Effects of S-Emopamil, Nimodipine, and Mild Hypothermia on Hippocampal Glutamate Concentrations After Repeated Cerebral Ischemia in Rabbits

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Background and Purpose: We sought to determine the effects of two different calcium channel antagonists, S-emopamil and nimodipine, on hippocampal glutamate and glycine release and to compare their effects to those of mild hypothermia.

Methods: New Zealand White rabbits were subjected to two 7.5-minute episodes of global cerebral ischemia at a 1-hour interval produced by neck tourniquet inflation (20 psi) combined with hypotension during halothane anesthesia. Hippocampal extracellular concentrations of glutamate and glycine were monitored using in vivo microdialysis. Animals were randomized to receive either S-emopamil (1 mg·kg\(^{-1}\)·min\(^{-1}\) bolus, 0.1 mg·kg\(^{-1}\)·min\(^{-1}\) infusion), nimodipine (10 µg·kg\(^{-1}\)·min\(^{-1}\) bolus, 1 µg·kg\(^{-1}\)·min\(^{-1}\) infusion), hypothermia (32°C), or saline (control) before ischemia. Drug infusion and hypothermia were continued throughout the study periods.

Results: In all four groups, both ischemic episodes resulted in significant elevations of hippocampal extracellular concentrations of glutamate and glycine (baseline vs peak, \(P<.01\) in all groups). However, glutamate levels were significantly lower in the S-emopamil (\(P=.0001\)) and hypothermia (\(P=.0003\)) groups compared with the control group after the onset of the first ischemic episode through 1 hour after the second ischemic episode. There was no significant difference between the four groups in the concentrations of glycine. There was no significant difference between the peak concentrations of glutamate or glycine after each ischemic period.

Conclusions: These results suggest that pres ischemic administration of S-emopamil, but not nimodipine, attenuates the increase in hippocampal extracellular concentrations of glutamate in the peri-ischemic period in this model, and that this effect is also observed when mild hypothermia is instituted before ischemia. Decreased concentrations of glutamate after ischemic episodes may be a possible mechanism for the observed neuroprotective properties of S-emopamil and mild hypothermia (32°C). (Stroke 1993;24:1228-1234)

Key Words • calcium channel blockers • cerebral ischemia • glutamates • rabbits

Although many cellular mechanisms of neuronal damage after global brain ischemia have been proposed, a final common pathway to ischemic neuronal damage is thought to involve calcium toxicity.\(^1\) Calcium enters neurons through two major routes: the N-methyl-D-aspartate (NMDA) receptor–operated ion channel and the voltage-sensitive calcium channel (VSCC).\(^1\) The administration of VSCC antagonists has been shown to reduce ischemic neuronal damage in vivo,\(^2\) although the exact cellular mechanisms of their actions are not known.

Glutamate, a known neurotoxin, increases in the extracellular space during ischemia by increased release and/or decreased uptake.\(^3\) In vitro studies have suggested that glutamate release from a specific transmitter pool is calcium dependent and operates only in the first few minutes after the onset of anoxia because it requires the presence of high-energy phosphates, which are rapidly depleted during anoxia.\(^3,5,6\) As the ischemic duration increases, increased extracellular potassium may disturb the glutamate uptake system, resulting in decreased uptake of glutamate and/or glutamate release from cytoplasm of both nerve terminals and glial cells by the reversal of the uptake system.\(^3\) As ischemic time increases, this mechanism then becomes predominant because it is energy independent.\(^3\) VSCC antagonists could potentially decrease the calcium-dependent release of glutamate if administered before the onset of global ischemia. If the ischemic duration is appropriately brief, it may be possible to observe the inhibitory effect of VSCC antagonists on the extracellular concentration of glutamate during ischemia.

The paradigm of repeated global ischemia is also of interest in this regard. Although each ischemic episode may be brief and not injurious to neurons, repeated ischemic insults can have a cumulative effect and result
in neuronal damage. The mechanisms of this phenomenon are still unknown, but alterations in the amounts of excitatory neurotransmitters released might be involved. Because it is known that VSCC antagonists bind more avidly to partially depolarized channels, it is possible that the VSCC antagonists might show greater efficacy in interfering with glutamate release during repeated ischemia.

