Effects of Bifemelane Hydrochloride (MCI-2016) on Acetylcholine Level Reduced by Scopolamine, Hypoxia and Ischemia in the Rats and Mongolian Gerbils

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Accepted April 24, 1985

Abstract—Effects of bifemelane hydrochloride (MCI-2016) on acetylcholine (ACh) level in the cerebral cortex and hippocampus of rats and Mongolian gerbils were examined. In normal rats, MCI-2016 (30 mg/kg, i.p.) slightly increased ACh content in the cerebral cortex. Scopolamine (1 mg/kg, i.p.) or hypoxia (95% N₂ + 5% O₂, 9 min) decreased ACh level and pretreatment of MCI-2016 attenuated the decrement of ACh level in the rats. ACh level in the brain of Mongolian gerbils was significantly decreased following ligation of bilateral carotid arteries. In this case, MCI-2016 also attenuated the decrement of ACh level. These results suggest that improvement by MCI-2016 of behavioral impairment observed in the animals treated with scopolamine, hypoxia or ischemia may be, at least partly, attributed to the amelioration of decreased ACh level in the brain.

Our recent investigations indicated that bifemelane hydrochloride (MCI-2016) 1) improved the scopolamine-induced amnesia (1 and A. Tobe et al., unpublished data) 2) protected the spontaneous motor activity reduced by hypoxia (2), 3) prolonged the survival time in hypoxia or KCN-induced anoxia (3), and 4) had EEG arousal action (4). MCI-2016 potentiated the effects of physostigmine, and the effects of MCI-2016 were antagonized by atropine. These results led us to anticipate that the cholinergic mechanism is partly involved in the improving effects of MCI-2016. In order to ascertain neurochemically the cholinomimetic action of MCI-2016, effects of MCI-2016 on the acetylcholine (ACh) content in the brain of rats treated with scopolamine or hypoxia were examined.

It is known that the collateral blood supply from the vertebral-basilar system of the Mongolian gerbil (M. gerbil) is insufficient to prevent the cerebral infarction when the carotid flow is blocked (5). We previously reported that MCI-2016 prolonged survival time and inhibited the reduction of norepinephrine (NE) and serotonin (5-HT) contents in bilaterally carotid-artery-ligated M. gerbils (6). In the present study, the change of ACh level following cerebral ischemia and the effect of MCI-2016 were also examined.

Materials and Methods

Animals: Male Wistar rats (175–250 g) and male M. gerbils (80–90 g) were purchased from Japan Laboratory Animals, Inc. and housed in groups of 5 or 6 with a 12 hr-light-dark cycle (light on 7:00 A.M.).

Drugs: Bifemelane hydrochloride (MCI-2016, 4-(o-benzylphenoxy)-N-methylbutylamine hydrochloride) was synthesized in our laboratory. Scopolamine hydrobromide was purchased from Sigma. These were dissolved in saline, which was used as a control.

Time course of effects of MCI-2016 on ACh content in normal rats: MCI-2016 was
administered at 15 or 30 mg/kg intraperitoneally or at 100 mg/kg orally at 1, 2 and 4 hr prior to the sacrifice by microwave irradiation (Toshiba TMW-6402A, 4.8 kW, 1.1 sec).

Scopolamine treatment: Scopolamine (1 mg/kg, i.p.) was injected 30 min before sacrifice. MCI-2016 (30 mg/kg, i.p.) or saline was given 30 min prior to the administration of scopolamine.

Exposure to hypoxic condition: MCI-2016 (50 mg/kg, p.o.) or saline was administered daily for 5 days. At 1 hr after the last treatment, rats were exposed at ambient pressure to a gas mixture with a low oxygen content (95% N2+5% O2) by placing the rats in a small chamber (30×20×20 cm), and the gas was maintained at a steady flow of 10 liter/min for a period of 9 min. Rats were killed by microwave irradiation 1 hr after the exposure to hypoxia.

Ischemia: M. gerbils were used. MCI-2016 (25 mg/kg, i.p.) or saline was injected 30 min before the ligation. Cerebral ischemia was performed as previously described (6). Briefly, under light ether anesthesia, bilateral carotid arteries were ligated with great caution to avoid vagal irradiation or injury. Animals were sacrificed by microwave irradiation 1 hr after the ligation.

Determination of ACh: After the microwave irradiation, the cerebral cortex and hippocampus were removed and weighed. ACh was extracted by the method of Toru and Aprison (7), with some modifications. Tissues were homogenized in 6–10 vol. of 15% 1 N formic acid mixed in acetone (v/v) and centrifuged at 12,000 rpm for 20 min. Aliquots of the supernatant were evaporated and were diluted with leech Locke-Ringer's solution and adjusted to pH 7.2 with 0.1 N NaOH. The leech Locke-Ringer's solution had the following composition: 86.3 mM NaCl, 3.08 mM KCl, 1.17 mM CaCl2, 0.68 mM MgCl2, 1.36 mM NaHCO3, 6.22 mM glucose and 50 nM eserine salicylate. Bioassay of ACh was performed by the micro-organ bath method of Kadota and Nagata (8) using dorsal muscle strips of the Japanese medical leech.

