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Different Modulation of Cholinergic Neuronal Systems by Dynorphin A (1–13) in Carbon Monoxide-Exposed Mice

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ABSTRACT. The effects of dynorphin A (1–13), a κ-opioid receptor agonist, on the content of acetylcholine (ACh) and high K^+-induced release of endogenous ACh were studied in mice exposed to carbon monoxide (CO). Mice were exposed to CO 3 times at 1-hr intervals and used 7 days after CO exposure. Administration of dynorphin A (1–13) (1.5 and 5.0 nmol/mouse, intracerebroventricularly) 15 min before killing significantly increased the ACh content in the striatum and hippocampus of control mice, but had no effect on the ACh content in CO-exposed mice. Dynorphin A (1–13) did not change the choline acetyltransferase (EC 2.3.1.6) activity in control or CO-exposed mice. The high K^+-induced endogenous ACh release from hippocampal slices in CO-exposed mice was significantly lower than that of controls, although exposure to CO did not affect the basal release of endogenous ACh from hippocampal slices compared with controls. Dynorphin A (1–13) caused dose-dependent decreases in high K^+-induced release of endogenous ACh from hippocampal slices in control mice. This inhibitory effect of dynorphin A (1–13) was blocked by co-perfusion with nor-binaltorphimine, a selective κ-opioid receptor antagonist. On the other hand, dynorphin A (1–13) did not decrease high K^+-induced release of endogenous ACh from hippocampal slices in CO-exposed mice. These results suggest that dysfunction of the cholinergic system occurred after exposure to CO, and as a result the inhibitory effects of dynorphin A (1–13) may be blocked in CO-exposed mice. BIOCHEM PHARMACOL **57**;11:1321–1329, 1999.

KEY WORDS. dynorphin A (1–13); carbon monoxide; cholinergic system; kappa opioid receptor; hippocampal slices; acetylcholine release

The opioid peptides and receptors have been shown to be present in specific neuronal pathways within the hippocampus [1–3] and are likely to act as neurotransmitters in this region [4, 5]. The potential roles of the endogenous dynorphin system in regulating hippocampal functions such as learning processes and in the regulation of neuronal excitability have been suggested by the effects of peptides applied into the brain [6–11], by increases in dynorphin peptide levels in age-impaired animals [12], and by alterations in dynorphin peptide levels following changes in the physiological activity of hippocampal neurons [13]. However, how endogenous dynorphins exert their effects in the hippocampus is unclear despite extensive electrophysiological studies [5, 14].

Multi-infarct dementia may be caused by a deficiency in the supply of oxygen and glucose to the brain as a result of impaired brain circulation. Transient ischemic attack is also known to induce a deficiency in the supply of oxygen and produce very slow but irreversible neuronal damage in the hippocampal CA1 subfield [15]. Furthermore, CO† has also been reported to cause hypoxia and deterioration of memory function [16, 17], and memory deficits develop insidiously in the days following recovery from CO intoxication in humans [18].

To investigate dementia and the development of learning and memory impairment, various hypoxic and ischemic animal models have been widely used. We have shown that ischemia and CO exposure produced impairment of spontaneous alternation performance and passive avoidance response in mice in a delayed manner (delayed amnesia) [19–21]. Moreover, delayed neuronal damage can also be produced after CO exposure in mice [22]. Using this model, we have demonstrated that these animals exhibit dysfunction of central cholinergic neurons in the frontal cortex, striatum, and hippocampus, which are important brain regions involved in learning and memory [19]. In addition, nefiracetam and tacrine, which activate cholinergic neuronal transmission, improve the memory deficiency induced

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† *Abbreviations:* ACh, acetylcholine; CO, carbon monoxide; ChAT, choline acetyltransferase; ACSF, artificial cerebrospinal fluid; HPLC-EC, high performance liquid chromatography with electrochemical detection; AUC, area under the curve; and NMDA, *N*-methyl-D-aspartate.

by CO exposure [23, 24]. Therefore, CO exposure can provide an amnesic model for the investigation of memory deterioration with cholinergic neuronal dysfunctions [25].

Although investigations of learning and memory have focused primarily on cholinergic neurotransmission, as mentioned above, reports of increased dynorphin A (1–8)-like immunoreactivity in the hippocampus of aged rats [12] and κ-opioid receptor density in the brain of Alzheimer's patients [26] suggest that disruption of opioidergic neurotransmission may also play a role in the cognitive deficits associated with Alzheimer's disease and aging. Recent studies have indicated that neuropeptides modulate learning and memory processes in experimental animals. Dynorphin A (1–13), a κ-opioid receptor agonist, improves the scopolamine- and mecamylamine-induced impairments of spontaneous alternation in mice [10, 27] and carbachol-induced impairments of learning and memory in rats [8]. Moreover, dynorphin A (1–13) ameliorates CO-induced delayed amnesia in mice [9, 10]. However, the biochemical mechanisms underlying the ameliorating effect of dynorphin A (1–13) in CO-induced amnesia models have not yet been elucidated. We investigated the effects of dynorphin A (1–13) on the cholinergic neuronal systems in mice exposed to CO.