Recently it has been recognized that glycine is an important facilitator of glutamate’s action at the NMDA receptor. Indeed, the presence of glycine at the NMDA receptor has been demonstrated to be essential for the functional activation of the receptor by glutamate. Persistent elevation of the NMDA receptor has been demonstrated to be essential during repeated ischemia. It is possible that the VSCC antagonists might maintain the receptor by gluta-

Materials and Methods

The protocol was reviewed and approved by the Animal Care Committee of the University of California, San Diego. Eighteen New Zealand White rabbits weighing 2.68 ± 0.27 kg (mean ± SD) were anesthetized in a Plexiglas box with 5% halothane in oxygen. After intubation of the trachea with a 4.0-mm uncuffed wire reinforced endotracheal tube, the animals were mechanically ventilated with 1.5% halothane in oxygen to maintain normocapnia (PBaCO2, 35 to 40 mm Hg). Body temperature was monitored with an esophageal thermistor. After inflation with 0.25% bupivacaine, a catheter (PE-90) was inserted into the femoral artery for measurement of arterial blood pressure and arterial blood gases. The femoral vein was also cannulated for the administration of drugs during inflation of the neck tourniquet. An ear vein catheter was inserted for the administration of fluids (0.9% saline) and drugs. All rabbits initially received 40 mL·kg⁻¹ of 0.9% saline solution administered intravenously by an infusion pump over a 1-hour period. This was followed by a maintenance infusion at 4 mL·kg⁻¹·h⁻¹ throughout the study. The rabbit’s head was positioned in a stereotaxic frame, and a pneumatic tourniquet (2.5 inches in width; Zimmer) was secured loosely around the neck. After inflation with 0.25% bupivacaine, the cranium was exposed and burr holes were made bilaterally over the dorsal hippocampus (4 mm posterior and 4 mm lateral to the bregma) for the insertion of microdialysis probes (CMA-10, Carnegie Medicin, Sweden). A third burr hole was made 4 mm anterior and 3 mm lateral to the bregma over the right hemisphere for the insertion of a thermistor (23-gauge thermocouple, Physitemp Instruments) into the epidural space. The epidural tempera-
ture was servocontrolled to 36.5°C with a heat lamp and a warming pad. Biparietal needle electrodes were placed into the scalp for the continuous recording of the electroencephalogram (EEG). Monitored variables included mean arterial pressure, heart rate, arterial blood gases, hematocrit, blood glucose concentrations, esophageal and brain epidural temperatures, and the EEG. After the completion of these surgical preparations, the inspired halothane concentration was decreased to 1%.

Acceptable recovery rates for each microdialysis probe were verified by using 10⁻² mol/L dextrose solution in vitro before their insertion into the brain. The dura over the dorsal hippocampus was then incised, and microdialysis probes of concentric design (fiber length, 4 mm; diameter, 0.25 mm) were inserted vertically to a depth of 6 mm using micromanipulators. The probes were perfused with artificial cerebrospinal fluid (147 mmol/L NaCl, 2.3 mmol/L CaCl₂, 0.9 mmol/L MgCl₂, 4.0 mmol/L KCl) at a rate of 2 μL·min⁻¹. After implantation into the brain, the probes were perfused for at least 1 hour before collecting baseline samples of brain tissue microdialysate.

The rabbits were randomly assigned to one of the following groups. Group 1 (n=5) received a 1 mg·kg⁻¹ bolus of S-emopamil (dissolved in saline) over 15 minutes 55 minutes before ischemia, which was followed by an infusion of 0.1 mg·kg⁻¹·min⁻¹ throughout the study period. Group 2 (n=5) received a 10 μg·kg⁻¹·min⁻¹ bolus of nimodipine over 15 minutes 55 minutes before ischemia, which was followed by an infusion of 1 μg·kg⁻¹·min⁻¹ throughout the study period. Group 3 (n=4) started to be slowly cooled by the application of ice bags to the body surface 1 hour after insertion of the microdialysis probes. An epidural temperature of 32°C was achieved before collecting the first sample and was maintained throughout the study period. Group 4 (n=4) received a volume of saline that was equal to the total volume infused in groups 1 and 2.

Global cerebral ischemia was induced by lowering the mean arterial blood to less than 50 mm Hg by using trimethaphan boluses (10 mg) and the application of positive end-expiratory airway pressure. The neck tourniquet was then inflated to a pressure of 20 psi for 7.5 minutes. A tendency to hypertension during the first 3 minutes of ischemia was treated with additional doses of trimethaphan as needed to keep mean arterial pressure below 50 mm Hg. Global cerebral ischemia was verified in each rabbit by observation of an isoelectric EEG within 30 seconds after tourniquet inflation. Immediately after deflation of the tourniquet, a bolus and then an infusion of phenylephrine was administered to restore the mean arterial pressure to 75 mm Hg. After 1 hour of recirculation, a second, identical period of global ischemia was instituted.

At the end of the study period, the microdialysis probes were removed and the recovery rate for each microdialysis probe was determined again using 10⁻² mol/L dextrose solution in vitro. To verify the position of probes, 5 mL of Evans blue dye (2%) was administered intravenously. After the animal was euthanatized, the brain was removed and sectioned coronally to see the staining of the dye along the tracks of the probes.

