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Effects of I.C.V. Administration of Ethylcholine Aziridinium (AF64A) on the Central Glutamatergic Nervous Systems in Rats

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Changes in glutamatergic nervous activities following intracerebroventricular (icv) administration of ethylcholine aziridinium (AF64A) were studied in rats. The levels of total glutamate, those of glutamate in cerebrospinal fluid (CSF) and in extracellular fluid (ECF) of striatum, the activities of glutamine synthetase (GS), glutaminase and glutamate dehydrogenase (GDH) and the specific binding sites of [^{3}H]MK801 in striatum, hippocampus and frontal cortex were assessed a week after the infusion of AF64A (3 nmol) into lateral ventricle. The levels of total glutamate were significantly decreased in striatum, hippocampus and frontal cortex after AF64A treatment. Although the levels of glutamate in CSF weren't changed after AF64A treatment, the levels of glutamate in ECF of striatum were significantly decreased (62.6%). GS activities in striatum were significantly decreased. But, glutaminase activities in striatum were significantly increased. However, the activities of GS and glutaminase in frontal cortex and hippocampus weren't changed. Although GDH activities in frontal cortex were significantly decreased, those in striatum and hippocampus weren't altered. The striatal densities of [^{3}H]MK 801 binding sites were increased without changes in its affinity. Also, the specific binding sites of [^{3}H]MK801 were increased in frontal cortex but not in hippocampus. These results indicate that the glutamatergic nervous activities were altered with the infusion of AF64A into lateral ventricle. Furthermore, it suggest that the decreased levels of glutamate after AF64A treatment may affect the change in the other parameters of glutamatergic neuronal activities.

Key words : Ethylcholine aziridinium, Glutamate, Glutamine synthetase, Glutaminase, NMDA receptors

INTRODUCTION

The main symptom of the dementia is the intelligence impairments, especially memory disturbance. Also, the disturbances of the picture are dominant in the early symptoms. It has been reported that the number of cholinergic cell body in dementia was decreased and many parameters of cholinergic nervous system in hippocampus were altered (Bartus *et al.*, 1982; Colye *et al.*, 1984; Mash *et al.*, 1985; Sims *et al.*, 1983). In addition, it has been recently reported that impairments in glutamatergic nervous system might be involved in learning disturbance (Greenamyre and Maragos, 1993; Myhrer, 1993). Several researchers also reported that glutamatergic neurotransmission in neocortex and hippocampus was severely disrupted and the levels of glutamate were decreased in dementia (Maragos *et al.*, 1987; Palmer *et al.*, 1990).

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Ethylcholine aziridinium ion (AF64A) is a nitrogen mustard analog of choline which is selective, irreversible neurotoxin. The AF64A-treated animals have been reported to lose the memory (Chrobak *et al.*, 1988; Lim *et al.*, 1995; Nakahara *et al.*, 1988). Recently, Lim *et al.* (1995) reported that AF64A impaired the motivation as well as the latent memory in Morris water maze. Thus, this toxin has been proposed as a useful tool in the development of animal models of Alzheimer's disease and senile dementia of the Alzheimer type (Hanin *et al.*, 1982).

It has been reported that AF64A induced the decrease in choline uptake, the destruction of the presynaptic terminals in cholinergic nervous systems (Bartus *et al.*, 1982; Leventer *et al.*, 1987; Ransmayr *et al.*, 1992). It has also been reported that AF64A affect the other neuronal activities, such as dopaminergic, gabanergic and glutamatergic neuronal activities (Hortnagl *et al.*, 1991; Lim *et al.*, 1996; Meana *et al.*, 1992). However, few investigate the changes in the glutamatergic nervous activities after AF64A treat-

ment. To examine the usefulness of AF64A for the amnestic animal models, it is necessary to study the various parameters of glutamatergic nervous activities.

Therefore, the study is designed to determine whether AF64A induces the changes in the various activities of the central glutamatergic neuronal system.

MATERIALS AND METHODS

Animals and materials

Male Spraque-Dawley rats weighing 250-300 g were housed at 21±1°C, 50-60 RH on a 12h light/12h dark schedule. Animals were freely accessible on food and water.