Statistical analysis: Statistical differences were evaluated using the two-tailed Student's t-test. All values were expressed as the mean±S.E.M.

Results

Effects of MCI-2016 on ACh level in the cerebral cortex and hippocampus of rats:
The time course of effects of MCI-2016 on ACh level is shown in Table 1. ACh level in the cerebral cortex was gradually increased after the injection of MCI-2016 (30 mg/kg, i.p.), and at 4 hr, it was significantly higher than that of the control. In the case of oral administration of MCI-2016 (100 mg/kg), ACh content in the cerebral cortex changed in a similar manner, but this change was not statistically significant. On the other hand, ACh level in the hippocampus was scarcely

| Drugs               | Cerebral cortex | Hippocampus |
|---------------------|-----------------|-------------|
|                     | 1 hr            | 2 hr        | 4 hr        | 1 hr        | 2 hr        | 4 hr        |
| Control 1 (saline, i.p.) | 19.9±0.7       | 17.7±0.7    | 17.6±0.9    | 22.6±2.2    | 25.0±1.7    | 23.4±1.4    |
| MCI 2016 (15 mg/kg, i.p.) | 17.7±1.1       | 19.3±1.2    | 17.6±1.3    | 23.8±2.2    | 24.1±1.5    | 25.7±1.6    |
| MCI-2016 (30 mg/kg, i.p.) | 20.1±2.1       | 20.7±1.6    | 24.2±2.0*   | 23.5±2.7    | 21.5±1.2    | 25.3±2.2    |
| Control 2 (saline, p.o.) | 18.7±1.4       | 17.5±0.9    | 17.9±1.4    | 23.2±1.3    | 22.4±1.4    | 23.2±1.2    |
| MCI-2016 (100 mg/kg, p.o.) | 18.2±1.0       | 18.8±1.1    | 20.0±1.7    | 22.9±1.0    | 23.5±1.4    | 24.9±2.3    |

Each value represents the mean±S.E.M. of 6 experiments. *P<0.05 vs. control 1.
affected by the administration of MCI-2016.

**Effects of MCI-2016 on ACh level decreased by scopolamine:** In the rats treated with scopolamine (1 mg/kg, i.p.), ACh level was decreased to 74% and 70% of the control in the cerebral cortex and hippocampus, respectively (Fig. 1). The fall in the concentration of ACh in each region was attenuated by the pretreatment of MCI-2016 (30 mg/kg, i.p.).

**Effects of MCI-2016 on ACh level following exposure to hypoxic condition:** In the rats treated with MCI-2016 for 5 days (50 mg/kg, p.o.), ACh level in the cerebral cortex was slightly higher than that of the control (Fig. 2). One hr after exposure to the hypoxic condition, ACh contents in the cerebral cortex of the control group were reduced by 13%, compared to the non-hypoxic control group, and the decrement of ACh level was attenuated by the treatment of MCI-2016, and the level was significantly higher than that of hypoxic control group.

**Effects of MCI-2016 on ACh level in the M. gerbils with cerebral ischemia:** Following the ligation of the bilateral carotid arteries, ACh level was significantly decreased to 23% and 40% of the sham control in the cerebral cortex and hippocampus, respectively (Fig. 3). Pretreatment of MCI-2016 (25 mg/kg, i.p.) attenuated the decrease in ACh level in both regions. This dose of MCI-2016 produced no change of ACh level in the brain of normal M. gerbils (Table 2).

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**Fig. 1.** Effects of MCI-2016 on scopolamine-induced ACh depletion in the cerebral cortex and hippocampus. Rats were injected with saline or MCI-2016 30 min prior to the administration of saline or scopolamine and sacrificed 30 min after the second injection by microwave irradiation. White column: control. Black column: scopolamine (1 mg/kg, i.p.) treatment. Striped column: scopolamine (1 mg/kg, i.p.) and MCI-2016 (30 mg/kg, i.p.) treatment. Each value represents the mean±S.E.M. of 6 experiments. *P<0.05 vs. control.

