, LTP in layer II/III ($LTP_{II/III}$) does not depend on NO signaling,[14] while LTP in layer V (LTP_V) in the medial frontal cortex does.[15] These studies strongly suggest the importance of NO/cGMP signaling in synaptic plasticity.

Carbon monoxide (CO) facilitates cGMP formation as NO does, and is a candidate for a neural messenger.[16,17] Induction of LTP in the hippocampal area CA1 is facilitated by CO.[18] Although CO activates cGMP formation in the olfactory bulb,[17,19,20] CO suppresses NO-induced cGMP formation in cerebellar granule cells at low concentrations but not at high concentrations.[21] Therefore, CO may facilitate or suppress NO/cGMP-dependent synaptic plasticity depending on the dose. In the rat auditory cortex, marked LTP is elicited by tetanic stimulation.[22-24] We have already found that LTP_V in the auditory cortex is NO/cGMP-dependent while $LTP_{II/III}$ is not (unpublished observation). The purpose of the present study is to study the role of exogenous and endogeneous CO in the induction of LTP in the rat auditory cortex.

MATERIALS AND METHODS

Slice preparation

Wistar rats of both sexes (4-7 weeks old) were anesthetized with ether and immersed in ice-cold water, except for the nose, for 3 min to reduce the brain temperature. The whole brain was removed

Correspondence: Katsuei Shibuki, M. D., Ph. D. Department of Neurophysiology, Brain Research Institute, Niigata University, Asahi-machi 1, Niigata 951-8585, Japan.

immediately after decapitation. The location of the auditory cortex was determined to be that of area 41.[25] Coronal slices (400 μm thick) of the auditory cortex were prepared from the block including the auditory cortex in an ice-cold medium using a microslicer (Dosaka, DTK-2000). The composition of the medium, bubbled continuously with 95% O_2 and 5% CO_2, was (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26, and glucose 10. After incubation at 30℃ for at least 1 h, the slices were transferred to a small recording chamber, and the recordings were performed at 30℃.

Drugs

In pharmacological experiments, various drugs were added to the perfusion medium. N^G-nitro-L-arginine (NA), 8-bromo-cGMP (Br-cGMP) and succinylacetone (SA) were purchased from Sigma (St. Louis, USA). 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX) was obtained from Tocris (Bristol, UK). δ-Aminolevulinic acid (ALA) and bicuculline were obtained from Wako (Osaka, Japan) and RBI (Natick, USA), respectively. A standard CO medium (250 μM) was prepared by injecting 300 μl of CO gas into 50 ml of degassed saline in a glass syringe. The syringe was vortexed until the bubbles of CO gas disappeared. This standard CO medium was diluted to prepare CO media of various CO concentrations without exposing it to the air since CO escapes into the air quite rapidly. For application of CO to the slices, the CO medium contained in a glass syringe was injected at a rate of 20 μl/min into the tubing which supplied the external perfusion medium at a rate of 1 ml/min into the recording chamber (0.3 ml in volume). CO and Br-cGMP were applied to the slices during a period of tetanic layer IV stimulation and a few minutes before the stimulation. Other drugs were added to the perfusion mudium throughout the recording.

LTP recording

Field potentials in layer II/III and layer V in slices were elicited by layer IV stimulation. Layer IV was stimulated with biphasic current pulses, which were applied to the slices through the cut end of a Teflon-coated Ag wire placed on the surface of the slice (Fig. 1Aa and Ba). The current intensity of stimuli was adjusted between 200 and 500 μA to ensure that half-maximal responses were elicited. The duration of each pulse phase was 100 μs. Field potentials were recorded using an electrolytically polished Ag wire that was insulated with polyvinyl chloride except for the tip. After signals were passed through a band-pass filter between 0.2 Hz and 10 kHz, the recorded potentials were stored in a computer for later analysis. Field potentials were elicited at 20 or 30 s intervals, and 2 or 3 traces were averaged every minute to determine baseline responses. After stable baseline responses were recorded for at least 10 min, tetanic layer IV stimulation was carried out. To evoke $LTP_{II/III}$, 100 pulses at 100 Hz were applied to layer IV twice at an interval of 30 s. These stimulus parameters were not sufficient for evoking LTP_V. Therefore, 100 pulses at 200 Hz were applied to layer IV three times at 20 s intervals to evoke LTP_V. Tetanic stimulation at test stimulus intensities was usually insufficient to evoke marked LTP.[23,24] Therefore, we placed another stimulation electrode near the first electrode (distance between the tips <100 μm) and simultaneously applied tetanic stimulus pulses that were 1.5 times the intensity and 2 times the duration of test pulses. The amplitude of LTP was measured 30 min after the tetanic stimulation. Statistical significance in the obtained data was evaluated using the Mann-Whitney U-test.

