Amino Acids (1997) 12: 21–32

Amino
Acids

Guanidinoethane sulphonic acid interferes with the binding of [^{3}H]dizocilpine and neurotoxic action of AF64A

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Accepted March 5, 1996

Summary. The binding of [^{3}H]dizocilpine [[^{3}H]MK-801] to the N-methyl-D-aspartate receptor complex of well washed rat cortical membranes was reduced by guanidinoethane sulphonic acid (GES). Micromolar concentrations of GES, which were high relative to those of dizocilpine, inhibited in a concentration dependent manner the binding of [^{3}H]dizocilpine. The inhibitory effect of GES on [^{3}H]dizocilpine binding was slightly influenced by concentration of glutamate. The glutamate antagonist DL-2-amino-5-phosphonovaleric acid blocked the effect GES at concentrations higher relative to GES. The inhibitory effect of GES was still present during spermidine-induced stimulation of [^{3}H]dizocilpine binding. GES reduced the binding of the glycine antagonist [^{3}H]5,7-dichlorokynurenic acid with an IC_{50} of 530μM. Intraperitoneal injections of GES (0.2mmol/kg) protected against both amnesia and decrease in the choline acetyltransferase activity following local injections of the neurotoxin AF64A into the nucleus basalis magnocellularis. GES given to lesioned rats during the training period in the spatial learning task gradually improved the performance to the level of sham operated rats. It is concluded that GES interferes with the transmitter and the dizocilpine binding sites of the NMDA receptor complex and has the capacity to protect against neurotoxic brain damage.

Keywords: Amino acids – Guanidinoethane sulphonic acid – [^{3}H]Dizocilpine – Choline acetyltransferase – [^{3}H]Glycine – [^{3}H]5,7-Cl-Kynurenic acid – AF64A – Learning

Introduction

We have previously reported that guanidinoethane sulphonic acid (GES) *in vitro* at micromolar concentrations reduces the number of binding sites for [^{3}H]dizocilpine in rat cortical membranes (Liljequist, 1992). GES causes intensive convulsions when given intracerebroventricularly (Mori 1981; Toda et al., 1989; Watanabe et al., 1991; Yokoi et al., 1992) but following peroral

administration, weakly prevents pentylenetetrazol-induced convulsions (Bonhaus et al., 1985). The concentration of GES is high, 27 mmol/ml, in the serum of uremic patients and its concentration correlates with convulsion degree in experimental models (Matsumoto et al., 1976). GES has been used as a taurine uptake inhibitor to produce an experimental model of taurine deficiency (Huxtable et al., 1979; Hiramatsu et al., 1982; Marnela and Kontro, 1984). Recent studies have shown that GES interacts with the transport of glutamate. In cell culture, GES inhibits the uptake of glutamate (Nilsson et al., 1989). Brain microdialysis studies have demonstrated that the concentration of extracellular glutamate increases in the rat hippocampus following peroral subchronic treatment with GES (Lehman et al., 1987). Also, other amino acids such as glycine, alanine and serine, have been reported to be elevated in brain tissues following treatment with GES (Hiramatsu et al., 1989). These effects – increased concentration of glutamate in the extracellular space, and glycine, may favor cognitive functions by influencing the NMDA receptor complex (Liljequist, 1996, in press).

In the present study we have investigated if the inhibitory effect of GES on [^{3}H]dizocilpine binding is influenced by compounds which modify the transmitter and modulatory sites of the N-methyl-D-aspartate (NMDA) sensitive subtype of the glutamate receptor family. This receptor contains distinct recognition sites for glutamate and NMDA, glycine, MK-801 (and TCP), Mg^{++}, Zn^{++}, and polyamines including their agonists spermidine and spermine and antagonists DET and DA_{10} (Foster and Wong, 1987; Danysz et al., 1989; Ransom and Stec, 1988; Thomson, 1989; Monahan et al., 1989; Williams et al., 1990). We have measured whether the effect of GES on [^{3}H]dizocilpine binding is influenced by the polyamine agonist spermidine or its antagonist arcaine, or by the glycine site antagonist kynurenic acid. The possible influence on the glycine antagonist [^{3}H]5,7-dichlorokynurenic acid binding was also included in the experiments. We have also used a model of experimental amnesia to investigate the ability of GES to protect neurones and consequent amnesia following toxin-infusions into the nucleus basalis magnocellularis (Bartus et al., 1985; Brandeis et al., 1986; Dunnett et al., 1991; Smith, 1988).