MATERIALS AND METHODS

Animals

Seven-week-old male ddY mice (Japan SLC) were kept in a regulated environment (23 ± 1°, 50 ± 5% humidity), with a 12-hr light–dark cycle (light on 8 a.m.–8 p.m.) and given food and tap water *ad lib*. Experimental protocols concerning the use of laboratory animals were approved by the committee of Meijo University and followed the guidelines of the Japanese Pharmacological Society (Folia Pharmacol. Jpn., 1992, 99: 35A) and the interministerial decree of May 25th, 1987 (the Ministry of Education).

CO Exposure

Each mouse was put into a transparent plastic vessel (diameter 6 cm, height 10 cm) with a pipe feeding into it and two holes at the bottom to remove air. Mice were exposed to pure CO gas 3 times at 1-hr intervals at a rate of 10 cm^3/min until clonic convulsions were observed and maintained in that state for 6 or 10 sec in the vessel [23]. CO exposure therefore lasted for between 40 and 60 sec. Under these conditions, the mortality rate ranged from 10 to 20%. Previously, we showed that CO exposure induced hypothermia [22]. Thus, in the present study, mice were kept on a hot plate (KN-205D, Natsume) for 2 hr to maintain their body temperature at 38–39°.

Drugs

Dynorphin A (1–13) was purchased from Peptide Institute. Nor-binaltorphimine dihydrochloride (nor-binaltorphimine) was purchased from Research Biochemicals International. For the measurement of the content of ACh and ChAT activity in the mouse brain, dynorphin A (1–13) was dissolved in 0.9% saline solution and administered into the lateral ventricle (intracerebroventricularly, i.c.v.) of the mouse brain according to the method of Haley and McCormick [28] in a volume of 5 μL/mouse under brief ether anesthesia. For the measurement of ACh release from hippocampal slices, drugs were dissolved in ACSF.

Assay for ACh and Choline Content

Mice were exposed to CO and killed 7 days later. Dynorphin A (1–13) (0.5, 1.5, and 5.0 nmol/mouse, i.c.v.) was administered 15 min before killing. The animals were killed by microwave irradiation (Toshiba Microwave Applicator, TMW-4012) for 0.85 sec at 5 kW, and were decapitated. The brain was quickly removed and the frontal cortex, striatum, and hippocampus were dissected out. The brain samples were rapidly frozen at −110° and stored in a deep freezer at −80° until assayed.

The tissue was homogenized in 0.2 N perchloric acid containing 1.5 nmol ethylhomocholine as an internal standard. The homogenates were centrifuged at 18,600 *g* for 5 min and supernatants were adjusted to pH 3.0 using 1 M sodium acetate. Twenty μL samples were assayed for ACh and choline by HPLC-EC. The HPLC system consisted of a delivery pump (EP-300, Eicom), degasser (DG-300, Eicom), column oven (ATC-300, Eicom), guard column (Eicom), analytical column (Eicompak AC-GEL 6.0 × 150 mm, Eicom), enzyme column (AC-ENZ, Eicom), and an electrochemical detector (ECD-300, Eicom) with a platinum electrode (WE-PT, Eicom). The electrode potential was set to +450 mV versus Ag/AgCl reference electrode. The mobile phase was 0.1 M phosphate buffer (pH 8.5) containing 925 mM 1-octanesulfonic acid sodium salt, 13.4 mM EDTA-2Na, and 593 mM tetramethylammonium chloride. The flow rate was set at 1.0 mL/min.

ChAT Activity

The effects of dynorphin A (1–13) on ChAT activity in CO-exposed mice were measured by the method of Kaneda and Nagatsu [29], with some modifications. The enzyme solution was prepared from the frontal cortex, striatum, and hippocampus of mice by homogenization in 12.5 mL of 25 mM sodium phosphate buffer (pH 7.4) per gram of wet weight followed by centrifugation at 20,000 *g* for 60 min at 4°. The supernatant was used as the enzyme solution. The standard incubation mixture consisted of the following components in a total volume of 200 μL (final concentrations in parentheses): 100 μL of 0.1 M sodium phosphate buffer, pH 7.4 (0.05 M), containing 10 mM choline chloride (5 mM); 0.4 mM acetyl-CoA (0.2 mM); 0.2 mM eserine hemisulfate (0.1 mM); 0.3 M sodium chloride (0.15 M); and 20 mM EDTA-2Na (10 mM) and 100 μL of enzyme solution in 25 mM sodium phosphate buffer, pH