RESULTS

$LTP_{II/III}$ and LTP_V in the auditory cortex

Field potentials in layer II/III in the auditory cortex are composed of early and late negative waves, which represent antidromic and trans-synaptic activation of pyramidal neurons, respectively.[22-24] Only the late negative waves showed clear $LTP_{II/III}$ lasting more than 30 min after tetanic layer IV stimulation (Fig. 1Ab). The amplitude of $LTP_{II/III}$ was 70±8% (mean ±SEM, n=10) (Fig. 1C).

Layer IV stimulation elicited field potentials of two negative waves in layer V. Only the late negative waves were blocked by 10 μM CNQX, a blocker of non-NMDA glutamate receptors (data not shown). Tetanic layer IV stimulation produced LTP_V of the late negative waves (Fig. 1Bb). The amplitude of LTP_V was 38±5% (n=12) (Fig. 1D).

Layer-specific effect of CO on LTP

To elucidate the roles of CO in neocortical LTP, we studied the effect of CO (40 nM) on the induction of $LTP_{II/III}$ and LTP_V. The amplitude of $LTP_{II/III}$ induced in the presence of 40 nM CO (74±9%, n=7) was not significantly different from that in control experiments (Fig. 1Ac and C). In contrast, the LTP_V amplitude elicited in the presence of 40 nM CO (8±2%, n=10) was significantly smaller than that in

control experiments ($p<0.001$) (Fig. 1Bc and D). To determine the dependence of the suppression of LTP_V on CO dose, we changed the CO concentration between 8 nM and 5 μM (Fig. 2). While no clear effect of CO (8 nM-5 μM) was found on $LTP_{II/III}$, a significant suppression of LTP_V was observed with 40 nM and 200 nM CO ($p<0.001$). However, no significant effect of CO was found at 8 nM, 1 μM or 5 μM.

CO suppresses NO-induced cGMP formation in cerebellar granule cells at low concentrations.[21] Therefore, the suppression of LTP_V by CO may be explained by an insufficient production of cGMP. To test this possibility, we studied the effect of 40 nM CO on LTP_V elicited in the presence of Br-cGMP. Although LTP_V was significantly suppressed by 10 μM NA, an inhibitor of NO synthase, the LTP_V amplitude elicited in the presence of 10 μM NA plus 1 mM Br-cGMP ($28\pm5\%$, $n=6$) was significantly larger than that recorded in the presence of NA alone ($p<0.001$, Fig. 3A), and not significantly different from that in control experiments. The LTP_V elicited in the presence of NA and Br-cGMP was not affected by 40 nM CO (LTP_V amplitude, $27\pm3\%$, $n=6$) (Fig. 3B). These results suggest that the suppression of LTP_V by 40 nM CO is mediated by the suppression of an NO-induced cGMP formation.

Suppression of LTP_V by endogenous CO

If the endogenous CO level is around 40 nM, it is expected that LTP_V is suppressed by endogenous CO. To test this possibility, we elicited LTP_V in the presence of 30 μM ALA, which is the first intermediate and the product of the rate-limiting enzyme reaction in heme synthesis.[26] The amplitude of LTP_V in the slices incubated with 30 μM ALA for at least 30