Materials and methods

Animals

Adult male Spraque-Dawley rats weighing 250 ± 10 g were used. They were maintained on ad lib food and water under standard temperature- and humidity-controlled laboratory conditions with a 12 hour light (09.00 pm–09.00 am) -dark schedule. The behavioral experiments were performed during the dark period. The animals received intraperitoneal GES injections in doses of 3.08 and 30.83 mg/kg 45 min before the training. After a three day wash-out period these rats were decapitated and their cortices and hippocampi were collected for ChAT analysis. Untreated animals were used for receptor binding experiments.

Surgical procedure to lesion the nucleus basalis magnocellularis (NBM)

The rats were anaesthetized with chloral hydrate (350 mg/kg i.p.) and placed in a stereotaxic instrument (David Kopf, Tujunga, CA, USA). The stereotaxic coordinates were A −0.9, V −7.7, L +/−2.8 according to Paxinos and Watson Atlas (Paxinos and Watson, 1982). Ethylcholine aziridium, AF64A was activated in basic solution and infused within a few hours bilaterally into the NBM (during 2–3 min; 57.5 ng/nucleus; 0.25 nmol/μl) using a Sage syringe pump. Sham controls were operated likewise except that toxin influsions were excluded. One group received two GES injections, 30.8 mg/kg each, the first 45 min before and the second 3 h 45 min after toxin infusion. The behavioral experiments were started two weeks after the operation and no drug treatments were performed during the recovery period.

Apparatus and testing procedure in learning experiments

A circular water maze was used to test learning acquisition (Liljequist and Winblad, 1993). The maze was constructed of PVC, filled with water, and maintained at water temperature 25 ± 2°C. A platform was hidden under the surface of the water. Each rat was allowed to swim freely until they climbed onto the platform. Most of the animals found the platform within 60 seconds. If the animal did not climb onto the platform within 60 seconds they were placed on it. Four training trials were conducted each day for 5 consecutive days with a 30 second interval between trials. The drugs were injected intraperitoneally, in blind fashion, 45 minutes before the learning test.

All experimental protocols were approved by the institutional ethical committee and met the quidelines of the responsible governmental agency.

Biochemical assays

Choline acetyltransferase (ChAT) activity

After the completion of learning experiments, frontal cortices and hippocampi were dissected to measure choline acetyltransferase (ChAT) activity according to the method described by Fonnum et al. (1987).

Protein concentration

The concentration of protein was measured according to the method described by Bradford (1976).

Receptor binding

[3H]Dizocilpine

[3H]Dizocilpine receptor binding assays were performed according to the method described by Grant et al. (1990) and Liljequist and Winblad (1993). Rat cortices were homogenized in 0.32 M sucrose and homogenates were centrifuged for 10 minutes at 1,000 × g. Pellets were washed 3 times with distilled water and stored at −20°C for few days prior to performing binding assays. On the day of the binding assay, pellets were resuspended in 5 mM Tris HCl buffer (pH 7.4) and washed, four times by repeated centrifugation and resuspension in fresh buffer. Between washings, the membrane preparations were incubated for 20 min at 23°C. Binding assays were performed in Tris HCl buffer containing in addition 10, 100 or 1,000 μM glutamate, 10 μM glycine, 1 mM ethylenediaminotetra-acetic acid, 0.43 mM $MgCl_2$ and 2 nM [3H]MK-801. 100 μM of MK-801 was used for the determination of nonspecific binding. After incubating for 90 min at 23°C, the filters were washed 3 times with 5 ml of buffer and bound radioactivity was counted by liquid scintillation spectrometry.