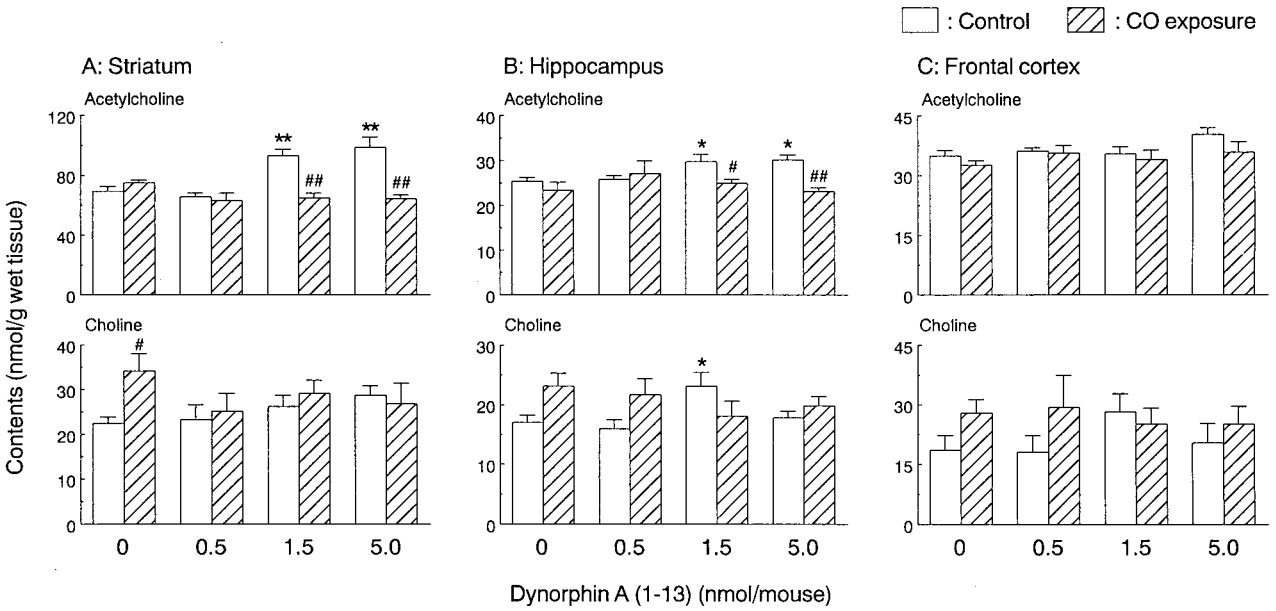

FIG. 1. Effects of dynorphin A (1–13) on the content of ACh and choline in the striatum, hippocampus, and frontal cortex of mice. Mice were exposed to CO and killed 7 days later. Mice were treated with dynorphin A (1–13) (0.5, 1.5, and 5.0 nmol/mouse, i.c.v.) 15 min before killing. Each value is the mean ± SEM of 6–11 mice. *$P < 0.05$, **$P < 0.01$ versus corresponding drug-free control (Bonferroni test). #$P < 0.05$, ##$P < 0.01$ versus corresponding non-CO-exposed group (Student's *t*-test).

7.4. Incubation was carried out at 37° for 20 min, and the reaction was stopped with 50 μL of 1 M perchloric acid in an ice bath. After 10 min, 6 μL of 20 mM isopropylhomocholine as an internal standard was added, and the reaction mixture was centrifuged at 18,600 *g* for 5 min. The supernatants were adjusted to pH 3.0 using 1 M sodium acetate, and 20 μL samples were assayed for resultant ACh by HPLC-EC, as described above. As the mobile phase, 0.1 M phosphate buffer (pH 8.5) containing 1.23 mM 1-decanesulfonic acid sodium salt, 13.4 mM EDTA-2Na, and 593 mM tetramethylammonium chloride was used. The flow rate was 0.6 mL/min. The protein content was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

Measurement of ACh Release from Hippocampal Slices

Mice were decapitated 7 days after CO exposure and the brain was quickly removed. The hippocampus was dissected out and slices 0.5 mm thick were prepared. The hippocampal slices were placed in a perfusion chamber (0.1 mL volume, Brandel) and perfused at a rate of 0.125 mL/min with ACSF containing 2 μM choline chloride and 10 μM eserine hemisulfate salt. The composition of the ACSF was as follows: 118 mM NaCl, 4.7 mM KCl (in high K^+ ACSF, 82.7 mM NaCl, 40 mM KCl), 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.1 mM glucose, pH 7.4. All media were saturated with 95% O_2 − 5% CO_2 and maintained at 37°. After a 60-min conditioning perfusion, perfusates were collected at 2-min intervals throughout the subsequent experiments. After collection of 3 fractions to determine whether basal release was stable, the slices were stimulated twice (S1, S2) with high K^+ ACSF for 2 min each. Drugs were applied for a period of 6 min at 4 min prior to S2 stimulation. To the perfusates was added 6 μL of 0.1 μM isopropylhomocholine as an internal standard followed by assay for ACh by HPLC-EC as described above.