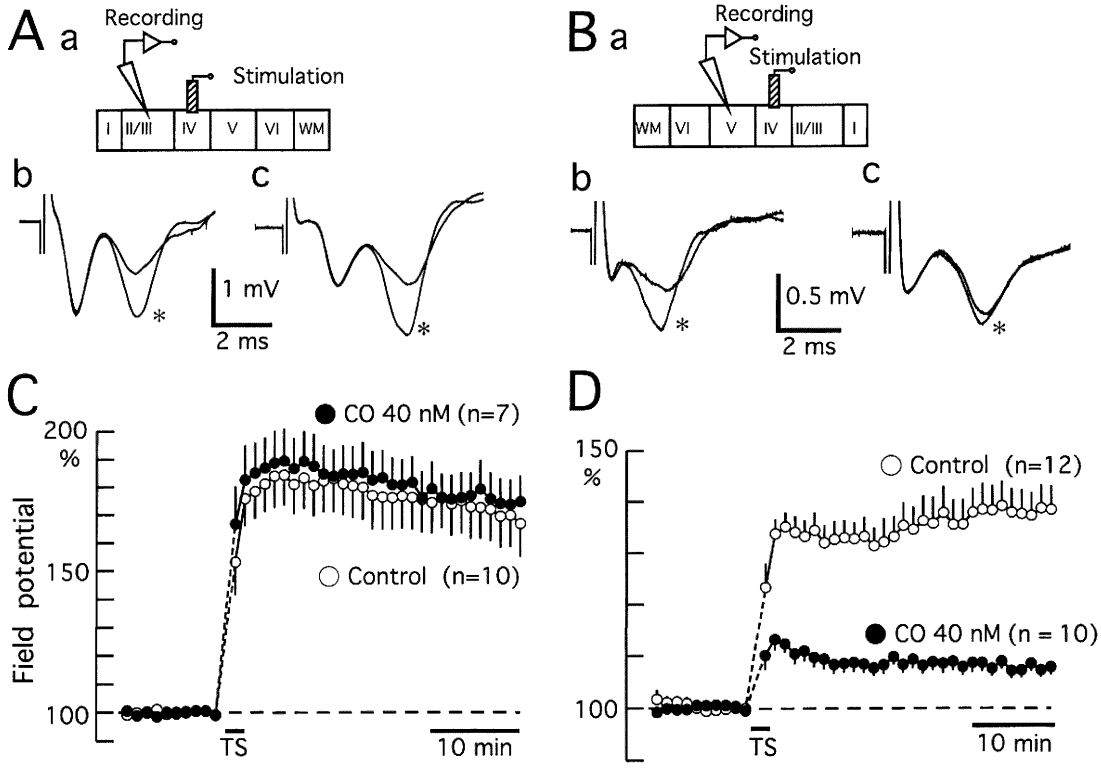

Fig. 1. Differential effect of 40 nM CO on $LTP_{II/III}$ and LTP_V. **Aa.** Experimental setup for $LTP_{II/III}$ recording. Cortical layers (I-VI) and white matter (WM) are shown together with recording and stimulating electrodes. **Ab and Ac.** Field potentials in layer II/III before and 30 min after (*) tetanic layer IV stimulation (TS), which was applied to the slices in the absence (**Ab**) or presence of 40 nM CO (**Ac**). Calibration in **Ab** is also applicable to **Ac**. **Ba.** Experimental setup for LTP_V recording. **Bb and Bc.** Traces recorded in layer V before and 30 min after (*) TS, which was applied to the slices in the absence (**Bb**) or presence of 40 nM CO (**Bc**). Calibration in **Bb** is also applicable to **Bc**. **C.** $LTP_{II/III}$ elicited in the presence of 40 nM CO (*closed circles*). Mean$\pm$SEM are shown. Control $LTP_{II/III}$ is also shown (*open circles*). **D.** LTP_V elicited in the presence of 40 nM CO (*closed circles*) and control LTP_V (*open circles*).

min (7±3%, n=12) was significantly smaller than that in the control experiment ($p<0.001$) (Fig. 4A). $LTP_{II/III}$ was not affected by ALA ($LTP_{II/III}$ amplitude:66±19%, n=7, data not shown).

Heme synthesis and the subsequent facilitation of endogenous CO production from ALA are blocked by SA.[27] Therefore, we tested the effect of ALA on LTP_V in the presence of 10 μM SA (Fig. 4B). LTP_V was slightly suppressed, but not significantly, by 10 μM SA alone. However, the LTP_V amplitude recorded in the presence of 30 μM ALA plus 10 μM SA (26±3%, n=9) was significantly larger than that recorded in the presence of ALA alone ($p<0.001$, Fig. 4B).

It has been reported that ALA inhibits GABA release.[28] Bicuculline (1 μM), a $GABA_A$ receptor antagonist, showed no significant effect on LTP_V (LTP amplitude:31±3%, n=7) (Fig. 4C), and the suppression of LTP_V by 30 μM ALA was not affected by 1 μM bicuculine (Fig. 4C). Therefore, $GABA_A$ receptors are unlikely to be involved in the suppression of LTP_V by ALA, although the contribution of $GABA_B$ receptors is still possible.