Acetylcholine mustard-HCl was purchased from Research Biochemical Inc. (Wayland, MA). Ethylcholine aziridinium (AF64A) is synthesized according to the method of Mantione *et al.* (1983) with a minor modification. [^{3}H]MK801 (20.3 Ci/mmol) and [^{14}C] Glutamate (261.6 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Animal treatment

For the administraion of AF64A, all surgical procedures were performed on male Spraque-Dawley rats with initial weights 250-300 g. Rats were anethetized with Equithsin and mounted in a David Kopf stereotaxic apparatus. The skull was exposed and a guide cannula was implanted according to Paxino and Watson (1986) through the dural surface into the lateral ventricle with respect to bregma at the following coordinates : A -0.8, L±1.4, V -4.4 mm. A stainless steel obturator was inserted into the guide cannula. Penicillin 30,000 I.U. was administered to protect from infection after surgery. Before the infusion of AF64A, the rats were allowed to recover from surgery for 4 days, housed singly in their cages. AF64A was infused in both ventricle with the rate of 0.5 μl/min (3 nmol/each side) at four days after the surgery. The dose of AF64A was chosen according to Meana *et al.* (1992). The control groups were infused with the artificial cerebrospinal fluid. According to our previous report (Lim *et al.*, 1995), the rats were sacrificed seven days after the infusion of AF64A.

For the determination of glutamate in cerebrospinal fluid (CSF) and ECF of striatum, the microdialysis probe was implanted seven days after infusion of AF 64A in lateral ventricle and striatum using the following coordinates : A -0.8, L -1.4, V -4.4 mm and A +1.0, L -2.4, V -2.5 mm, respectively. The loop was perfused with artificial CSF (0.13 M NaCl, 1.77 mM $CaCl_2$, 2.65 mM KCl, 0.98 mM $MgCl_2$, and 0.25 mM ascorbic acid, pH 7.3). The flow rate of artificial CSF was 2 μl/min. After the 60 minutes of the equilibration period, dialysates were collected for 40 min.

Determination of glutamate and other amino acids

Seven days after infusion of AF64A into lateral ventricle, rats were sacrificed by decapitation and each brain region was dissected out rapidly according to Glowinski and Iversen (1966). The tissues were then homogenized in cold methanol/water (50:50 v:v) using a Polytron homogenizer in ice bath. The homogenates were centrifuged at 20,000×g for 20 min at 4°C and the supernatant was collected, diluted with cold methanol/water (50:50 v:v) for the determination of total glutamate. The concentrations of glutamate and other amino acids in each brain region were determined according to Ellison *et al.* (1987). The supernatant of tissue homogenate and the collected dialysates of CSF and ECF of striatum were treated with o-phthaldialdehyde derivatizing agent according to the method of Shoup *et al.* (1984). Fifty microliter was injected into HPLC-ECD system (Waters system). Separations were achieved using a C18 reverse type column (Rainin instrument 15 cm in length) and 0.1 M sodium phosphate buffer containing 37% methanol was used with mobile phase. The concentrations of glutamate and other amino acids were determined by direct comparison of sample peak heights to those of an external standard containing the amino acids. Since aspartate, glycine and glutamine are the another excitatory neurotransmitter, the amino acid to bind to NMDA receptor and a metabolite of glutamate, respectively, their changes in levels are also included (Nicolls and Attwell, 1990; Watkins *et al.*, 1990; Young and Fagg, 1990).

Determination of glutamine synthetase (GS) activity

The activity of GS was determined following the method of Patel *et al.* (1982) with a minor modification. The dissected tissues were washed in cold imidazole-saline buffer containing 20 mM imidazole-HCl (pH 6.8), 5.4 mM KCl, 137 mM NaCl, 5.5 mM glucose and homogenized in 10 mM imidazole-HCl (pH 6.8) including EDTA. Fifty microliter assay buffer composed of 50 mM imidazole-HCl (pH 6.8), 15 mM $MgCl_2$, 10 mM ATP, 10 mM L-[U-^{14}C]glutamate (0.8 mCi/mmol), 4 mM NH_4Cl, 1 mM β-mercaptoethanol, 1 mM ouabain and homogenates was incubated for 30 min at 37°C. After incubation, the reaction was terminated by adding 1 ml of ice-cold water and then loaded immediately into a column (Dowex AG-1 X8, acetate form and Amberlite IRP-G9). Columns were washed with 5 ml deionized water and the eluates were transferred to vials containing scintillation solution and counted with liquid scintillation spectrophotometer.

