Effects of Nefiracetam on Cerebral Adenylyl Cyclase Activity in Rats with Microsphere Embolism-Induced Memory Dysfunction

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The effects of nefiracetam on the cerebral adenylyl cyclase (AC) activity of animals with microsphere embolism-induced memory dysfunction were examined. Sustained cerebral ischemia in the right cerebral hemisphere was induced by an injection of microspheres into the right internal carotid artery of rats. To examine learning and memory function, the water maze test was performed from day 7 to day 10 after the operation. The escape latency of the microsphere-embolized (ME) rat in the water maze task was longer than that of the sham-operated (Sham) rat, suggesting that spatial memory dysfunction occurred in the ME rat. Gsα and Gi1,2α protein levels in the cerebral cortex, striatum and hippocampus of the ME rat, when determined on day 11, were similar to those of the Sham rats. The basal AC activity in the striatum, but not in the other two regions, of the ME rat decreased. The AC activity in the presence of 10 μM colforsin daropate (Col), a direct stimulator of AC, was increased by approximately 20-fold in sham animals and 7–10-fold in the ME rat. Treatment of the ME rat with 10 mg/kg/d nefiracetam p.o. from day 1 to day 10 after the operation shortened the escape latency, restored the basal AC activity in the striatum, and reversed the Col-induced increases in AC in these three regions without any changes in the cerebral Gsα and Gi1,2α protein levels. These results suggest that nefiracetam-mediated activation of AC activity may contribute to the improvement of memory and learning function in sustained cerebral ischemia.

Key words adenylyl cyclase; nefiracetam; memory function; cerebral ischemia; G protein

Adenylyl cyclase (AC) is a key enzyme for producing for cAMP that is the second messenger for intracellular signal transduction in various kinds of cells. Several reports have shown that AC may play an important role in learning and memory function. For example, Guillow et al. have shown the enhancement of AC activity after learning in the barpressing task, and an increase in Ca2+-sensitive AC activity after a spatial learning test. Thus, it is possible that changes in AC activity may affect learning and memory function in animals and humans.

Nefiracetam, N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, has been developed as a nootropic drug in animals and humans. Several investigators have shown the anti-amnesic effect of nefiracetam on scopolamine-, benzodiazepine-, and cycloheximide-treated animals. Furthermore, Yamada et al. (1999) have shown that treatment of the rat with nefiracetam improved β-amyloid-induced impairment of learning and memory function. The effects of nefiracetam are conceived to be mediated through the cholinergic system and/or GABAergic system. The exact mechanism underlying the anti-amnesic effect of this drug, particularly concerning the signal transduction system, is unknown. Recently, Itoh et al. have suggested that nootropic effects of nefiracetam may be exerted via an increase in intracellular cAMP using the mice administered morphine. It remains unclear, however, whether nefiracetam may modify cerebral AC activity.

In the present study, we attempted to explore the profile of AC activity, an initial step of the AC/cAMP/protein kinase A (PKA) signal transduction pathway, in animals with cerebral ischemia-induced learning and memory dysfunction, and then to examine the effects of nefiracetam. We used microsphere embolism-induced cerebral ischemia as a cerebral ischemic model. In previous studies, we showed that microsphere embolism produced wide-spreading emboli in the ipsilateral hemisphere, resulting in cerebral infarction and also in the impairment of cerebral energy metabolism and neurotransmitter metabolism. Furthermore, this model revealed spatial memory dysfunction in the water maze task.

MATERIALS AND METHODS

Animals Male Wistar rats (Charles River Japan, Atsugi, Japan), weighing 180—220 g, were used in the present study. The animals were conditioned to an environment of 23±1 °C, a constant humidity of 55±5%, and a cycle of 12-h light/12-h darkness, and given free access to food and tap water according to “The Guide for the Care and Use of Laboratory Animals” as promulgated by the National Research Council. The protocol of this study was approved by “The Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Science.”