Statistical Analysis

The data are presented as the means ± SEM. In the ACh release experiments, the total amount of ACh release (AUC) for each stimulation was determined as the sum of ACh release of 5 fractions after S1 stimulation minus the basal release (S1-AUC), and the sum of ACh release of 5 fractions after S2 stimulation minus the basal release (S2-AUC). Basal release was determined as a trapezoidal area of the 4th and 8th or the 12th and 16th fractions. The effects of drugs on ACh release are expressed as the ratio of S2-AUC/S1-AUC. One-way ANOVA followed by Bonferroni's test was used for statistical evaluation. Comparison of two groups was carried out using the Student's *t*-test.

RESULTS

Effects of Dynorphin A (1–13) on the Content of ACh and Choline in the Mouse Brain

The content of ACh did not change 7 days after CO exposure in the striatum, hippocampus, and frontal cortex compared with the control mice (Fig. 1, A, B, and C). In the striatum of CO-exposed mice, there was a significant increase in the choline content compared with the controls (Fig. 1A).

TABLE 1. Effects of dynorphin A (1–13) on the choline acetyltransferase activity in the striatum, hippocampus, and frontal cortex of mice

Dose (nmol/mouse)	Acetylcholine formed (nmol/hr/mg protein) Control	(N)	CO exposure	(N)
Striatum				
0	319.8 ± 21.1	(10)	353.0 ± 17.1	(10)
0.5	365.9 ± 14.7	(10)	351.6 ± 19.0	(10)
1.5	344.4 ± 17.1	(10)	368.7 ± 17.3	(10)
5.0	312.4 ± 14.1	(10)	337.8 ± 20.1	(10)
Hippocampus				
0	56.3 ± 1.4	(10)	61.8 ± 2.3	(10)
0.5	58.7 ± 1.8	(10)	57.1 ± 2.4	(10)
1.5	57.9 ± 2.4	(10)	60.3 ± 2.5	(10)
5.0	58.6 ± 2.1	(10)	55.4 ± 1.5	(10)
Frontal cortex				
0	64.4 ± 2.8	(10)	67.3 ± 4.2	(10)
0.5	66.1 ± 5.0	(10)	65.5 ± 4.0	(10)
1.5	69.6 ± 4.8	(10)	72.4 ± 5.1	(10)
5.0	67.3 ± 3.9	(10)	61.5 ± 3.6	(9)

Mice were exposed to CO and killed 7 days later. Mice were treated with dynorphin A (1–13) (0.5, 1.5, and 5.0 nmol/mouse, i.c.v.) 15 min before killing. Each value is the mean ± SEM of 9–10 mice.

Administration of dynorphin A (1–13) (1.5 and 5.0 nmol/mouse) significantly increased the ACh content in the striatum and hippocampus in non-CO-exposed mice (Fig. 1, A and B). Pretreatment with nor-binaltorphimine (4.9 nmol/mouse, i.c.v.) blocked the increment of ACh content in the striatum (control 69.5 ± 3.3, dynorphin A (1–13) alone 93.0 ± 4.5, nor-binaltorphimine + dynorphin A (1–13) 70.9 ± 3.6, N = 11) and hippocampus (control 25.5 ± 0.8, dynorphin A (1–13) alone 29.9 ± 1.6, nor-binaltorphimine + dynorphin A (1–13) 25.8 ± 1.7, N = 10–11). However, dynorphin A (1–13) did not change the ACh content in the striatum, hippocampus, or frontal cortex of CO-exposed mice (Fig. 1, A, B, and C). The content of ACh in the striatum and hippocampus of CO-exposed mice after administration of dynorphin A (1–13) (1.5 and 5.0 nmol/mouse) was significantly lower than that of the corresponding drug-treated groups (Fig. 1, A and B).

Effects of Dynorphin A (1–13) on ChAT Activity in the Mouse Brain

The ChAT activities in the striatum, hippocampus, and frontal cortex 7 days after CO exposure were not changed compared with the control group (Table 1). Dynorphin A (1–13) (0.5, 1.5, and 5.0 nmol/mouse) also had no effect on the ChAT activities in either control or CO-exposed mice (Table 1).