[^{3}H]Glycine and [^{3}H]5,7-Cl-Kynurenic acid binding

[^{3}H]Glycine binding assays were performed according to the method described by Kishimoto et al. (1981) and the binding of [^{3}H]5,7-Cl-kynurenic acid according to the method by Canton et al. (1992) with slight modifications. In [^{3}H]glycine binding assays, synaptic membranes were incubated for 20 min at 4°C with labeled glycine and test compounds in 50 mM Tris-citric acid buffer. [^{3}H]5,7-Cl-kynurenic acid binding was performed in HEPES-KOH buffer with a 30 min incubation time. For [^{3}H]glycine binding assays, the reactions were terminated by high-speed centrifucation and the pellets washed three times. The Microfuge tubes were cut off, the pellet dissolved in Protosol and the radioactivity counted by liquid scintillation spectrometry. The [^{3}H]5,7-Cl-kynurenic acid binding assay was terminated by filtration. Unlabeled compounds were used for displacement.

Drugs

Guanidinoethane sulphonic acid (GES) was synthesized at the Department of Organic Chemistry, Royal College of Techonology, Stockholm (Liljequist et al., 1990). MK-801 was a generous gift from Merck Sharp and Dohme, U.K. Ethylcholine aziridium (AF64A) was purchased from Research Bochemicals Inc., Natick, MA, U.S.A., [^{3}H]dizocilpine [[^{3}H]MK-801], [^{3}H]glycine [^{3}H]5,7-Cl-kynurenic acid, 5,7-chlorokynurenic acid (5,7-Cl-KYNA), arcaine (1,4-bis[guanidino]butane) and DL-2-amino-5-phosphonovaleric acid (APV) were purchased from Tocris Neuramin Ltd., U.K. Spermidine was purchased from Aldrich, Milwaukee, U.S.A., and glycine and all other chemicals from Sigma, St. Louis, MO, U.S.A.

Statistics

Data from binding experiments were analyzed by using the EBDA software program of McPherson, data from learning experiments were analyzed for main effects using a one- and two-way analysis of variance followed by individual post-hoc comparisons using Fisher Least Square Difference (PLSD), Scheffe and t-tests.

Results

The influence of GES on [^{3}H]dizocilpine binding was modulated by compounds which interact with transmitter recognition sites of the NMDA receptor complex. To demonstrate the inhibition, the receptor had to be activated by the addition of glutamate and glycine in the binding buffer.

We tested the possibility that the effect of GES on the binding of dizocilpine may be due to a direct inhibitory action at the binding site of MK-801. This was done by incubating different concentrations of GES with unlabeled dizocilpine. The IC_{50} value for dizocilpine was 11 nM (Fig. 1A). A dose of 1 mM GES completely inhibited the binding of 100 nM and lower concentrations of dizocilpine (Fig. 1B).

We have previously found that the inhibition produced by GES is dependent on the presence of transmitter agonists in the incubation medium. Experiments were carried out to determine whether changes in glutamate concentration alone are of importance. The inhibition of binding of [^{3}H]dizocilpine by GES was measured in the presence of 10, 100 and 1,000 μM glutamate. The addition of glutamate 10 μM increased [^{3}H]dizocilpine binding