Determination of glutaminase activity

The activity of glutaminase was determined following the method of Bergmeyer (1974) with a minor modification. The tissues were homogenated in deionized water. The 0.25 ml of homogenates were mixed with the 0.25 ml of 40 mM L-glutamine and then incubated at 37°C for 20 min. After incubation, the 0.5 ml of 15% trichloroacetic acid was added in assay mixture and the assay mixture was centrifuged at 12, 000 rpm for 5 min. Glutaminase activities were determined at 400 nm using UV spectrophotometer.

Determination of glutamate dehydrogenase (GDH) activity

The activity of GDH was determined according to the method of Patel *et al.* (1982) with a minor modification. The assay mixture (1 ml) contained (final concentrations) : 90 mM phosphate buffer, pH 8.0, 1 mM EDTA, 0.16% Triton X-100, 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate and 10 mM 2-oxoglutarate. The assay was carried out at 25°C and oxidation of NADH was measured at 340 nm for 10 min using UV spectrophotometer.

Determination of [^{3}H]MK801 binding sites

Membranes for NMDA receptor binding assays were prepared according to Foster and Wong (1987). The dissected tissues were homogenized in 15 volumes of ice-cold 0.32 M sucrose at low speed. The homogenates were centrifuged at 1,000×g for 10 min and the supernatant was centrifuged at 20,000×g for 20 min and then crude synaptozomal pellet was obtained. This crude synaptozomal pellet was resuspended in deionized water and dispersed with the Polytron homogenizer for 30 sec. The suspension was centrifuged at 8,000×g for 20 min. The supernatants, including the buffy layer, were collected, resuspended in deionized water and centrifuged at 48, 000×g for 20 min and then the pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -70°C until use. The frozen membrane preparation was thawed, suspended in 75 volumes of 5 mM Tris-HCl buffer (pH 7.4) and centrifuged 3 times at 48, 000×g for 20 min at 4°C and the final pellet was suspended in 5 mM Tris-HCl buffer (pH 7.4) containing 1 μM glycine and 30 μM glutamate.

Binding sites using [^{3}H]MK801 was determined according to Ebert *et al.* (1991). Aliquots (200 μl) of the membrane preparations (200-250 μg/tube) were incubated with [^{3}H]MK801 for 4h at 25°C. Nonspecific binding was determined by adding 100 μM unlabeled MK801. After incubation, the samples were filtered through Whatman GF/B fiber filters and washed twice with 5 ml of ice-cold 5 mM Tris-HCl buffer (pH 7.4) and then transferred to vials containing scintillation solution and counted with liquid scintillation spectrophotometer.

Determination of protein concentration

The protein contents of tissue homogenates were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistics

The statistical significance of differences was determined using Student's t-tests.

RESULTS

The levels of total glutamate and other amino acids in various brain regions 7 days after AF64A infusion are shown in Table I. The concentrations of glutamate were significantly decreased in striatum, hippocampus and frontal cortex by 41.6, 42.3 and 33.6%, respectively. Also, in striatum, the concentrations of total glutamine, glycine and taurine were significantly decreased by 77.1, 52.3 and 33.3%, respectively. In hippocampus, the concentrations of total aspartate, glycine and taurine except glutamine were decreased by 43.5, 41.5 and 34.0%, respectively. Furthermore, the concentrations of various amino acids in frontal cortex were differently decreased; aspar-

Table I. Effects of i.c.v. administration of AF64A on the concentrations of glutamate and other amino acids in the various rat brain regions

