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Neurotoxicity Induced by Glutamate in Glucose-Deprived Rat Hippocampal Slices is Prevented by GMP

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Guanosine-5′-monophosphate (GMP) was evaluated as a neuroprotective agent against the damage induced by glutamate in rat hippocampal slices submitted to glucose deprivation. In slices maintained under physiological conditions, glutamate (0.01 to 10 mM), Kainate, alpha-amino-3-hydroxi-5-methylisoxazole-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), or L-2-amino-4-phosphonobutanoic acid (L-AP4) (100 μM) did not alter cell membrane permeability, as evaluated by lactate dehydrogenase (LDH) release assay. In slices submitted to glucose deprivation, GMP (from 0.5 mM) prevented LDH leakage and the loss of cell viability induced by 10 mM glutamate. LDH leakage induced by Kainate, AMPA, NMDA or 1S,3R-ACPD was fully prevented by 1 mM GMP. However, glutamate uptake was not altered in slices submitted to glucose deprivation and glutamate analogues. Glucose deprivation induced a significant decrease in ATP levels which was unchanged by addition of glutamate or GMP. Our results show that glucose deprivation decreases the energetic charge of cells, making hippocampal slices more susceptible to excitotoxicity and point to GMP as a neuroprotective agent acting as a glutamatergic antagonist.

KEY WORDS: GMP; neuroprotection; glutamate; glucose deprivation; LDH release; hippocampal slices.

INTRODUCTION

Glutamate is the main excitatory neurotransmitter in mammals. However, excessive activation of glutamate receptors leads to neuronal degeneration and death. Excitatory amino acid neurotoxicity has been proposed to contribute to the pathogenesis of neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Huntington's diseases, and is also involved in the neuronal damage found in cerebral ischemia (for review, see 1).

During cerebral ischemia (2), hypoglycemia (3), and cerebral trauma (4), the extracellular concentration of glutamate increases substantially, due probably to an exaggerated release or to an impairment of its high-affinity uptake system (5). Neuronal death associated with these brain disorders can be effectively prevented by the administration of glutamate receptor antagonists (6,7). However, clinical trials have shown that irreversible blockade of glutamate receptors is prejudicial to ischemic or

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traumatic recovering (8). Therefore, the search for molecules that modulate glutamatergic transmission continues.

Besides their well defined intracellular roles, as modulating G-proteins activity and ribosomal activation to protein synthesis, guanine nucleotides display important extracellular roles. GTP and the guanine nucleoside, guanosine, were shown to act as trophic factors to neurons and proliferating factors to astrocytes (9) and GMP is shown to be released from cultured astrocytes after hypoxia/hypoglycemia (10). A highlight extracellular role of these compounds is their modulatory effects on the glutamatergic system. Guanine nucleotides decrease the binding of glutamate and glutamate analogues to their receptors (11). Moreover, they inhibit glutamate-induced physiological cell responses (12,13) and it was shown they are present in the extracellular space, through evaluation of the cerebrospinal fluid content in humans (14). We have also reported that guanine nucleotides bind to a purified Kainic acid (KA) receptor, in a binding site without hydrolytic activity, resembling an interaction site to guanine nucleotides (15). Specific extracellular binding sites to guanosine have also been demonstrated (16). Furthermore, guanine nucleotides were found to protect against glutamate receptors-mediated neurotoxicity. *In vivo* studies have shown that administration of GMP prevented NMDA-mediated seizures (17,18) and cell death in the rat striatum (19). GMP also reduced neuronal damage in hipocampal slices submitted to hypoxia (20) or ischemia *in vitro* (21).

Experimental *in vitro* models for ischemia have advantages in studying cellular pathophysiological responses. Deprivation of oxygen and/or glucose from brain tissue preparations reproduces several pathological states induced by brain energy failure. Incubation of brain slices in a low oxygen and/or glucose-deprived medium causes a rapid reduction of cellular ATP content, release of neurotransmitters, and increase in cytosolic calcium concentration (1) which might provoke loss of neural homeostasis and degeneration. In the present study, vulnerability of hippocampal slices preparations to glucose deprivation in the presence of glutamate or glutamate receptor agonists was assessed by measuring lactate dehydrogenase (LDH) release, MTT (thiazolyl blue) reduction, glutamate uptake and intracellular ATP levels. An increased leakage of LDH, a parameter used to observe cell swelling (22), and a decreased cellular viability measured by MTT reduction, were observed in glucose-deprived hippocampal slices submitted to glutamate or glutamate receptor agonists. Glucose deprivation reduced intracellular ATP levels, which was not exacerbated by addition of glutamate. Exogenous added GMP, a guanine nucleotide which does not bind to G-proteins, prevented the increased LDH leakage and decreased cell viability provoked by glutamate and glutamate receptor agonists during glucose deprivation but had no effect on glutamate uptake or recovering of ATP content.