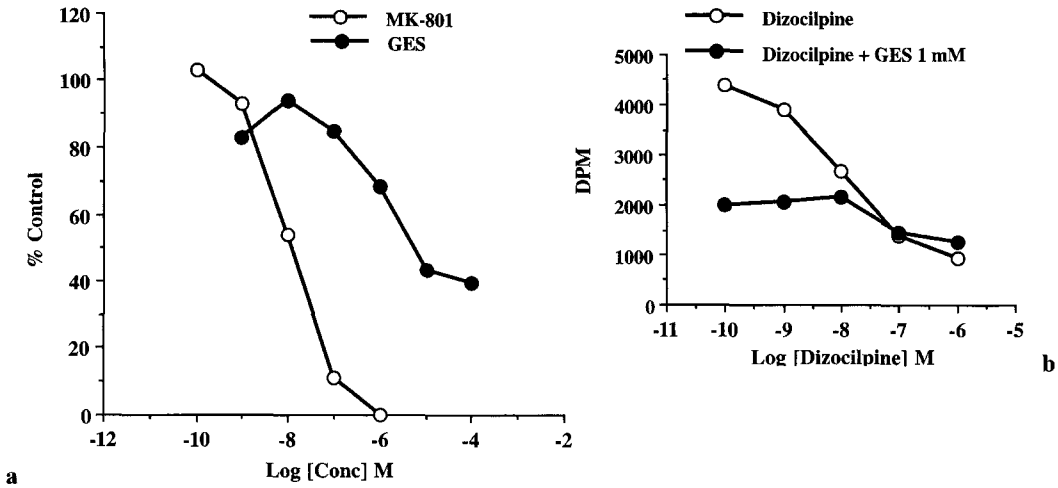

Fig. 1. Effects of different concentrations of unlabeled MK-801 and GES (**a**) and in combination with 100μM of guanidinoethane sulphonic acid (**b**) on [^{3}H]MK-801 binding on synaptosomal membranes prepared from rat cortex. Values are means from three separate experiments each performed in triplicate, error bars less than 15%

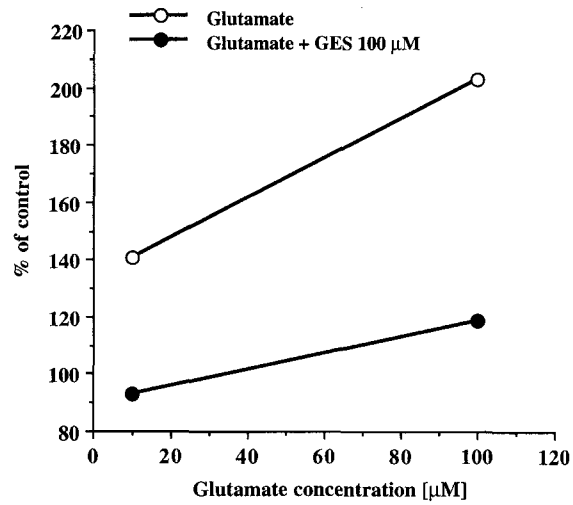

Fig. 2. Effects of 10 and 100μM of glutamate alone and in combination with 100μM of guanidinoethane sulphonic acid on [^{3}H]MK-801 binding on synaptosomal membranes prepared from rat cortex. Values are representative from three separate experiments each performed in triplicate

to 140% of control (Fig. 2). The decrease caused by GES was partly, but not completely influenced by varying concentrations of glutamate. In the presence of 10μM of glutamate and 100μM of GES the binding of [^{3}H]dizocilpine was stimulated by 93%. The same dose of GES, in the presence of 100μM of glutamate, inhibited the binding of [^{3}H]dizocilpine by 64%. Glutamate concentrations higher than these did not enhance but started to inhibit [^{3}H]dizocilpine binding.

Table 1. Effect of guanidinoethane sulphonic acid (GES) on [^{3}H]glycine binding on rat cortical membranes

	[^{3}H]Glycine binding
Control	63.39 ± 5.35 pmol/mg
GES 1 mM	39.24 ± 3.92 pmol/mg

The results are presented as mean ± SEM of three different assays each performed in triplicate. Between groups the difference is significant at the $p < 0.007$ level.

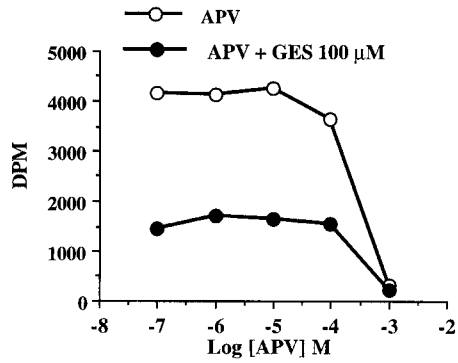

Fig. 3. Effects of different concentrations of Dl-2-amino-5-phosphonovaleric acid alone (*APV*) and in combination with 100 μM of guanidinoethane sulphonic acid on [^{3}H]MK-801 binding on synaptosomal membranes prepared from rat cortex. Values are means from three separate experiments each performed in triplicate

