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In vivo Quinolinic Acid Increases Synaptosomal Glutamate Release in Rats: Reversal by Guanosine

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Glutamate, the main excitatory neurotransmitter in the mammalian central nervous system (CNS), plays important role in brain physiological and pathological events. Quinolinic acid (QA) is a glutamatergic agent that induces seizures and is involved in the etiology of epilepsy. Guanine-based purines (GBPs) (guanosine and GMP) have been shown to exert neuroprotective effects against glutamatergic excitotoxic events. In this study, the influence of QA and GBPs on synaptosomal glutamate release and uptake in rats was investigated. We had previously demonstrated that QA "*in vitro*" stimulates synaptosomal L-[^{3}H]glutamate release. In this work, we show that i.c.v. QA administration induced seizure*s* in rats and was able to stimulate synaptosomal L-[^{3}H]glutamate release. This *in vivo* neurochemical effect was prevented by i.p. guanosine only when this nucleoside prevented QA-induced seizures. I.c.v. QA did not affect synaptosomal L-[^{3}H]glutamate uptake. These data provided new evidence on the role of QA and GBPs on glutamatergic system in rat brain.

KEY WORDS: Glutamate release and uptake; Guanosine; Quinolinic acid; Synaptosomal preparations; Seizures.

INTRODUCTION

Quinolinic acid (QA), an endogenous metabolite of tryptophan, and the importance as a neurotoxin was first evident from work by Lapin (1978), who demonstrated that the administration of QA to mice caused convulsion (1). QA is an agonist of *N*-methyl-D-aspartate (NMDA) receptors, a synaptosomal glutamate releaser and an inhibitor of vesicular and astrocytic glutamate uptake (2–4). Thus, QA overstimulates the glutamatergic system, induces seizures and is involved in the etiology of epilepsy (5,6). Additionally, accumulation of QA in the brain seems to be involved in the ethiopathology of convulsions and occurs in patients with hepatic encephalopathy, acquired immune deficiency syndrome (AIDS)-related neurological disorders, and Huntington's disease (4).

In the mammalian central nervous system (CNS), glutamate is the principal neurotransmitter mediating excitatory synaptic events, being essential for normal brain functions (7). The removal of glutamate from the synaptic cleft is an important mechanism for modulating glutamate actions, and also for maintaining its extracellular concentration below neurotoxic levels (8,9). Glutamate uptake processes involve two transport systems located at distinct cellular levels: high affinity Na^+-dependent carriers located mainly at astrocytic cell membranes (8–11), and a low affinity Na^+-independent carrier

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located at the membrane of synaptic vesicles (12,13). The coordinated actions of both systems effectively maintain glutamate concentration in the synaptic cleft at low micromolar levels.

Extracellular guanine-based purines (GBPs), namely the nucleotides GTP, GDP, GMP and the nucleoside guanosine, have been shown to exert trophic effects on neural cells (14) as well as to modulate the glutamatergic system. Concerning their effects on the glutamatergic activity, i*n vitro* GBPs inhibit the binding of glutamate and analogs (15–17), prevent cell responses to excitatory amino acids (15–18) and increase the glutamate uptake by astrocytes (19–21), which is a physiological process involved in neuroprotection. *In vivo*, GBPs protected against seizures induced by glutamatergic agents in rats and mice, such as quinolinic acid, kainate and α-dendrotoxin (15,22–26). Additionally, GBPs present amnesic and anxiolytic effects in mice (25–27). These behavioral effects seem to be related, at least partially, to antagonism of the glutamatergic system. Recent studies suggested that neuroprotective GBPs effects against toxic overstimulation of the glutamatergic system were exerted specifically by guanosine (21,24).

Considering the involvement of QA and the glutamatergic system in seizures/epilepsy, and the modulation exerted by GBPs on this system, the purpose of this work was to evaluate the effects of QA-induced seizures and *in vivo* guanosine on synaptosomal L-[^{3}H]glutamate uptake and release in rats.

EXPERIMENTAL PROCEDURES

Drugs

Guanosine and quinolinic acid (QA) were obtained from Sigma Chemicals (St Louis, MO, USA). [^{3}H]Glutamate was purchased from Amersham International (UK). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). All solutions were dissolved in saline 0.9% and buffered to pH 7.4 when necessary. All other reagents were of analytical grade.