Induction of Microsphere Embolism in Rat Brain Microsphere-induced cerebral embolism was performed by the method of Miyake et al. with minor modification. In brief, rats were anesthetized with 40 mg/kg pentobarbital i.p. The right carotid and pterygo-palatine arteries were ligated with surgical strings. A needle connected to a polyethylene catheter (3 Fr, Atom Co., Tokyo Japan) was inserted into the right common carotid artery. Seven hundred microspheres with a diameter of 47.5±0.5 μm (New England Nuclear, Boston, MA, U.S.A.), suspended in 20% dextran solution, were injected into the right carotid artery through the cannula. The needle was removed, then the needle bore was closed with surgical glue. Thereafter, the blood was recirculated. Rats that underwent a sham operation were injected with the same volume of vehicle without microspheres.
Fifteen hours after the operation, the neurological deficits of microsphere-embolized (ME) rats were scored on the basis of paucity of movement, truncal curvature, and forced circling during locomotion, which were considered to be typical symptoms of stroke in rodents.\(^{19,20}\) The score of each item was rated from 3 to 0 (3, very severe; 2, severe; 1, moderate; 0 normal). Animals with a total score of 7—9 points were used in the present study.

**Treatment with Nefiracetam** After examination of stroke-like symptoms of microsphere-injected rats at 15 h after the operation, the animals were randomly divided into the two groups. Nefiracetam at a dose of 10 mg/kg, suspended with 0.5% carboxymethyl cellulose, was administered into the stomach by gavage, and the administration was continued up to 10 d after the operation. The vehicle was administered to the untreated group. The dose employed in the present study was based on findings described by others.\(^5\) and our dose–response measurements were assessed in a preliminary study: treatment with 3 mg/kg/d. p.o., shortened the escape latency to a lesser degree than that with 10 mg/kg/d.

The water maze test and the examination of biochemical variables were conducted at least 2 h after daily administration of the agent.

**Water Maze Task** Water maze task of the ME and Sham rats was examined as described previously.\(^{17}\) Briefly, the animals were released into a circular pool with a diameter of 1.7 m, which was filled with a 30 cm depth of water at 23 °C. When the rat reached the platform, it was kept there for 30 s. The test was started from day 7 to day 10 after the operation, since the neurological deficits described above were appreciably attenuated by day 7. The visible platform test was performed on day 11 to ensure that animals had no visual, motor, and sensory deficits that would impede learning. Three out of 19 ME rats (2 for the ME group and 1 for nefiracetam-treated ME (MN) group) were eliminated due to circling during locomotion, which were considered to be typical symptoms of stroke in rodents (Fig. 1). The protein concentration was determined in the presence of 10 \(\mu\)M Col to stimulate adenylate cyclase in the isolated membrane.

**Isolation of Membrane Fraction** To determine the cerebral Gs and Gi proteins or to measure adenylyl cyclase activity after the water maze test on day 11, the membrane fraction was prepared according to the methods of McMahon and our dose–response measurements were assessed in a preliminary study: treatment with 3 mg/kg/d. p.o., shortened the escape latency to a lesser degree than that with 10 mg/kg/d.
Changes in Body Weight  Changes in body weights of the sham-operated, nefiracetam-treated sham-operated, ME and nefiracetam-treated ME rats are shown in Fig. 2. The operated animals showed a decrease in the body weight on days 1 to 3. Thereafter, the body weights gradually increased with time after the operation. There were significant differences in body weight between the sham-operated and microsphere embolized groups (two-way ANOVA, followed by Scheffe’s t-test). Treatment with nefiracetam did not enhance significantly body weight gain.

Escape Latency in the Water Maze Task  Figure 3 shows the escape latency in the water maze task of the ME and Sham rats on days 7 to 10 after the operation. The time to reach the goal in the water maze test was markedly shortened from the 2nd trial of day 7 in the Sham rat. Although the time to reach the goal in the ME rat was also shortened at the 3rd trial of day 7, the degree of time shortening was smaller than that of the Sham group. In all trials of days 8 to 10, the escape latency of the ME group was always longer than that of the Sham group. When the ME rat was treated with nefiracetam, the escape latency shortened from the 3rd trial of day 7 to the final trial of day 10. The escape latency of the Sham rat treated with the agent was similar to that of the untreated Sham rat.