Calcium Dependence of the Release of Endogenous ACh

The demonstration of depolarization-evoked, calcium-dependent ACh release from nervous tissue preparations represents an important criterion for the identification of putative neurotransmitters. Exposure of hippocampal slices in superfusion to 40 mM KCl as a depolarizing agent elicited a large increase in endogenous ACh release (Fig. 2). The dependence on calcium ions was studied by omitting calcium during the depolarizing stimulus. The results clearly showed that at least 60% of the release of endogenous ACh evoked by 40 mM KCl from rat hippocampal slices was calcium-dependent.

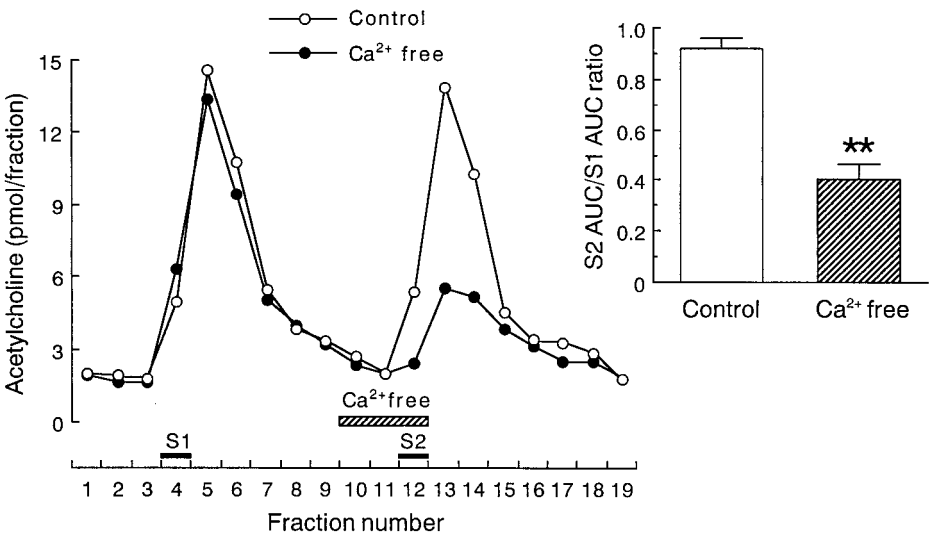

FIG. 2. Effect of Ca^{2+}-free buffer on the KCl-induced release of endogenous ACh from mouse hippocampal slices. Slices were stimulated with high K^+ buffer for 2 min at the 4th (S1) and 12th (S2) fractions (bars). Ca^{2+}-free buffer containing 1 mM EGTA was superfused for a 6-min period, beginning at 4 min before S2 stimulation (hatched bar). The total amount of ACh released (AUC) by each stimulation is the sum of the ACh content from the 4th to 8th fractions minus the basal release (S1 AUC), and the sum of the ACh content from the 12th to 16th fractions minus the basal release (S2 AUC). Each value is the mean ± SEM of 8 mice. The inset shows the S2 AUC/S1 AUC ratio. **$P < 0.01$ versus control (Student's *t*-test).

TABLE 2. Basal and KCl-induced release of endogenous acetylcholine from hippocampal slices in mice

Dose (nmol/mouse)	Control	CO exposure
	Acetylcholine (pmol/fraction)	
Basal release	1.890 ± 0.074	2.046 ± 0.090
	S1 AUC (% × min)	
KCl-induced release	3943.8 ± 220.1	2749.1 ± 150.1*

Mice were exposed to CO and killed 7 days later. Slices were stimulated with high K^+ buffer for 2 min at the 4th (S1) fraction. The total amount of ACh released (AUC) by the stimulation is the sum of ACh content from the 4th to 8th fractions minus the basal release (S1 AUC). Each value is the mean ± SEM of 55–56 mice. *$P < 0.01$ versus control (Student's *t*-test).

Effects of CO Exposure on Basal Release and KCl-induced ACh Release from Hippocampal Slices

CO exposure did not affect the basal release of endogenous ACh from hippocampal slices compared with controls (Table 2). In contrast, the high K^+-induced release of endogenous ACh from hippocampal slices in CO-exposed mice was significantly lower than that of control mice (Table 2).

Effects of Dynorphin A (1–13) on High K^+-induced Release of Endogenous ACh from Hippocampal Slices

Dynorphin A (1–13) (10^{-10} to 10^{-5} M) caused dose-dependent decreases in high K^+-induced release of endogenous ACh from hippocampal slices in control mice (Fig. 3). S2-AUC/S1-AUC ratios at concentrations of dynorphin A (1–13) of 10^{-8} to 10^{-5} M were significantly lower than those in the drug-free control group (Fig. 3B). The decrease in the S2-AUC/S1-AUC ratios at 10^{-6} M dynorphin A (1–13) was blocked by co-perfusion with norbinaltorphimine (10^{-5} M), a selective κ-opioid receptor antagonist (Fig. 4).