EXPERIMENTAL PROCEDURES

Animals. Male immature Wistar rats (18–21 days of postnatal age) maintained on a 12 h light–12 h dark schedule at 25 °C, with food and water *ad libitum*, were obtained from our local breeding colony. Experiments followed the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) and were approved by local Ethical Committee for Animal Research (CEUA/UFSC).

Materials. GMP, glutamate, NMDA, luciferin-luciferase assay kit were purchased from Sigma Chemicals, USA. KA and AMPA were from RBI, USA. 1S,3R-ACPD and L-AP4 were from Tocris Cookson, UK. L-[^{3}H]Glutamate was purchased from Amersham, Pharmacia Biotech Inc., USA. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Slices preparation and incubation. Rats were killed by decapitation and hippocampi were rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB) of the following composition (in mM): 122 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 0.4 KH_2PO_4, 25 $NaHCO_3$ and 10 D-glucose. The buffer was bubbled with 95% O_2-5% CO_2 up to pH 7.4 throughout the experiments. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4°C, and two slices per tube were allowed to recover for 30 min in KRB at 37°C. After pre-incubation, slices were incubated with glutamate (0.001 to 10 mM) for 1 h and aliquots of the bathing medium were withdrawn and frozen. In another set of experiments, slices were exposed to glutamate (10 mM), N-methyl-D-aspartate (NMDA), KA, alpha-amino-3-hydroxi-5-methylisoxazole-propionate (AMPA), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) or L-2-amino-4-phosphonobutanoic acid (L-AP4, 100 μM) for 1 h and at the end of this period, incubation medium were replaced to KRB gassed with 95% O_2-5% CO_2 and slices maintained for additional 3 h (reperfusion time). Aliquots of the bathing medium in each of the 4 h of incubation were withdrawn and frozen. Incubation was terminated by placing the tubes in an ice-cold bath and slices were homogenized to measure the protein content by the method of Lowry (23), using bovine serum albumin as standard. In order to obtain the glucose-deprived situation, hippocampal slices were incubated in a glucose-deprived buffer (GD) with the same salt composition of KRB, whereas 10 mM D-glucose was replaced by 10 mM 2-deoxy-glucose (2dg). Incubation was started by replacing the KRB to GD or to GD containing glutamate or glutamate receptor agonists and slices were maintained without further oxygenation. When added, GMP was pre-incubated for 10 min.

Lactate dehydrogenase (LDH) release assay. Cell membrane permeability was determined by the LDH release assay, using a spectrophotometric (340 nm) assay kit (Doles Reagentes, BR), modified from Whitaker (24). Total LDH activity was determined after adding a final concentration of 10% Triton X-100 and disrupting the slices by homogenization with a Tissue Tearor (Biospec). Results were expressed as a percentage relative to the 100% of LDH activity. Data from different experiments were pooled by normalizing to the amount of LDH released from disrupted slices. The variability due to differences in slice size was minimal.

MTT reduction assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction was measured to determine cell viability (25). The tetrazolium ring of MTT is cleaved by various dehydrogenases in active mithocondria and then precipitates as a purple formazan product. Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 20 min at 37°C. The medium was then aspirated, the precipitated formazan was solubilized with dimethyl sulfoxide and viable cells were quantified spectrophotometrically at a wavelength of 550 nm.

Glutamate uptake. Glutamate uptake was evaluated as previously described (26). After incubation in glucose deprived medium in the presence or absence of glutamate receptors agonists and/or GMP, hippocampal slices were incubated for 15 min at 35°C, in Hank's balanced salt solution (HBSS), composition in mM: 1.29 $CaCl_2$, 136.9 NaCl, 5.36 KCl, 0.65 $MgSO_4$, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 5 Hepes. Uptake was assessed by adding 0.33 μCi/ml L-[^{3}H]glutamate with 100 μM unlabeled glutamate. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS immediately followed by addition of 0.1% NaOH/0.01% SDS, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[^{3}H]glutamate through scintillation counting. Sodium-independent uptake was determined by using choline chloride instead of sodium chloride, being subtracted from the total uptake to obtain specific uptake. Results were expressed as nmol of L-[^{3}H]glutamate taken up per milligram of protein per minute.