Animals

Male adult Wistar rats (250–350 g) were kept on a 12 h light/dark cycle (light on at 7:00 am) at a constant temperature of 22 ± 1 °C. They were housed in plastic cages (five per cage) with commercial food and tap water *ad libitum*. Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout.

Surgical Procedure and Treatments

Animals were anesthetized with sodium thiopental (40 mg/kg, 1 ml/kg, i.p.). In an stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 9 mm guide cannula was placed at 0.9 mm posterior to bregma, 1.5 mm right from de midline and 1.0 mm above the lateral brain ventricle. Through a 2 mm hole made at the cranial bone, the cannula was implanted 2.6 mm ventral to the superior surface of the skull, and fixed with jeweler acrylic cement. Experiments were performed 72 h after surgery. A 30 gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a micro syringe. The tip of the infusion cannula protruded 1,0 mm beyond the guide cannula aiming the lateral brain ventricle. Animals were treated with an i.p. infusion of vehicle (saline 0.9%) or guanosine (7.5 mg/kg). After 30 min, an i.c.v. infusion of either 4 μl of vehicle or QA (39,2 mM, the lowest dose causing seizures in all control animals) was performed. Rats were observed for 10 min in plexiglas chambers for the occurrence of tonic-clonic seizures lasting more than 5 s (according to Ref. 1). Animals not displaying seizures during these 10 min were considered protected. Latency to first seizure, number and time of seizures were also measured. Immediately after behavioral observation, rats were sacrificed by decapitation and the brains used for synaptosomal preparation.

Synaptosomal Preparations

Animals were decapitated and the forebrain was used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley and colleagues (28). Synaptosomes were used in the same day of preparation. These preparations contain 5% contamination with inner and outer mitochondrial membranes fragments, microsomes, myelin, as well as neural and glial plasma membranes (29).

Measurement of Protein Content

Protein content was determined according to Lowry (30), using serum bovine albumin as standard.

Measurement of Lactate Dehydrogenase (LDH) Activity

In order to evaluate the integrity of the synaptosomal preparations after the incubation in the presence of QA or guanosine, an aliquot of the supernatant was withdrawn and frozen for determination of LDH leakage. LDH activity was evaluated by using an assay kit (Doles Reagents, Brazil), which measure the amount of a colored complex derived from the NADH formed by the enzymatic reaction, using a spectrophotometric method (510 nm).

L-[^{3}H]Glutamate Uptake by Synaptosomal Preparations

Synaptosomal preparations were washed twice in 3 volumes of 0.3 M sucrose with 15 mM Tris/acetate buffer (pH 7.4) and centrifuged at 13,000 × *g* for 15 min. The final pellet was suspended in 0.3 M sucrose with 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS, pH 7.4 (Hepes/Cl buffered salt solution,

composition in mM: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0), in the presence of 100 nM L-[^{3}H]glutamate (Amersham International, U.K., specific activity 53 Ci/mmol), for 1 min at 37 °C. The reaction was stopped by filtration through GF/B filters. The filters were washed 3 times with 3 ml of ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in a Wallac scintillation counter. Specific L-[^{3}H]glutamate uptake was calculated as the difference between uptake obtained in the incubation medium, as described above, and uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (nonspecific uptake).

L-[^{3}H]Glutamate Release from Synaptosomal Preparations

L-[^{3}H]glutamate release was measured according to Migues et al. (31), with minor modifications. Synaptosomal preparations were incubated in HBSS, pH 7.4, for 15 min at 37 °C in the presence of L-[^{3}H] glutamate (final concentration, 500 nM). Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 13,000 × *g* for 1 min. Supernatants were discarded, and the pellets were washed 4 times in HBSS by centrifugation at 13,000 × *g* for 1 min at 4 °C. In order to measure the basal L-[^{3}H]glutamate release, the final pellet was suspended in HBSS and incubated for 60 s at 37 °C. The experiments were performed with animals receiving i.c.v administration of QA, with or without i.p. administration of guanosine. In control group was administrated only i.c.v. vehicle (saline 0.9%). Incubation was terminated by immediate centrifugation (16,000 *g* for 1 min at 4 °C). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released L-[^{3}H]glutamate was calculated as a percentage of the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period. K^+-stimulated L-[^{3}H]glutamate release was assayed as described for basal release, except that the incubation medium contained 40 mM KCl (NaCl decreased accordingly) in order to induce synaptosomal depolarization.