**Fig. 2.** Effects of MCI-2016 on hypoxia-induced ACh depletion in the cerebral cortex and hippocampus. Rats were administered saline or MCI-2016 for 5 days and exposed to the hypoxic condition (95% N₂+5% O₂, 9 min) 1 hr after the last injection and then sacrificed by microwave irradiation 1 hr after the hypoxia treatment. White column: control. Striped column: MCI-2016 (50 mg/kg, p.o.) treatment. Each value represents the mean±S.E.M. of 10 experiments. *P<0.05 vs. hypoxia-control.
Table 2. Effects of MCI-2016 on ACh level in the cerebral cortex and hippocampus of M. gerbils

|                     | Cerebral cortex | Hippocampus |
|---------------------|----------------|-------------|
| Control             | 12.7±1.5       | 17.5±1.4    |
| MCI-2016 (25 mg/kg, i.p.) | 13.1±3.8       | 20.4±3.5    |

Each value represents the mean±S.E.M. of 8 or 9 experiments. MCI-2016 was injected 1.5 hr prior to sacrifice by microwave irradiation.

Discussion

We investigated the change of ACh level by scopolamine, hypoxia or ischemia, because these treatments are thought to be concerned in impairment of cholinergic neurons in the central nervous system (9–11).

The effects of MCI-2016 on ACh level were also examined. ACh level was decreased by scopolamine or hypoxia and MCI-2016 attenuated the decrease in ACh level by these treatments. We reported previously that MCI-2016 ameliorated the scopolamine induced deficit of spontaneous alternation behavior (1) and of passive avoidance response (A. Tobe et al., unpublished data), and it protected spontaneous motor activity reduced by hypoxia (2). Therefore, improvement by MCI-2016 of scopolamine induced memory impairment and of spontaneous motor activity reduced by hypoxia may be, at least partly, attributed to the attenuation of decreased ACh content in the brain.

The mechanism by which scopolamine decreased ACh level may be as follows: ACh release is stimulated by blocking the muscarinic receptors and the rate of ACh synthesis does not keep up the increased rate of ACh release, resulting in decrement of ACh content. In the case of hypoxia, Gibson et al. (10) and Gibson and Duffy (12) reported that ACh synthesis was impaired in mild anemic hypoxia (injection of NaNO2, 75 mg/kg, s.c.) or in mild hypoxic hypoxia (exposure to 85% N2+15% O2). Thus, the decrement of ACh level observed in this study by hypoxia may be due to the impairment of ACh synthesis. Consequently, attenuating effects of MCI-2016 on the decrease in ACh level by scopolamine or hypoxia may be involved in the inhibition of ACh release or improvement of ACh synthesis impairment. However, the former seems unlikely because our previous studies indicated MCI-2016 may activate cholinergic neurons in the central nervous system (1–3). Furthermore, MCI-2016 was observed to
enhance high K+ induced ACh release in vitro (K. Saito et al., unpublished data). Then, MCI-2016 may improve the ACh synthesis impairment. ACh synthesis in the brain is regulated by many factors such as the supplies of choline and acetyl CoA or choline acetyltransferase activity. To clarify the precise mechanism of MCI-2016, the effects of MCI-2016 on these factors are currently being investigated.

The sensitivity of MCI-2016 to ACh level seems to be higher in the cerebral cortex than in the hippocampus, because MCI-2016 increased ACh level in the cerebral cortex but not in the hippocampus of normal rats and protected the decrease in ACh content by hypoxia only in the cerebral cortex.

It is well known that bilateral occlusion of the carotid arteries produces cerebral ischemia in the M. gerbils, and several investigators (13-15) including our group (6) have reported that changes occur in NE, 5-HT and dopamine (DA). However, there is no report concerning ACh level following bilateral occlusion of the M. gerbils. In the present study, we showed that ACh level was significantly decreased in the brain 1 hr after bilateral occlusion and that pretreatment of MCI-2016 attenuated this decrement. In the previous data, MCI-2016 attenuated NE and 5-HT contents decreased by ischemia (6). The decrement of ACh content was much greater than that of NE or 5-HT. It has been reported that in mild hypoxia, ACh level (16) but not NE and 5-HT (17) is decreased in the brain. ACh synthesis also may be more sensitive to hypoxia than energy metabolism (10, 12). Thus, the protective effect of MCI-2016 on decreased ACh level following ischemia may be one of the beneficial properties. The possibility of cholinergic, noradrenergic and serotonergic interaction in the action of MCI-2016 is also suggested. However, it is uncertain at present whether MCI-2016 influences equally these neurons or primarily affects one of them, for example, cholinergic neurons, resulting in activation of the other neurons. MCI-2016 did not affect the blood pressure elevation following ligation of the carotid arteries of rats (18) and improved the abnormal EEG which appeared after recirculation (19).

It is interesting that MCI-2016 at a dose which did not change the ACh level in normal animals improved the decrease in the level by the treatments such as scopolamine, hypoxia or ischemia. MCI-2016 may be particularly effective under hypofunction of neurons in the brain.

In summary, pretreatment of MCI-2016 attenuated the decrease in ACh level by scopolamine, hypoxia or ischemia. This effect, in part at least, may be involved in the improvement by MCI-2016 of amnesia or impairment of spontaneous motor activity or survival time following the above treatments.

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