		Glu	Asp	Gln	Gly	Tau
Striatum	Control	39.18±6.02	18.82±4.44	4.34±0.76	8.89±0.58	15.27±2.27
	Treated	22.92±1.56*	14.01±1.17	0.99±0.12**	4.24±0.32**	10.17±0.43*
Hippocampus	Control	38.25±5.12	34.29±4.36	3.55±0.55	6.09±0.84	11.44±1.25
	Treated	22.05±1.23*	19.35±1.18*	2.39±0.55	3.56±0.20*	7.54±0.32*
Frontal cortex	Control	33.93±1.53	33.61±1.23	4.37±0.58	5.15±0.28	11.78±0.50
	Treated	22.50±1.43**	20.23±1.45**	2.11±0.26*	3.33±0.12**	7.47±0.39**

Rats were sacrificed a week after the infusion of AF64A (3nmol) into lateral ventricle. Values are in nmole/mg protein. Each value represents the mean±S.E.M. of 5 different rats. Significances of difference compared with control group; *p<0.05, **p<0.01

Table II. Effects of i.c.v. administration of AF64A on the concentrations of glutamate and other amino acids in cerebrospinal fluid and extracellular fulid of striatum

		Glu	Asp	Gln	Gly	Tau
CSF	Control	2.42±0.31	1.68±0.24	19.34±1.35	6.85±0.95	2.11±0.18
	Treated	2.77±0.61	3.38±0.65*	8.71±2.57*	4.97±0.45	1.63±0.16
ECF of Striatum	Control	3.99±0.74	2.34±0.47	22.97±2.89	12.64±2.73	2.55±0.40
	Treated	1.49±0.22*	2.11±0.75	19.27±5.37	6.94±2.34	1.94±0.53

Microdialysis was performed a week after the infusion of AF64A. Each value represents the mean±S.E.M. of 3 or 6 different rats for CSF and 3 or 5 different rats for ECF of striatum. Values are in μM. Significance of difference compared with control group; *p<0.05

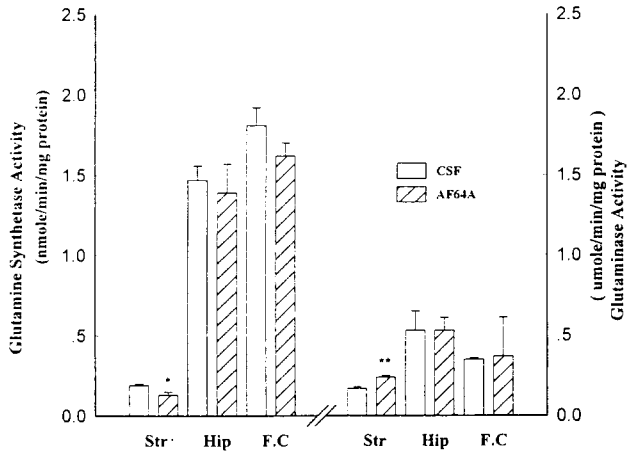

Fig. 1. Effects of i.c.v. administration of AF64A on activities of GS and glutaminase in various rat brain. Rats were sacrificed a week after the infusion of AF64A into lateral ventricle. The values are mean±S.E.M. of different determinations; 4 or 6 for GS and 4 or 5 for glutaminase. *, ** significantly different when compared to control; *p<0.05, * *p<0.01. Abbreviation : Str, striatum; Hip, hippocampus; F.C, frontal cortex