If the suppressive effect of ALA was mediated by ALA-stimulated production of endogenous CO, it is expected to become obscure in the presence of excess CO, since exogenous CO has no significant effect on LTP_V at concentrations higher than 1 μM (Fig. 2). The LTP_V amplitude elicited in the presence of 30 μM ALA plus 1 μM CO (23±2%, n=7) was significantly larger ($p<0.001$) than that recorded in the presence of 30 μM ALA alone (Fig. 4D) and was not significantly different from that recorded in the normal medium. These results strongly suggest that endogenous CO produced in the presence of 30 μM ALA is sufficient to suppress LTP_V.

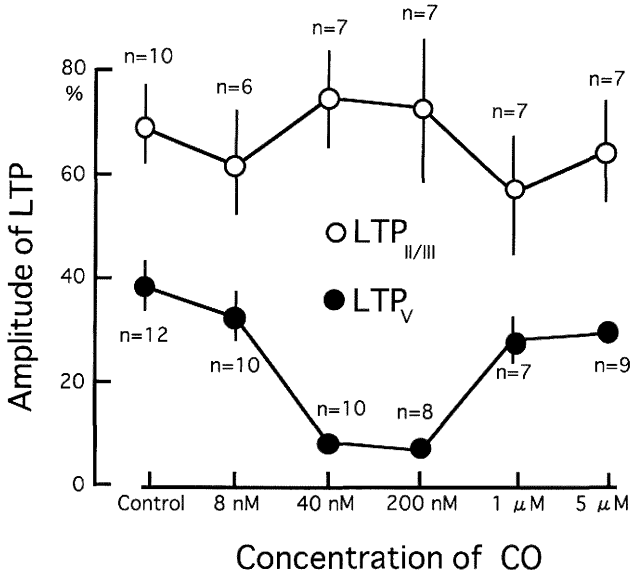

Fig. 2. Mean and SEM of the amplitude of $LTP_{II/III}$ (*open circles*) and LTP_V (*closed circles*) elicited in the absence or presence of CO (8nM-5 μM). Significant suppression of LTP_V ($p<0.001$) was found at CO concentrations of 40 nM and 200 nM.

DISCUSSION

In this study, we found that LTP_V but not $LTP_{II/III}$ was blocked by 40 nM CO. CO (40 nM) did not

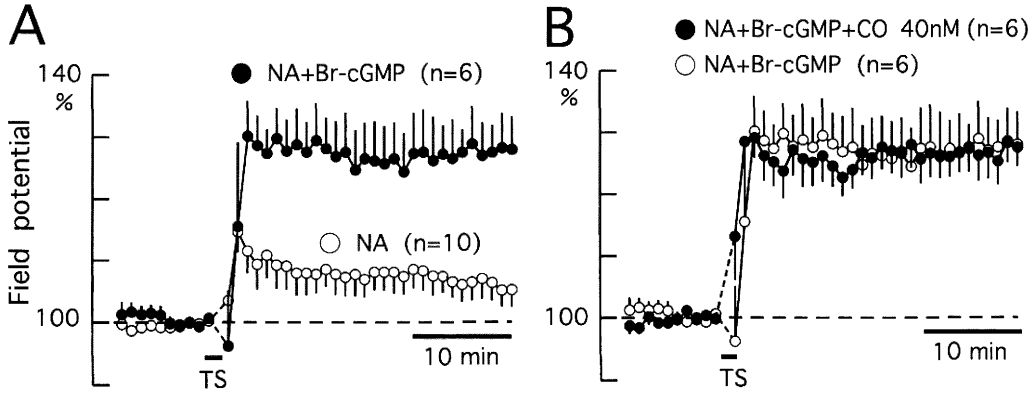

Fig. 3. Interaction between NO/cGMP signaling and CO. **A.** LTP_V recorded in the presence of 10 μM NA alone (*open circles*), and LTP_V recorded in the presence of 10 μM NA plus 1 mM Br-cGMP (*closed circles*). **B.** LTP_V recorded in the presence of NA and Br-cGMP (*open circles*, the same data shown in **A**) was not suppressed by 40 nM CO (*closed circles*).

suppress LTP_V elicited in the presence of 1 mM Br-cGMP. LTP_V was significantly suppressed by 30 μM ALA, which is expected to facilitate endogenous CO production. The principal conclusion deduced from these results is that exogenous or endogenous CO inhibits the induction of LTP_V via suppressing NO-induced cGMP formation. The rationale behind our conclusion is discussed below.