G Proteins  Table 1 shows changes in Gsα and Gi1/2α proteins in the cerebral cortex, striatum, and hippocampus of the right hemisphere of the ME and Sham rats. The Gsα protein in these regions of ME rats was similar to that of Sham rats. Gsα protein content of the ME and Sham rats was not affected by treatment with nefiracetam. There were no changes in Gsα protein content in these regions of the left hemisphere, regardless of microsphere embolism or sham-operation, or treatment with or without the agent.

The cerebral Gi1/2α protein in the three regions of the ME rat was similar to that of the Sham rat. Gi1/2α protein content of the ME rat and the Sham rat was not affected by treatment with nefiracetam. There were no changes in Gi1/2α protein content in these regions of the left hemisphere, regardless of microsphere embolism or sham-operation, or treatment with or without the agent.

Basal AC Activity  Figure 4 shows changes in the basal AC activity of the cerebral cortex, striatum, and hippocampus of the ME and Sham rats. In the ME rat, the striatal AC activity decreased to approximately 50% of the Sham rat, whereas the cerebrocortical and hippocampal AC activities of the ME rat were similar to those of the Sham rat. There were no changes in AC activities in the three regions of the left hemisphere, regardless of whether they were ME or Sham rats.

When the ME rats were treated with nefiracetam, the stri-
In the present study, ME-induced sustained cerebral ischemia impaired the learning and memory function of rats, similarly to previous observations. This memory dysfunction was improved by daily treatment with nefiracetam starting from 15 h after the operation. As described in the Introduction, several investigators have proposed a possible nootropic effect of nefiracetam in various amnesic animals using the passive avoidance test upon simultaneous treatment or pre-treatment with nefiracetam. The present study has provided evidence for more profound improvement of the spatial memory function in a water maze test. This may be another profile of this agent concerning learning and memory function of the sustained ischemic animal.

We found that the basal AC activity in the striatum was higher than that in any other brain region examined, and that the basal AC activity in the striatum of the ME rat on day 11 after the operation was decreased, whereas the cerebrocortical and hippocampal basal activities were not altered. Presumably, high AC activity in the striatum is due to the presence of dopaminergic neurons. The findings suggest that the striatal dopaminergic neuron may be much more vulnerable to cerebral ischemia. Although a reduction in basal activity occurred, delayed treatment with nefiracetam restored the activity almost to the Sham level.

Col-stimulated AC activity was measured to determine whether the AC activity might respond to direct stimulation. The ME animal showed a marked reduction in Col-stimulated AC activity in the three brain regions examined. Also, in this case, the decline in striatal AC activity was most evident. The delayed treatment reversed the Col-stimulated AC activity almost to the Sham level. These findings suggest that ME results in a reduction in intrinsic AC activity in brain regions, which may be associated with an impairment of learning and memory function as described below. Nefiracetam may improve the reduction in intrinsic AC activity and/or preserve the integrity of AC.

The AC activity is coupled directly with various types of G proteins, such as Gsα and Gi1/2α. We measured the cerebral Gsα and Gi1/2α proteins of the ME and Sham rats. Cerebral Gsα and Giα proteins of the ME rat were similar to those of the Sham rat, indicating that no appreciable changes in G proteins were seen after microsphere embolism. Treatment of the ME or Sham rat with nefiracetam also did not alter the cerebral G proteins. Therefore, it is unlikely that changes in cerebral Gsα and Giα protein levels contribute to ischemia-induced changes in the cerebral AC activity in the ME rat.

Several reports have shown the possibility that the AC/cAMP/PKA signal transduction is involved in learning and memory function, and that this route connects to the nuclear CREB and stimulates the transcription of proteins that may be related to learning and memory function. For example, cAMP content increased after the performance of an inhibition avoidance learning task, and overexpression of the R(AB) inhibitory subunit of PKA in mice resulted in deficits in spatial learning and in those in long-term, context-dependent fear conditioning. In addition, the expression of an activating CREB isoform enhanced memory formation. These findings suggest that the cAMP/PKA/CREB pathway may largely contribute to the acquisition of learning and
memory. Although the exact mechanism and proteins necessary for the mechanism remain unclear, activation of AC may play an important role in the formation of learning and memory in microsphere-embolized animals. Taken together, the nootropic effect of nefiracetam on memory and learning may be, at least in part, exerted via an improvement of AC activity in the brain afflicted with sustained ischemia induced by microsphere embolism.

REFERENCES