On the other hand, dynorphin A (1–13) at 10^{-10} to 10^{-6} M did not decrease high K^+-induced release of endogenous ACh from hippocampal slices in CO-exposed mice (Table 3). Only the highest concentration of dynorphin A (1–13) (10^{-5} M) significantly decreased ACh release as indicated by the S2-AUC/S1-AUC ratio (Table 3). To elucidate the difference in ACh release between control and CO-exposed mice, the S2-AUC/S1-AUC ratio was compared. The ratio of dynorphin A (1–13) at 10^{-8} to 10^{-6} M of CO-exposed mice was significantly higher compared with that of control (Table 3). There was no significant difference at a high concentration of dynorphin A (1–13) (10^{-5} M) (Table 3).

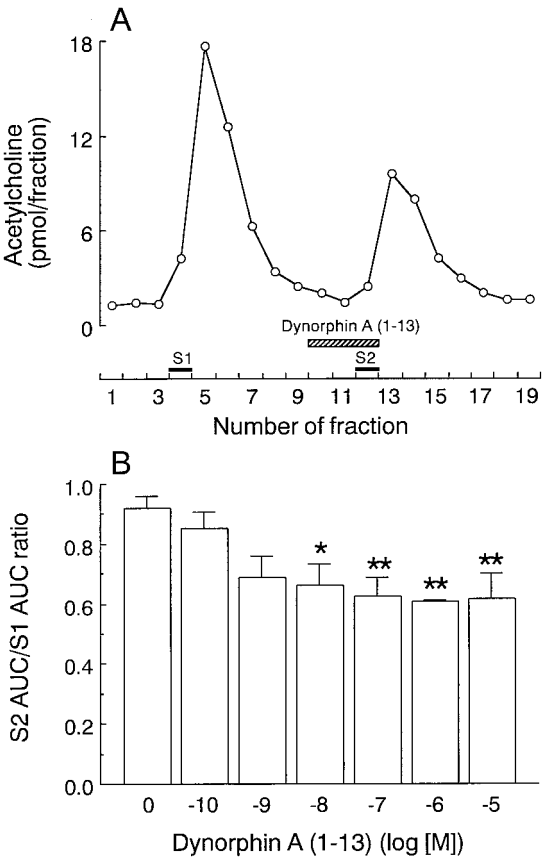

FIG. 3. Effects of dynorphin A (1–13) on the Ca^{2+}-dependent KCl-induced release of endogenous ACh from mouse hippocampal slices. (A) shows the data from dynorphin A (1–13) 10^{-6} M and (B) the S2 AUC/S1 AUC ratio. Slices were stimulated with high K^+ buffer for 2 min at the 4th (S1) and 12th (S2) fractions (bars). Dynorphin A (1–13) ($10^{-10} - 10^{-5}$ M) was applied for a 6-min period beginning at 4 min before S2 stimulation (hatched bar). The total amount of ACh released (AUC) by each stimulation is the sum of ACh content from the 4th to 8th fractions minus the basal release (S1 AUC), and the sum of those from the 12th to 16th fractions minus the basal release (S2 AUC). Each value is the mean ± SEM of 6–10 mice. *$P < 0.05$, **$P < 0.01$ versus drug-free group (Bonferroni test).

DISCUSSION

In the present study, dynorphin A (1–13) significantly increased the ACh content in the striatum and hippocampus of normal mice. Previous studies have shown that the κ-opioid receptor agonist ethylketocyclazocine reduced the release of ^{3}H[ACh] from slices of the rabbit hippocampus, and that the effect of ethylketocyclazocine was antagonized by (−)MR2266, a κ-opioid receptor antagonist [30]. In another investigation, dynorphin A (1–13) and U-50,488H, a selective κ-opioid receptor agonist, caused a dose-dependent decrease in high K^+-evoked ACh release from guinea pig striatal and hippocampal slices, whereas the κ-opioid receptor agonist was not effective in the rat [31]. However, the concentrations of the κ-agonists used in this latter study were very high (1–10 μM U-50,488H) in comparison with those needed to inhibit K^+-evoked dopamine release, i.e. 10–100 nM. These results suggest that activation of κ-opioid receptors decreases ACh release in a species-specific manner. Rommelspacher and Kuhar [32] reported that brain levels of ACh reflected changes in the firing rate of cholinergic neurons; an increase in firing rate was accompanied by a decrease in the level of this transmitter. Based on these prior studies, our results suggest that