Statistical Analysis

For behavioral data, statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures and Student's *t*-test for latency to first seizure, time and number of seizures. For uptake assays, statistical significance was assessed by Stundent's *t* test or ANOVA followed by Duncan's multiple range test when appropriate. All experiments were performed at least in triplicates and the mean was used for the calculations. Any difference with $P < 0.05$ was considered significant.

RESULTS

QA-induces Seizures in Rats

The administration of i.p. guanosine 7.5 mg/kg was able to prevent 50% of seizures induced by QA (Fig. 1). No significant effect was observed in latency to first seizure, duration and number of seizures (data not shown).

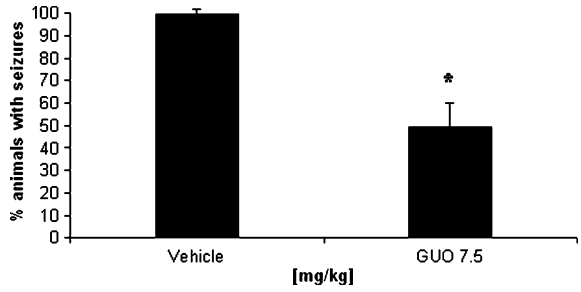

Fig. 1. Protection by guanosine against the convulsing effect of QA (GUO). n = 20–30 animals/group. * = $P < 0.05$ (Fisher exact test), as compared with vehicle group.

Effect of i.c.v. QA and i.p. Guanosine on Synaptosomal Glutamate Uptake

In order to evaluate whether QA or guanosine interferes with the glutamate uptake, we assayed L-[^{3}H]glutamate uptake into synaptosomal preparations obtained from rat brain. Figure 2 shows that glutamate uptake by synaptosomes was not affected by i.c.v. QA or i.p. guanosine (with or without seizures), when compared to control (saline 0.9%).

Effect of i.c.v. QA and i.p. Guanosine On Synaptosomal Glutamate Release

The effect of i.c.v. QA or i.p. guanosine on L-[^{3}H]glutamate release at basal (physiological extracellular K^+ concentration) or depolarized (40 mM KCl) conditions was evaluated. Neither QA nor guanosine was able to alter K^+-stimulated glutamate release. However, in rats presenting QA-induced seizures, there was an increase in the basal release, from 12% to 20% (Fig. 3). This effect was abolished by i.p. guanosine only when it displayed anticonvulsant activity.

Measurement of LDH Leakage

Synaptosomal preparations obtained from animals displaying or not seizures did not show any significant leakage of the cytosolic marker LDH, when compared with control (saline 0.9%) (data not shown), indicating no disruption of cell membranes in our assay conditions.

DISCUSSION

In presynaptic terminals, glutamate is located in cytoplasmatic and vesicular compartments. Although

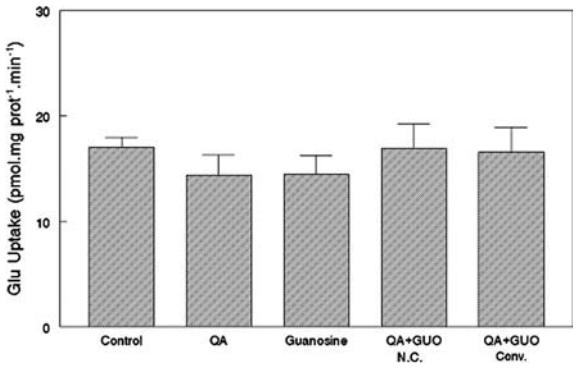

Fig. 2. Effect of i.c.v QA administration and i.p. injection of the guanosine (GUO) (7.5 mg/kg) on L-[^{3}H] glutamate uptake into brain synaptosomal preparation of rats. Glutamate uptake is expressed as pmol.mg of protein^{-1}.min^{-1}. Data are mean ± SEM from 6 independent experiments performed in triplicates. No statistically significant differences were observed between control (saline 0.9%) and other groups. N.C. = no convulsion; Conv. = convulsion.