LTP_V in the auditory cortex

We have found that NOS inhibitors do not affect $LTP_{II/III}$, while they significantly suppress the induction of LTP_V (unpublished observation). The suppressive effect of NOS inhibitors on LTP_V was antagonized by Br-cGMP (Fig. 3A), indicating that the suppression of LTP_V results from that of NO-induced cGMP formation. Because NO-induced cGMP formation has been demonstrated in pyramidal neurons,[29-31] the target of NO signaling is probably pyramidal cells in layer V. The pyramidal neurons in layer V project to subcortical structures such as the cochlear nucleus[32] and the inferior colliculus.[33] Therefore, LTP_V in the auditory cortex may serve to provide the long-term facilitation of feedback mechanisms in auditory information flow.

Involvement of CO in induction of LTP_V

HO has two isoforms,[34] of which HO-2, a constitutive isoform, is abundant in the neocortex.[35,36] It has been reported that endogenous CO produced by HO-2 may be involved in the induction of hippocampal LTP.[18] However, metaloporphyrins, which are used to block HO activity, have non-specific side effects such as the suppression of NOS[37] and guanylate cyclase activities,[38] or cytotoxic effects.[39] Knockout of the gene encoding HO-2 in mice produced no apparent be-

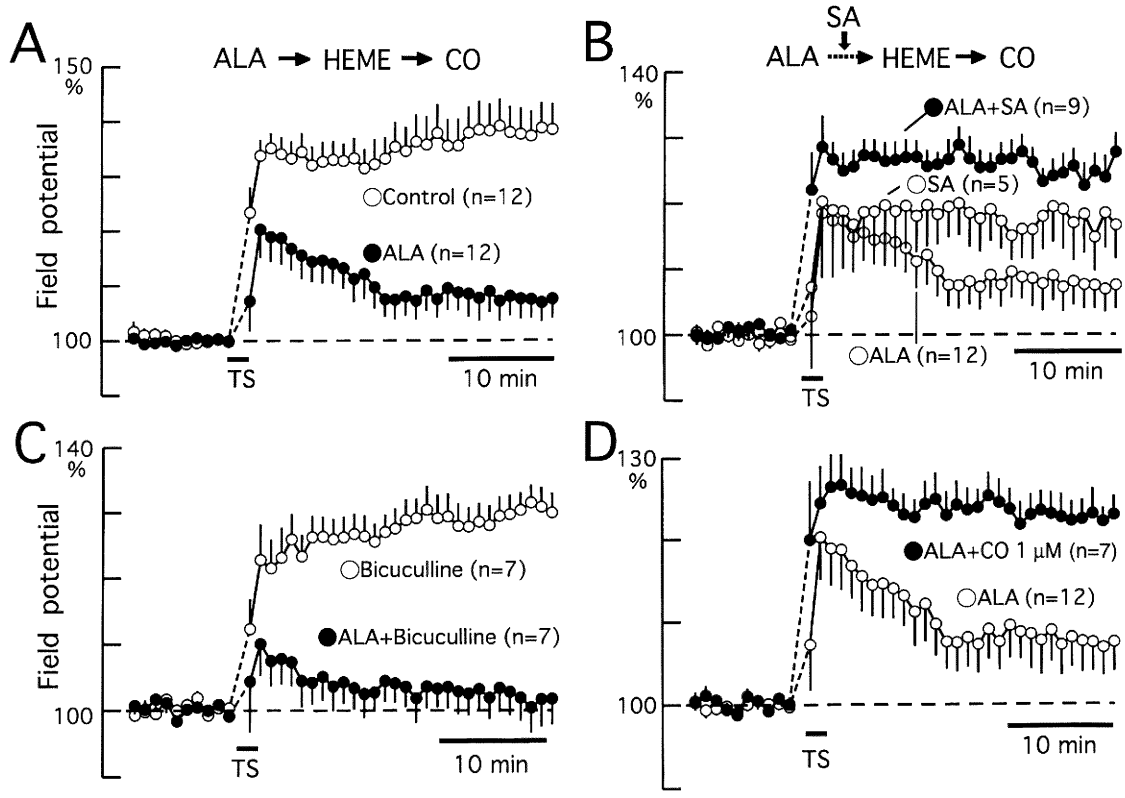

Fig. 4. Effect of ALA on LTP_V. **A.** LTP_V recorded in the slices incubated with 30 μM ALA for at least 30 min (*closed circles*). For comparison, the control LTP_V (*open circles*) is also shown. ALA is expected to facilitate endogenous CO production via the formation of heme. **B.** LTP_V recorded in the presence of 30 μM ALA plus 10 μM SA (closed circles, ALA+SA), 30 μM ALA alone (*open circles*, ALA; the same data shown in **A**) or 10 μM SA alone (*open circles, SA*). SA is expected to supperss Heme and CO synthesis from ALA. **C.** LTP_V recorded in the presence of 1 μM bicuculline (*open circles*) and 30 μM ALA plus 1 μM bicuculline (*closed circles*). **D.** LTP_V elicited in the presence of 30 μM ALA (open circles; the same data shown in **A**) and 30 μM ALA plus 1 μM CO (*closed circles*).

havioral abnormality or differences in hippocampal LTP.[40]

Although CO alone is a much weaker stimulant of cGMP formation than NO, it was recently reported that CO modulates NO-induced cGMP formation.[21] A characteristic feature of this modulation is that CO suppresses NO-induced cGMP formation at low concentrations while CO facilitates cGMP production at high concentrations. The characteristic CO dose-dependence was also observed in this study with respect to the suppression of LTP_V by CO (Fig. 2).