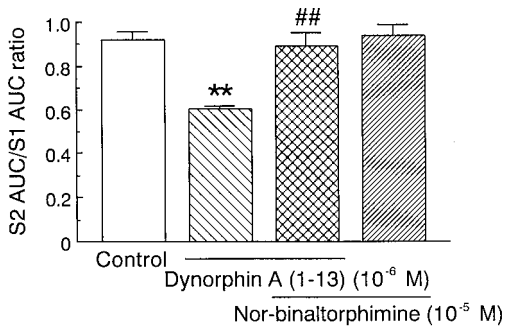

FIG. 4. Effects of dynorphin A (1–13) alone and in combination with nor-binaltorphimine on the KCl-induced release of endogenous ACh from mouse hippocampal slices. Slices were stimulated with high K^+ buffer for 2 min at the 4th (S1) and 12th (S2) fractions. Dynorphin A (1–13) (10^{-6} M) and/or nor-binaltorphimine (10^{-5} M) were applied for a 6-min period beginning at 4 min before S2 stimulation. The total amount of ACh released (AUC) by each stimulation is the sum of ACh content from the 4th to 8th fractions minus the basal release (S1 AUC), and the sum of those from the 12th to 16th fractions minus the basal release (S2 AUC). Each value is the mean ± SEM of 6–8 mice. **$P < 0.01$ versus drug-free control group, ##$P < 0.01$ versus dynorphin A (1–13)-treated group (Bonferroni test).

in the mouse, dynorphin A (1–13) decreased ACh release, and as a result, the ACh content increased. These increases in ACh content induced by dynorphin A (1–13) were not observed in CO-exposed mice. Dynorphin A (1–13) did not affect the ChAT activities in control or CO-exposed mice. Therefore, CO exposure seems to cause functional changes in the cholinergic neuronal systems that are differentially modulated by dynorphin A (1–13). To test this hypothesis, ACh release from hippocampal slices was measured in normal and CO-exposed mice.

The results of the present studies show that exposure of mice to CO caused a reduction in the high K^+-induced release of endogenous ACh from hippocampal slices without affecting the basal ACh release. Other presynaptic ACh markers, such as ACh content and ChAT activity, did not change after CO exposure. Similar observations were made after ischemia by Ishimaru *et al.* [33], who also showed a reduction in K^+-induced release of ACh by hippocampal cholinergic neurons at the 4th, 7th, and 14th day after reperfusion. Thus, as suggested earlier [25], CO exposure, like ischemia, causes a dysfunction of cholinergic nerve terminals.

In agreement with previous results, high concentrations of dynorphin A (1–13) inhibited the high K^+-induced ACh release from hippocampal slices, an action that was completely blocked by co-perfusion with the selective κ-opioid receptor antagonist, nor-binaltorphimine. Since the concentration of nor-binaltorphimine used in the present experiment was slightly high, we cannot rule out the possibility that the reversing effect by nor-binaltorphimine is mediated via not only κ- but also μ-opioid receptors. The decrease in the high K^+-induced ACh release of dynorphin A (1–13) was attenuated in hippocampal slices of CO-exposed mice, suggesting that CO exposure may down-regulate κ-opioid receptors that modulate ACh release. Although dynorphin A (1–13) did not modify the basal release of ACh release from hippocampal slices in CO-exposed mice (Fig. 4), our previous results suggest that κ-opioid receptors may not regulate cholinergic neurons positively under resting conditions, because a κ-opioid receptor antagonist did not change ACh release in normal rats [7]. Instead, these receptors may affect ACh release only when cholinergic neurons are stimulated, such as after depolarization, or impaired, such as after exposure to CO or to drugs which decrease ACh release [7, 8].

Cerebral ischemia causes delayed neuronal damage as a result of the excess release of excitatory amino acids such as glutamate and aspartate [34–36]. Cerebral ischemia and CO exposure also induced cholinergic dysfunction in the hippocampus and other brain regions susceptible to hypoxia [25, 37]. On the other hand, Fried and Nowak [38] reported that transient bilateral carotid artery occlusion reduced dynorphin A immunoreactivity in the hippocampus by 30–40% as early as 1 hr after recirculation, and this change was maintained at 50% of the control level for at least 1

TABLE 3. Effects of dynorphin A (1–13) on the Ca^{2+}-dependent KCl-induced release of endogenous acetylcholine from hippocampal slices of control and CO-exposed mice