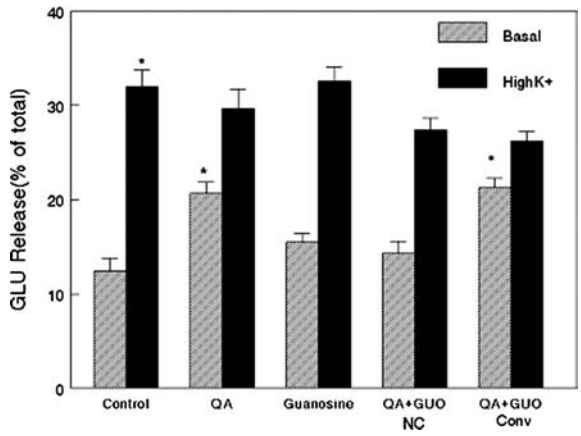

Fig. 3. Effect of i.c.v QA administration on basal and K^+-stimulated L-[^{3}H]glutamate release from rat brain synaptosomes and its reversal by i.p. injection of the guanosine (GUO) (7.5 mg/kg). Glutamate release is expressed as percentage of total radioactivity content. Data are mean ± SEM from 6 independent experiments performed in triplicates. (*) Represents values significantly different from the control group (saline 0.9%); $P < 0.05$, by ANOVA followed by Duncan's multiple range test. N.C. = no convulsion; Conv. = convulsion.

the meaning of basal glutamate release (cytoplasmatic) is controversial, the vesicular, Ca^{2+}-dependent, K^+-stimulated glutamate release after presynaptic depolarization is clearly involved in the glutamatergic activity (8). The actions of the neurotransmitter glutamate released, by acting on neuronal and astrocytic receptors, are ended by its uptake mainly by astrocytes (8,9).

Moreover, there is evidence for presynaptic NMDA auto receptors at glutamatergic nerve terminals (3,32), which could modulate the glutamate releasing. These autoreceptors are probably involved in the QA effect observed in this study. By stimulating basal glutamate release, without affecting glutamate uptake, QA probably contributes to increase the glutamate concentration in the synaptic cleft, leading to neurotoxicity induced by glutamate.

Concerning previous studies from our group related to QA, the present observation that *in vivo* QA did not alter glutamate uptake in synaptosomal preparations is compatible with our previous *in vitro* results (3). However, it differs from other studies, which show that QA decreases the uptake by astrocytes (3) and by synaptic vesicles (2). There is a possible explanation for these differences, depending on preparation used. Glutamate uptake by synaptosomal preparations is mainly due to the presence of ''gliosomes''(vesicles derived from astrocytes) attached to presynaptic terminals. These ''gliosomes'' do not present the same properties of astrocytes, which could be the reason for different effects of QA on glutamate uptake.

Moreover, in studies with cortical slices obtained from young rats, QA-induced seizures decrease glutamate uptake (33), fact that could increase extracellular glutamate concentrations. Interestingly, another results from our group using synaptic vesicles from rats showed that QA-induced seizures increased glutamate uptake and decreased GABA uptake (34). It is reasonable to suppose that these effects could not be a cause, but a consequence of seizures. However, this could lead to an increase of the vesicular glutamate content, further increasing its quantal release, augmenting synaptic strength (35).

Here, guanosine abolished QA effects on synaptosomal glutamate release only when blocking seizures, suggesting that this neurochemical effect is related to its actions as anticonvulsant. Concerning this fact, we previously demonstrated that guanosine *in vivo* reversed the QA-induced glutamate uptake decrease in cortical slices from rats (33), only when protecting against QA-induced seizures. Thus, the anticonvulsant effect of guanosine could involve at least partially the decrease of extracellular glutamate concentrations to physiological levels.

Thus, the present study adds new data about the role of QA-induced seizures and GBPs on the glutamatergic system. The enhanced synaptosomal glutamate release could contribute to increase extracellular glutamate concentration, which ultimately could lead to overstimulation of the glutamatergic system. These effects may be related to the neurotoxicity and seizures induced by the glutamatergic agent QA in rats.

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