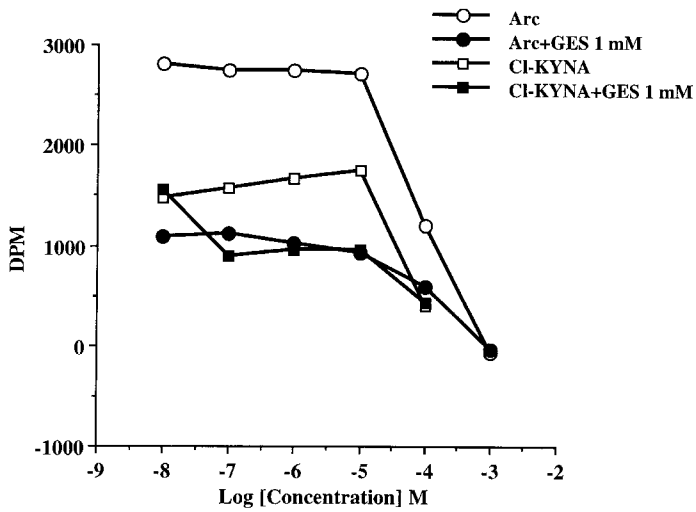

Fig. 4. Effects of different concentrations of arcaine (*Arc*) alone and kynurenic acid alone (*Cl-KYNA*) and in combination with 100 μM of guanidinoethane sulphonic acid on [^{3}H]MK-801 binding on synaptosomal membranes prepared from rat cortex. Values are means from three separate experiments each performed in triplicate

We compared the inhibitory effect of APV alone and in the presence of GES on the binding of dizocilpine. The highest concentration of APV, 1 mM, inhibited the effect of 100 μM GES by 70–100% (Fig. 3). Similar patterns were obtained for the glycine site antagonist chlorokynurenic acid (Fig. 4) which also, only at high concentrations; masked the inhibition after GES. Binding studies using [^{3}H]glycine (Table 1) and [^{3}H]-5,7-Cl-kynurenic acid support these results. GES 1 mM displaced [^{3}H]glycine by 61.90%. GES reduced the [^{3}H]-5,7-Cl-kynurenic acid binding with an IC_{50} value of 530 μM (Fig. 5). These results show that CES is able to influence the glycine and its antagonist binding at high micromolar concentrations.

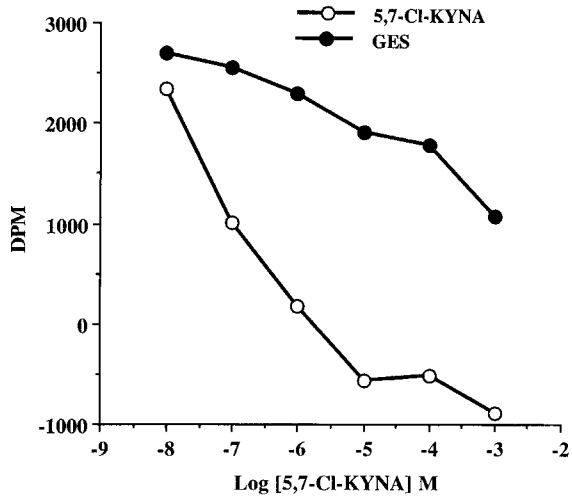

Fig. 5. Effects of different concentrations of kynurenic acid and 100 μM of guanidinoethane sulphonic acid on [^{3}H]5,7-Cl-kynurenic acid binding on synaptosomal membranes prepared from rat cortex. Values are means from three separate experiments each performed in triplicate

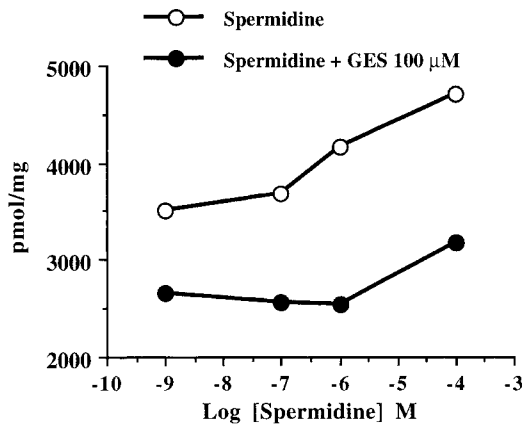

Fig. 6. Effects of different concentrations of spermidine alone and in combination with 100 μM of guanidinoethane sulphonic acid on [^{3}H]MK-801 binding on synaptosomal membranes prepared from rat cortex. Values are means from three separate experiments each performed in triplicate

[^{3}H]Dizocilpine binding was stimulated when assays were carried out with different concentrations of spermidine. Concentrations higher than 100μM inhibited [^{3}H]dizocilpine binding. Spermidine had little effect in reversing the inhibition produced by GES. Addition of 100μM GES inhibited binding at all concentrations of spermidine (Fig. 6).