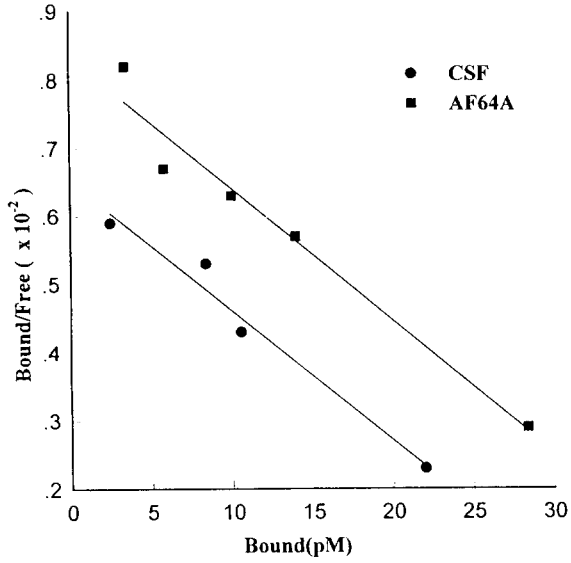

Fig. 2. The specific Scatchard plot of [^{3}H]MK801 binding sites in rat brain. Rats were sacrificed a week after AF64A infusion into lateral ventricle. The values are the average of 2 determinations, done in duplicate. K_d is 4.53 vs 5.17 nM. B_{max} is 450.7 vs 803.9 pmole/g protein.

Table III. Effects of i.c.v. administration of AF64A on GDH activities in the various rat brain regions

Regions	Control	Treated
Striatum	0.41±0.02	0.49±0.05
Hippocampus	0.32±0.03	0.43±0.04
Frontal cortex	0.30±0.04	0.12±0.02*

Rats were sacrificed a week after the infusion of AF64A into lateral ventricle and each brain region was dissected out. The values are the mean±S.E.M. for 3 or 5 different determinations. The units are in μmole/min/mg protein. *significantly different when compared to the control value, *p<0.05

tate 39.8%; glutamine 51.7%; glycine 35.3%; taurine 36.5%.

The concentrations of glutamate and other amino acids in CSF and ECF of striatum after AF64A infusion are shown in Table II. In CSF, the concentrations of aspartate were significantly increased by 101.1%. Furthermore, those of glutamine were significantly decreased by 54.9%. However, those of other amino acids including glutamate weren't altered. In ECF of striatum, the concentrations of glutamate were significantly decreased by 62.6%. But, those of other amino acids weren't altered.

Changes in the activities of GS and glutaminase in various brain regions after AF64A treatment are shown in Fig. 1. The activities of GS in striatum in AF 64A-treated groups were significantly decreased by 31.5% compared with those in control (0.19±0.01 vs 0.13±0.02 nmole/min/mg protein). But, there was no change in other regions examined. The activities of glutaminase in striatum in AF64A-treated groups were significantly increased by 41.1% compared with those in control (0.17±0.01 vs 0.24±0.01 μmole/min/mg protein). But, there was no change in other regions.

The activities of GDH in various brain regions after AF64A treatment are shown in Table III. The activities of GDH in frontal cortex were significantly decreased by 60.2%. However, those in AF64A-treated groups weren't altered in striatum and hippocampus.

Changes in the characteristics of striatal NMDA re-

Table IV. Effects of i.c.v. administration of AF64A on NMDA receptor binding of [^{3}H]MK801 in hippocampus and frontal cortex

Regions	Control	Treated
Hippocampus	2.25±0.015	2.09±0.067
Frontal cortex	1.85±0.066	2.33±0.004 **

Rats were sacrificed a week after the infusion of AF64A into lateral ventricle. The units are in pmole/mg protein. The values are the mean±S.E.M. of 4 different determinations, done in duplicate. **significantly different when compared to control; **p<0.01

ceptors by administration of AF64A is shown in Fig. 2. The densities of [^{3}H]MK801 binding sites in striatum were increased by 78.4% without changes in its affinity (K_d 4.53 vs 5.17 nM and B_{max} 450.7 vs 803.9 pmole/g protein). In frontal cortex, the specific binding sites of [^{3}H]MK801 were significantly increased by 25.9% (Table IV). However, there was no change in hippocampus.

DISCUSSION

The present results demonstrate that the activities of the glutamatergic nervous system are changed after the intracerebroventricular administration of AF64A. The total and striatal extracellular levels of glutamate are decreased. The striatal activities of GS and glutaminase are decreased and increased, respectively. However, the activities of GDH is not changed in striatum. The maximum binding density of NMDA receptor in striatum is increased.