	S2 AUC/S1 AUC ratio		
Concentrations [M]	**Control**	**CO**	*P* values
Dynorphin 0	0.919 ± 0.040 (8)	0.876 ± 0.037 (6)	0.606
Dynorphin 10^{-10}	0.852 ± 0.054 (6)	0.860 ± 0.069 (6)	0.423
Dynorphin 10^{-9}	0.686 ± 0.071 (6)	0.824 ± 0.057 (6)	0.109
Dynorphin 10^{-8}	0.661 ± 0.069* (8)	0.941 ± 0.092 (6)	0.039‡
Dynorphin 10^{-7}	0.624 ± 0.062† (10)	0.971 ± 0.030 (8)	0.001§
Dynorphin 10^{-6}	0.605 ± 0.009† (6)	0.866 ± 0.026 (6)	0.004§
Dynorphin 10^{-5}	0.617 ± 0.082† (7)	0.648 ± 0.026* (7)	0.848

Mice were exposed to CO and killed 7 days later. Slices were stimulated with high K^+ buffer for 2 min at the 4th (S1) and 12th (S2) fractions (bars). Dynorphin A (1–13) (10^{-10} – 10^{-5} M) was applied for a 6-min period beginning at 4 min before S2 stimulation (hatched bar). The total amount of ACh released (AUC) by each stimulation is the sum of the ACh content from the 4th to 8th fractions minus the basal release (S1 AUC), and the sum of those from the 12th to 16th fractions minus the basal release (S2 AUC). Each value is the mean ± SEM of 6–10 mice.

*$P < 0.05$, † $P < 0.01$ versus drug-free group (Bonferroni test); ‡$P < 0.05$, §$P < 0.01$ versus corresponding dynorphin concentrations (Student's *t*-test).

week. Dynorphin release is evoked by cerebral ischemia [39]. Taken together, these results suggest that κ-opioid as well as cholinergic systems in the hippocampus are damaged by hypoxic conditions. Our recent studies demonstrated that exogenously administered dynorphin A (1–13) and U-50,488H improved CO-induced delayed amnesia [6, 9, 10]. Therefore, a reduced dynorphin A level in the brain may also contribute to learning and memory impairments after CO exposure. Experiments to evaluate this hypothesis by measuring dynorphin content and dynorphin mRNA levels are currently in progress in our laboratory.

Dynorphin was found to have dual effects on NMDA synaptic currents, increasing currents at low concentrations and decreasing them at high concentrations. Only the inhibitory action of dynorphin was sensitive to naloxone, indicating that this effect was mediated by an opioid receptor [14]. These workers demonstrated that exogenously applied dynorphin had a biphasic concentration–response relationship. At high concentrations, exogenous dynorphin inhibited the NMDA receptor-mediated synaptic current, and at low concentrations dynorphin enhanced the current [14]. Several studies have suggested that dynorphin may play a role in the regulation of NMDA receptors [40–43]. In these studies, selective antagonists to the NMDA receptor complex blocked the effects of exogenously applied dynorphin. The results of these experiments suggested that dynorphin may potentiate NMDA receptor function. However, several other studies have demonstrated that dynorphin has inhibitory actions in the central nervous system. We have recently reported that dynorphin A (1–13) and U-50,488H could not improve NMDA receptor antagonist-induced impairment of spontaneous alternation behavior [10] and step-through-type passive avoidance behavior [44].

It has been reported that dynorphin A (1–13) exerts so-called 'non-opioid effects' [45]. We have recently shown that a des-[tyrosine^1]-dynorphin analog, dynorphin A (2–13), improved scopolamine- and CO-induced learning and/or memory impairment [46]. Taken together with the present results, dynorphin A (1–13) may have both κ-opioid receptor-mediated and 'non-opioid effects', and both mechanisms may be involved in the ameliorative effects of dynorphin A (1–13).

Our previous study indicated that dynorphin A (1–13) (0.5–5.0 nmol, i.c.v.) did not affect ACh release in the hippocampus of normal rats using an *in vivo* microdialysis technique [7, 47]. However, dynorphin A (1–13) ameliorated galanin-induced impairments of learning and memory accompanied by abolition of reductions in ACh release via κ-opioid receptors [7]. We proposed that dynorphin A (1–13) activates cholinergic systems directly and/or indirectly only when the cholinergic systems are impaired by CO exposure, and thereby improves CO-induced delayed amnesia [9]. The results of this and of previous studies suggest that dysfunction of the cholinergic system occurs after exposure to CO, and as a result dynorphin A (1–13) modulates cholinergic neuronal systems differently in CO-exposed mice. This might be one of the mechanisms by which dynorphin A (1–13) ameliorates CO-induced impairments of learning and memory.

Although the results of this and of previous studies with dynorphin indicate that the role of endogenous dynorphin in the central nervous system is extremely complex, κ-opioid receptor agonists might be effective in the treatment of various forms of cognitive disturbances related to dysfunction of the presynaptic cholinergic system, with beneficial effects on learning and memory. However, considerable research is still necessary to fully understand the potential utility of κ-opioid receptor agonists in the treatment of cognitive dysfunction.

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