Measurement of ATP levels. After incubation, hippocampal slices were homogenized in 2% Trichloroacetic acid, centrifuged for 3 min at 12,000 *g* and supernatants were neutralized to pH 7.4 with Tris 1 M. Intracellular ATP content was determined by using a quantitative bioluminescent (luciferin–luciferase) assay kit (27).

Statistical Analysis. Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary. Differences among groups with or without glutamate or glutamate receptor agonists in the GD situation were measured using a Spearman's test. Each experiment was carried out in triplicate.

RESULTS

Effect of glutamate and glutamate receptor agonists on LDH leakage from hippocampal slices incubated under physiologic conditions. The effect of glutamate or its receptor agonists on cell membrane permeability of hippocampal slices incubated for 1 h under physiological conditions is shown in Fig. 1. Increasing glutamate concentrations (from 0.01 to 10 mM) showed no LDH leakage different from control slices (data not shown). Synthetic glutamate receptor agonists were used in order to prevent the high affinity glutamate uptake by glial cells (28). KA, NMDA or AMPA, the ionotropic glutamate receptor agonists; 1S,3R-ACPD, a metabotropic glutamate receptor agonist acting on metabotropic receptor groups I and II; and L-AP4 which acts on group III; (all analogues used at 100 μM) did not increase LDH leakage under this physiological condition, as compared to control slices. LDH leakage observed in the three following hours after glutamate or glutamate receptor agonists incubation was always lower than values observed in the first hours and was also not significantly different from the respective control group (Fig. 1).

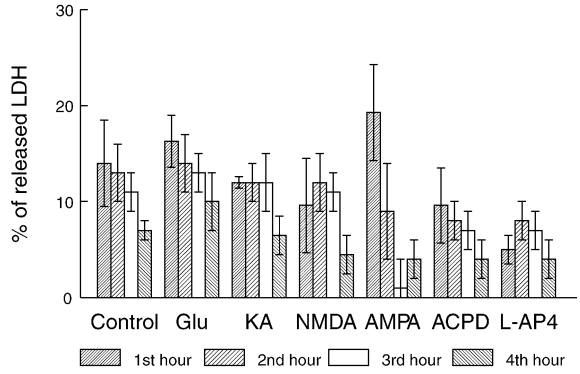

Fig. 1. Effect of glutamate and glutamate receptor agonists on the LDH leakage in hippocampal slices incubated under physiological conditions. Glutamate (Glu, 10 mM) or glutamate receptor agonists (KA, NMDA, AMPA, 1S,3R-ACPD, L-AP4; 100 μM) were incubated for 1 h and LDH leakage was evaluated in the end of this first h and in the three following h of incubation (legend bars). The percentage of LDH released from slices was calculated as the ratio of total LDH, determined after adding a final concentration of 10% Triton X-100 and disrupting the slices by homogenization. Specific activity of total LDH related to two hippocampal slices was 1697.48 ± 294.42 nmol/min/mg protein. Data represent means ± standard deviations of at least four determinations carried out in triplicates. Results were analyzed by one-way ANOVA.

Effect of glutamate and GMP on LDH leakage and cell viability in glucose-deprived hippocampal slices. When incubating hippocampal slices under glucose deprivation (i.e., buffer with 2-deoxy-glucose and non-oxygenated slices, GD), glutamate (10 mM) induced an increase in the leakage of LDH (Fig. 2a) and a decrease in the cellular viability (Fig. 2b) related to control slices. The increased LDH leakage was prevented by GMP (from 0.5 mM) in a concentration-dependent