What is the molecular mechanism for the suppression of NO-dependent synaptic plasticity by CO? The suppression of LTP_V by CO might be attributed to toxic effects of CO on the neocortical slices. However, the main toxic effect of CO, the blockage of O_2 binding to hemoglobin,[41] is not critical in the slice preparation, in which O_2 is supplied by passive diffusion from the perfusion medium. Addition of Br-cGMP to the perfusion medium eliminated the suppressive effect of CO on LTP_V (Fig. 3B). NO-dependent LTP_V was suppressed by 40 nM CO whereas NO-independent $LTP_{II/III}$ was not (Fig. 1). These findings can be explained by the suppression of NO-induced cGMP formation by CO.[21] One problem, however, is the difference in the effective CO doses. The cGMP formation is suppressed at micromolar concentrations,[21] while in this study, 40 nM was sufficient to suppress LTP_V. Obviously, the CO sensitivity may be different between the neocortical slice preparations and cultured granule cells. Alternatively, the difference might be superficial, because CO dissolved in a solution escapes very rapidly into the air so that absolute concentrations of CO are very difficult to control. After taking into account the above considerations, we conclude that the suppression of LTP_V by CO in this study is attributed to the specific effect of CO on the suppression of NO-induced cGMP formation.

The suppression of LTP_V by ALA was antagonized by 1 μM CO (Fig. 4D). In this respect, it was very similar to the suppression of LTP_V by 40 nM CO. It has been demonstrated that the application of glycine facilitates the production of endogenous CO via the formation of ALA and heme.[20] Therefore, it is plausible that the effect of ALA can be attributed to the effect of endogenous CO produced from ALA. Other results in this study support this hypothesis. First, the suppression of LTP_V by ALA was antagonized by SA, which is an inhibitor of ALA dehydratase and blocks the synthesis of heme and CO from ALA.[27] Second, LTP_V was suppressed by ALA or 40 nM CO, while $LTP_{II/III}$ was not. Although ALA inhibits GABA release,[28] the suppression of LTP_V was not affected by bicuculline. All these results are consistent with the idea that the suppression of LTP_V by ALA is mediated by endogenous CO produced from ALA.

The regulatory mechanism for HO-2 activity is not well known.[42] The supply of a substrate such as ALA for HO-2 may be regulated by various stimuli. HO-1, another isozyme of HO, is induced by a number of stimuli.[35,43-45] Because of the importance of CO shown here and in other studies, it will be necessary to elucidate the mechanisms controlling endogenous CO production to understand more thoroughly the physiological roles of CO signaling.

Acknowledgments. We thank Y. Tamura and N. Taga for their technical assistance. This work was supported by grants from the Japanese Government, Toyota RIKEN and the Uehara Foundation.

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