It has been reported that AF64A, irreversible neurotoxin, impaired the choline uptake in the cholinergic nervous terminals (Chrobak *et al.*, 1988; Nakahara *et al.*, 1988). It has been reported that the i.c.v. administration of AF64A produced a decrease in the glutamate levels in hippocampus (Abe *et al.*, 1992; Hotnagl *et al.*, 1991). The present results is agreed with those reports. In addition, the present results indicate that other amino acids in various regions were also affected by the i.c.v. infusion of AF64A. It is known that glycine has the potentiated effect on the central glutamatergic nervous system and aspartate is another excitatory amino acid in CNS (Watkins *et al.*, 1990; Young and Fagg, 1990). Thus, the present results suggest that the i.c.v. administration of AF64A induce the decrease in the glutamatergic as well as cholinergic neuronal activities.

Meana *et al.* (1992) reported that the extracellular glutamate levels in neostriatum were increased following the high dose intrastriatal administration of AF64A. The present results is disagreed with those reports. It has been reported that cortical cholinergic stimulations increase the striatal activities of glutamatergic nervous system (Dijk *et al.*, 1995; Gonzales *et al.*, 1993). It imply that the impaired cortical cholinergic neuronal activities might affect the striatal glutamatergic neuronal activities. Thus, the discrepancy between ours and Meana *et al.* (1992) may be due to the injection site of AF64A. It has been reported that glutamate is normally released by Ca^{2+}-dependent exocytosis and is reaccumulated directly into the nerve terminal or into adjacent glia (Nicolls and Attwell, 1990). Therefore, the present results suggest that the decreased glutamate levels in ECF of striatum may be partly due to either/both decrease in release of glutamate or/and increase in uptake of glutamate in glutamatergic nervous systems.

It has been reported that the released glutamate is replenished via glutamate-glutamine cycle in amino acidergic nervous system (Kugler, 1993). GS converts glutamate into glutamine in glial cells. And glutamine generated is diffused into extracellular space and enters the presynaptic terminals. That glutamine serves as a precursor for presynaptic cytoplasmic glutamate through the mitochondrial glutaminase (Nicolls and Attwell, 1990). Also, it has been reported that the formation of glutamate is closely related to glucose metabolism by the action of GDH (Kugler, 1993). The present results indicate that the i.c.v. treatment of AF 64A induces the decrease in the activities of GS and the increase in those of glutaminase. Thus, the present results imply that changes in the activities of GS and glutaminase may be contributed to reduce the degradations of glutamate and induce the synthesis of glutamate. The changes in both enzyme activities and levels of glutamate are somewhat controversial. However, it has been reported that glutaminase is subject to potent glutamate inhibition (Patel *et al.*, 1982). Therefore, it is possible that the decreased levels of glutamate as the present results are disinhibited the activity of glutaminase. Although the exact cause and consequences in the change of GS and glutaminase activities are not known, the present results suggest that the altered activities of metabolizing enzymes may be related to adjust the decreased levels of glutamate in the AF64A-treated rats. However, as the unaltered striatal activities of GDH, the i.c.v. administration of AF64A may not directly affect the glucose metabolism for the formation of glutamate in striatum.

The various changes in the subtypes of glutamate receptors have been reported in Alzheimer's disease ; either reduced (Greenmyre *et al.*, 1987), unaltered (Cowburn *et al.*, 1988), or increased (Ulas *et al.*, 1994). It has been reported that NMDA and non-NMDA receptor agents affect memory and learning function (Pettit *et al.*, 1994; Staubli *et al.*, 1994). It has been also reported that NMDA receptors are highly localized to areas of the brain, such as the hippocampus and frontal cortex, which have been implicated in learning and memory process (Monaghan

and Cotman, 1985). Although further studies of the change in the characteristics of non-NMDA receptors are needed, the present results indicate that NMDA receptors in striatum and frontal cortex are increased but not in hippocampus. It is well known that the chronic administration of either agonists or antagonists induces the down- or up-regulation of its receptors. Therefore, the increased NMDA receptor densities in striatum may be due to the reduced glutamate in ECF of striatum to maintain neuronal balance. Although the various parameters of central glutamatergic neuronal systems are changed by the AF 64A treatment, the learning behaviors by glutamatergic agents in the AF64A-induced animals are remained to be further elucidated in the usefulness of the model.

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