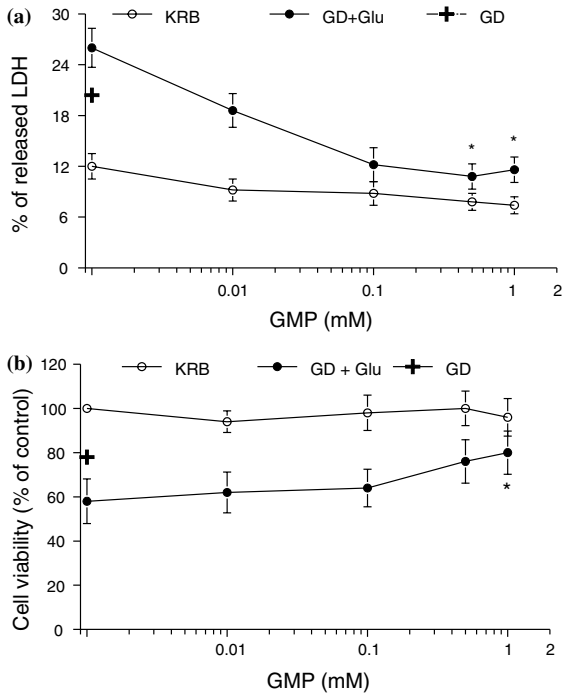

Fig. 2. Effect of GMP on LDH leakage and cellular viability in acute hippocampal slices incubated with glutamate under glucose deprivation. Increasing concentrations of GMP (0.01 to 1 mM) were incubated in physiological buffer (KRB) without glutamate; or submitted to glucose deprivation (GD) in the presence of 10 mM glutamate (GD + Glu). GD represents the glucose deprivation buffer (Krebs-Ringer bicarbonate buffer with 10 mM 2-deoxi-glucose) without glutamate. (a) Graph showing percentage of released LDH. (b) Graph showing percentage of cellular viability measured by MTT reduction assay. Data represent means ± standard deviations of at least six determinations carried out in triplicates. * indicates means significantly different from the GD group and the group GD + Glu without GMP; $P < 0.05$ (Duncan's test).

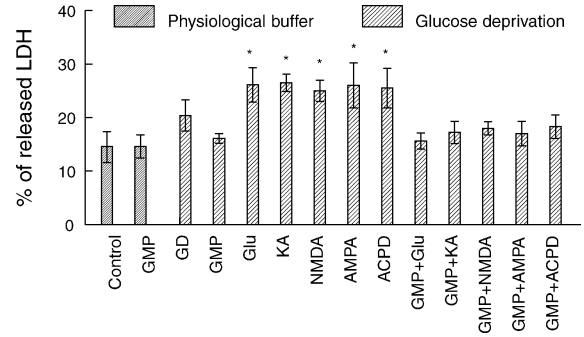

Fig. 3. Effect of glutamate, glutamate receptor agonists and GMP on the LDH leakage in acute hippocampal slices incubated under glucose deprivation. Glutamate (Glu, 10 mM), or KA, NMDA, AMPA, 1S,3R-ACPD (100 μM) were incubated for 1 h in the glucose-deprived buffer and LDH leakage was measured. When present, 1 mM GMP was added to the incubation buffer 10 min before glutamate or glutamate receptor agonists. Data represent means ± standard deviations of at least six determinations carried out in triplicates. * indicates means significantly different from control, GD group, and all groups in the presence of GMP; $P < 0.05$ (Spearman's test).

manner (Fig. 2a) and the decreased cell viability was prevented by 1 mM GMP (Fig. 2b).

Effect of glutamate receptor agonists and GMP on LDH leakage from glucose-deprived hippocampal slices. The glutamate receptor agonists, KA, NMDA, AMPA or 1S,3R-ACPD (100 μM), also induced an increase in LDH leakage from glucose-deprived hippocampal slices (Fig. 3). L-AP4 (100 μM) did not alter LDH leakage even in this glucose deprived situation (data not shown). KA, NMDA, AMPA, or ACPD did not significantly alter the LDH leakage at 10 μM concentration and the increase of concentration to 1 mM did not induce additional damage (data not shown) than the observed by using these glutamate receptor agonists at 100 μM (Fig. 3).

In glucose-deprived hippocampal slices, 1 mM GMP (added 10 min before glutamate or glutamate receptor agonists) prevented the increased LDH leakage which could be related to increased cell membrane permeability, probably due to cell swelling. Significant LDH release was observed only with the combination of a glucose-deprived buffer plus glutamate or glutamate receptor agonists (Fig. 3). However, the presence of 1 mM GMP abolished even the tendency in increasing LDH leakage observed when incubating slices in the glucose-deprived situation alone. Therefore, GMP acted as a neuroprotective agent against glucose deprivation in the presence of glutamatergic agonists. The levels of LDH released from slices evaluated after glutamate or glutamate receptor agonists incubation period, i.e., in the reperfusion time, decreased in the three following h of incubation, similar from the observed in the Fig. 1 (data not shown).