Arcaine, an antagonist at the polyamine binding site, inhibited the effect of spermidine (results not shown) and also influenced the binding of [^{3}H]dizocilpine. Arcaine at a concentration higher than 1mM masked the effect of GES on [^{3}H]dizocilpine binding (Fig. 4).

Learning

Two-way analysis of variance applied on learning data (Table 2) revealed a significant learning effect ($F = 71.8$; $p < 0.0001$) and a significant group effect ($F = 4.4$; $p < 0.002$) over testing days. One way analysis of variance for group differences on separate days showed significant group differences on days 3–5. Post hoc comparisons (Fisher PLSD; Scheffe) revealed that on day 3 sham group was better than the group which had received GES 30.83mg/kg ($p < 0.05$). On days 4 and 5, the sham operated rats performed better than lesioned rats. On the last day, all GES treated groups, when the compound was given in connection with toxin infusions or training, performed better than the lesion + sal group (all at the probability level 0.05 according to Fischer PLSD).

Choline acetyltransferase activity

Choline acetyltransferase activity (Table 3) was decreased in the projection area frontal cortex following toxin infusion. The lesioned group which received saline had a significantly lower ChAT activity in comparison with the sham operated group ($t = 2.5$; $p < 0.03$). The injection of GES before the toxin protected NBM from the toxic effects of AF64A ($t = 2.2$; $p < 0.05$).

Table 2. Latency (mean ± SEM seconds) to find the platform

	Lesion + Sal	GES + GES	GES 3.08	SHAM + Sal	GES 30.83
Day 1	38.2 ± 2.6	46.5 ± 4.1	39.0 ± 2.9	37.8 ± 4.1	44.1 ± 3.4
Day 2	23.8 ± 3.3	24.7 ± 6.6	18.3 ± 2.8	18.5 ± 2.3	26.9 ± 4.0
Day 3	20.4 ± 3.6	22.7 ± 4.9	15.6 ± 3.8	12.6 ± 2.3	25.7 ± 3.7*
Day 4	15.6 ± 3.5	14.8 ± 2.8	13.5 ± 2.4	8.3 ± 1.4†	11.6 ± 1.7
Day 5	15.2 ± 1.1	6.4 ± 0.9†	7.6 ± 0.8†	7.1 ± 1.1†	8.9 ± 1.2†

Spatial learning of NBM lesioned rats following the intraperitoneal injections with guanidinoethane sulphonic acid (GES) 3.08 and 30.83mg/kg given 45min pretraining. Two injections of 30.83mg/kg GES, the first 45min before and the second 3h 45min after the infusion of the toxin AF64A were given (group GES + GES). The sham operated rats received saline. The number of animals is 7–8 in each group. *differs from SHAM; †differs from Lesion + Sal.

Table 3. Choline acetyltransferase activity (mean ± SD nmol/min/mg protein)

Lesion + Sal	G + G	G 3.08	SHAM + Sal	G 30.83
1.9 ± 0.2	2.2 ± 0.4†,*	2.0 ± 0.2*	2.3 ± 0.4†	1.7 ± 0.1

Choline acetyltransferase activity in the frontal cortex of nucleus basalis magnocellularis lesioned rats (lesion + sal). One group received guanidinoethane sulphonic acid 30.83 mg/kg before and after the toxin infusion (G + G), * differs from SHAM + Sal; † differs from Lesion + Sal. Intraperitoneal injections of guanidinoethane sulphonic acid (G) 3.08 mg/μg and 30.83 mg/μg were given for five days.