Effect of glutamate receptor agonists and GMP on glutamate uptake into glucose-deprived hippocampal slices. Glucose deprivation did not alter glutamate uptake into hippocampal slices (Fig. 4). The addition of KA, NMDA, AMPA or ACPD induced a slight decrease on glutamate uptake which was not significantly different from the control slices. GMP (1 mM) also did not alter glutamate uptake into slices maintained in physiological or glucose-deprived situation (Fig. 4).

Effect of glutamate and glucose deprivation on intracellular ATP levels of hippocampal slices. Both glucose deprivation, glucose deprivation plus glutamate (10 mM) or glucose deprivation plus KA

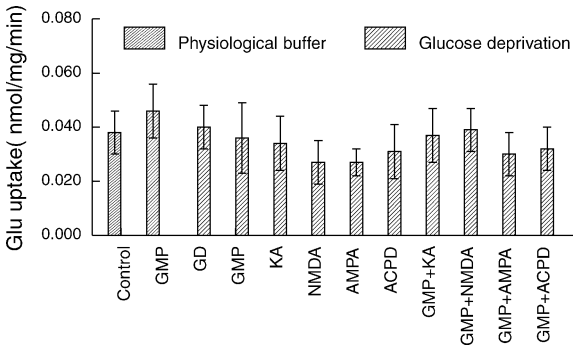

Fig. 4. Effect of glutamate receptor agonists and GMP on glutamate uptake in acute hippocampal slices incubated under glucose deprivation. KA, NMDA, AMPA, 1S,3R-ACPD (100 μM) were incubated for 1 h in the glucose-deprived buffer and glutamate uptake was measured as described in the Experimental Procedures. When present, 1 mM GMP was added to the incubation buffer 10 min before glutamate receptor agonists. Data represent means ± standard deviations of at least five determinations carried out in triplicates.

(100 μM) decreased ATP levels to approximately 16% of control values when compared to slices incubated under physiologic conditions. This decrease in the energetic charge promoted by glucose deprivation is not prevented by adding 1 mM GMP (Table I).

DISCUSSION

Incubation of hippocampal slices in the glucose-deprived situation was an useful tool in order to induce cellular damage potentiated by glutamate and glutamate receptor agonists. Therefore, when the injury promoted by glucose deprivation was exacerbated by glutamate receptors activation, an effective neuroprotective role of GMP against cell membrane permeabilization and loss of cell viability was demonstrated.

The absence of increasing LDH leakage observed in hippocampal slices submitted to physiological conditions, even in the presence of glutamate or glutamate receptor agonists (Fig. 1), can be interpreted as a mild damage evoked by glutamate receptors activation in such physiological condition, which could not be detected by the parameter used in this study. Efficient uptake of glutamate presumably contributes to its weak neurotoxicity in hippocampal slices. However, this can not fully explain the lack of toxicity induced by glutamate since its receptor agonists (which are not taken up by glutamate carriers) also had no effect in inducing LDH leakage under physiological conditions (Fig. 1). The neurotoxicity evoked by glutamate receptors activation involves an increased calcium influx, which leads to several responses as production of free radicals and alterations in membrane permeability (1). However, when glutamate uptake is functional, acute neurotoxicity in brain slices may be primarily linked to glutamate receptors overactivation and depends on incubation time, ionic and energetic balance (29). Accordingly, we have observed that cellular permeability was not affected by excitotoxic events induced by glutamate or glutamate receptor agonists when slices were incubated in standard ionic and energetic conditions (Fig. 1).

Glucose-deprived slices showed a tendency to increase LDH leakage when compared to slices incubated under physiologic conditions (Fig. 2a). However, cell damage was significant when slices were submitted to glucose deprivation and glutamate (Fig. 2) or glutamate receptor agonists (Fig. 3). The glutamate receptor agonists tested (at

Table I. Measurement of intracellular ATP levels in hippocampal slices submitted to glucose deprivation, glutamate and KA

Treatments	ATP levels (μM)	% of ATP (related to control slices)
Control (KRB)	1.780 ± 0.55 *	100
GMP (KRB)	2.426 ± 0.56 **	136
GD	0.291 ± 0.02	16
GMP (GD)	0.358 ± 0.08	20
Glu (GD)	0.285 ± 0.11	16
GMP + Glu (GD)	0.346 ± 0.06	19
KA (GD)	0.294 ± 0.04	16
GMP + KA (GD)	0.317 ± 0.12	17

Slices were incubated for 1 h in physiological buffer (KRB, Krebs-Ringer buffer) to obtain the Control situation, or in the presence of 1 mM GMP. Slices submitted to glucose deprivation (GD) were incubated in the presence of 1 mM GMP, 10 mM Glu, 100 μM KA, or GMP + Glu and GMP + KA for 1 h. The cellular levels of ATP were measured as described in Material and Methods from one hippocampal slice (approximately 284.0 ± 7.07 μg of protein) submitted to the treatments cited above. Data represent means ± standard deviations of at least nine determinations carried out in triplicate. * and ** indicate means significantly different from all other groups; $P < 0.01$.

100 μM) had the same order of magnitude of effect in enhancing LDH leakage when compared to slices incubated with glutamate (10 mM).

During glucose deprivation, the lack of energy and the failure of the energy dependent reuptake system might be the determinant factors to induced cellular damage (2–5). Glucose deprivation induced a prominent decrease in ATP levels in the hippocampal slices (Table I), however, glutamate uptake was not significantly impaired (Fig. 4). The release of glutamate from glucose-deprived slices presumably may account for a mild damage which is converted only in a slight LDH release tendency (Fig. 3). However, when these slices are exposed to extracellular added glutamate or glutamate analogues, an increased glutamate receptors activation and thus an increased loss of cell viability is observed, even if the reuptake system is functional. Therefore, the neuronal vulnerability to glutamate toxicity is affected by the extracellular glucose concentration (30), even when it is evaluated soon after glutamate exposition (Figs. 2 and 3). Studies using extracellular recordings of evoked spikes as a parameter of neuronal function in hippocampal slices, also demonstrated that glutamate can increase cell damage induced by glucose deprivation (31,32). However, we have previously shown that in slices submitted to an ischemic-like condition (glucose-deprived hippocamapal slices are also exposed to a respiratory chain inhibitor, potassium cyanide), when the cell damage is more severe, no additional effect is observed by the addition of KA (21).

Regarding to the protective effect of GMP, it has been shown that inhibition of cyclic GMP degradation by using phosphodiesterase inhibitors affords significant neuroprotection (33). Such neuroprotective action of cyclic GMP was demonstrated to be due to an extracellular action (34). However, we have previously reported that GMP *per se* inhibits cellular responses induced by glutamate (12,13). Moreover, guanine nucleotides inhibit NMDA and Kainate-induced neurotoxicity in cultured neurons (35). Here we are showing that extracellular added GMP prevented the damage induced by glucose deprivation in the presence of glutamatergic agonists. Considering that carriers to phosphorylated nucleotides in the plasmatic membrane were not yet demonstrated, the mechanism of action of GMP as a neuroprotective agent could be related to its ability to act as a glutamate receptor antagonist. Accordingly, the neuroprotective effects of GMP in hippocampal slices (21) and neuronal cultures (35) were similar to the effects observed with classical glutamate receptors antagonists. On the contrary, guanosine added to neuronal cultures displayed a protective role only if the enzyme responsible for its hydrolysis was not inhibited (36), indicating that the neuroprotective effect of guanosine would also be exerted by providing intermediates to maintain cell metabolism. Thus, guanine derivatives might provide doubled neuroprotective action: (i) an intracellular action, as shown with guanosine, by providing intermediates to replenish cell metabolism (and therefore normalizing the energetic charge of cells); and (ii) an extracellular action, by modulating glutamate-induced neurotoxic actions through displacement of glutamate binding to its receptors.

Since GMP did not recover the decreased energetic charge induced by glucose deprivation and glutamate or KA (Table I), its neuroprotective action must be occurring via interaction with glutamate receptors (21), although it might also be hydrolyzed extracellularly (37). Therefore, GMP effect occurs via blockade of glutamate receptors activation and do not involve interactions with glutamate uptake system or intracellular ATP content recovery.

The ultimate consequences of a hypoglicemic episode in an intact animal must represent the sum of all possible neurodegenerative and protective processes. Nonetheless, the pharmacological blockade of glutamate receptors by the endogenous modulator, GMP, represents one potentially effective therapy against excitotoxicity.

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