Sham rats had a higher ChAT activity than the NBM lesioned group which received GES 30.83 mg/kg during training ($t = 3.3$; $p < 0.008$). The administration of GES after lesion development did not influence ChAT activity (seen in comparison of GES 30.83 mg/kg administered before the toxin infusion with the same dose during training after the development of the lesion ($t = 3.0$; $p < 0.01$).

Discussion

GES is an endogenous compound occurring in mammals, and found in trace amounts in several tissues including the brain. A number of guanidine containing compounds, such as guanidinosuccinic acid and creatine have been implicated in neurological dysfunction states varying from sensory abnormalities to seizures and coma (DeDeyn et al., 1987). The physiological significance of GES is not well known at present. Its concentration has been found to increase during uremia in humans and in experimental convulsion models (Matsumoto et al., 1976, 1989) suggesting that it may be important in the etiology of epilepsy. Interactions with glutamate, glycine, serine and alanine (Hiramatsu et al., 1982; 1989) and catecholamines (Watanabe et al., 1991) invite the possibility to test cognitive functions (Liljequist et al., 1983; 1990; Liljequist and Winblad, 1993). Cognitive performance changes in close relation with NMDA receptors and even low concentrations of additional excitatory amino acids and modulatory compounds are able to induce variations in learning capacity (Liljequist, 1996). GES has been found to interact with the GABAergic ion-channel (Liljequist, 1993). MK-801, an antagonist at the ion-channel of NMDA preferring subtype of glutamate receptors, and GES have shown weak interactions in in vivo animal learning and activity studies and ex vivo binding experiments (Liljequist, 1990; Liljequist and Winblad, 1993).

The activation of NMDA receptors is influenced by many endogenous compounds in extracellular space, including redox reagents (Aizenman et al., 1989; 1990). A number of other compounds with a guanidine group have been documented no influence NMDA ion channel and glycine binding (Reynolds and Rothermund, 1992). The present experiments show that GES modify the binding of dizocilpine and the glycine and its antagonist binding. The effect of GES is evident under the influence of modulatory compounds.

We found that 100μM GES completely displaced dizocilpine at a concentration of 100nM and lower. The binding of MK-801 occurs in the ion channel, and high concentrations of GES relative to dizocilpine are needed. Dizocilpine has a high affinity to the TCP site but lacks clinical relevance because its heavy side effects. Of the compounds investigated here, interactions with glutamate may be of most physiological importance since the inhibitory action on the uptake of glutamate in *in vitro* experiments is evident at GES concentrations of 70–80μM (Nilsson et al., 1989).

The importance of NMDA receptor – guanidine interaction for physiological responses will depend on the concentrations reached in tissues or cerebrospinal fluid. The cerebrospinal fluid of uremic patients contains guanidine compounds in low micromolar concentrations (DeDeyn et al., 1987). The endogenous concentration of GES in the rat brain has been measured to be 3–5μg/g (Guidotti and Costagli, 1970; Mori et al., 1975) but it is not excluded that regional differences exist.

To test the functional consequences of the interference with the NMDA receptor complex, the efficacy of GES to preventing neurotoxic effects was tested in a learning paradigm. NMDA antagonists are effective anticonvulsants, and protect from neuronal damage under hypoxia and hypoglycemia by reducing the excessive release of excitatory amino acids. AF64A has been characterized to specially disrupt cholinergic neuron terminals but also to a some extent serotonergic and catecolaminergic neuron terminals (Nakamura and Ohno, 1995). The concentrations of ChAT decrease in the projection area for cholinergic neurones, frontal cortex, as documented earlier (review Smith, 1988) and also in the present study. The NBM lesion following AF64A was found to impair the rate of learning acquisition. Defects have been reported in other memory functions (Nakamura and Ohno, 1995, Brandeis et al., 1986). The injection of GES before the toxin infusion protected the neurons and no lesion was found as indicated by the unchanged ChAT activity and the learning data. Further studies and a verification based on histochemical examinations may enlighten the mechanims of action of CES.

Acknowledgements

The study was supported by Mary, Åke and Hans Ländell, and Karolinska Institute fonds.

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Received January 2, 1996