Samples of microdialysate from the dorsal hippocampus were collected as follows. After the initial bolus drug administration (eg, S-emopamil, nimodipine) or the achievement of 32°C epidural brain temperature,
Physiological Data and Doses of Drugs in a Rabbit Global Cerebral Ischemia Model

<table>
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<tr>
<th>Group</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>Heart rate (bpm)</th>
<th>pH</th>
<th>Paco$_2$ (mm Hg)</th>
<th>Po$_2$ (mm Hg)</th>
<th>Epidural temperature (°C)</th>
<th>Esophageal temperature (°C)</th>
<th>Blood glucose (mg/dl)</th>
<th>Total trimethaphan dose (mg/kg)</th>
<th>Total phenylephrine dose (µg/kg)</th>
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<td>138±21</td>
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Values are mean±SEM. bpm, Beats per minute. *P<.05 vs control group.

The means and SEMs for the concentrations of glutamate and glycine were calculated for each time period by using the data from each catheter as a separate data point. Data for amino acid concentrations were corrected using in vitro recovery rates for dextrose. To determine whether ischemia had any effect on the concentrations of glutamate or glycine, corrected (Bonferroni) paired t tests were used to compare basal (time=0) vs peak concentrations of each of the amino acids. To determine whether differences existed between groups over time, a two-way repeated analysis of variance (ANOVA) (groups vs time) was done. If this analysis demonstrated a significant (P<.05) group effect, a repeated-measures ANOVA was done by sequentially comparing each of the experimental groups with the control group in a paired fashion (eg, hypothermia vs control) to determine final probability values for differences between groups. To determine whether the second ischemic episode had any incremental effect on the concentrations of glutamate or glycine, corrected paired t tests were used to compare the first peak vs the second peak concentrations of each of the amino acids. Physiological data were tabulated and compared using the Kruskal-Wallis test at each point. Mann-Whitney U tests were done when indicated. Doses of trimethaphan and phenylephrine were also analyzed using the Kruskal-Wallis test. Differences associated with P<.05 were considered statistically significant.

**Results**

Physiological data are shown in the Table. There were no significant differences between the four groups for mean arterial pressure, pH, PaO$_2$, PacO$_2$, or blood glucose. Heart rates at 10 minutes after both reperfusion in the hypothermia and the S-emopamil groups were significantly lower than those of the control group.
After the loading doses of S-emopamil and nimodipine, phenylephrine was administered to maintain mean arterial pressure greater than 60 mm Hg before ischemia when it was needed (four animals in the emopamil group, 237±167 μg/kg [mean total dose±SD], and four animals in the nimodipine group, 166±101 μg/kg). The in vitro recovery rate of microdialysis probes for dextrose ranged from 18% to 32%.

The time course of changes in concentrations of glutamate and glycine in the dialysate of hippocampal probes is illustrated in Figs 1 and 2. There was no difference between the four groups in the concentrations of glutamate and glycine before ischemia. In all four groups, both ischemic episodes resulted in significant elevations of hippocampal extracellular concentrations of glutamate and glycine. However, there were significant differences between the control group and the S-emopamil group and between the control group and the hypothermia group in the concentrations of glutamate after the onset of the first ischemic episode.

**Fig 1.** Line graph shows mean±SEM corrected dialysate concentration of glutamate over time for the S-emopamil (n=5), nimodipine (n=5), hypothermia (n=4), and control (n=4) groups. Ischemia resulted in significant increases in glutamate concentrations in all four groups. *Significant difference between baseline (time=0) and peak value within all groups (P<.05). There were also significant differences between the S-emopamil group and the control group, and between the hypothermia group and the control group after the onset of the first ischemic episode through 1 hour after the second ischemic episode by two-way analysis of variance (level of significance between groups indicated by probability values). Arrows indicate onset of ischemia.

**Fig 2.** Line graph shows mean±SEM corrected dialysate concentration of glycine over time for the S-emopamil (n=5), nimodipine (n=5), hypothermia (n=4), and control (n=4) groups. Ischemia resulted in significant increases in glycine concentrations in all four groups. *Significant difference between baseline (time=0) and peak value within all groups (P<.05). There was no significant difference between the four groups by two-way analysis of variance. Arrows indicate onset of ischemia.
through 1 hour after the second ischemic episode. There was no significant difference between the four groups in the concentrations of glycine (two-way ANOVA, group vs time). There was no significant difference between the peak concentrations of glutamate or glycine after each ischemic period.

**Discussion**

The present study demonstrated that S-emopamil and mild hypothermia (32°C) significantly reduced the concentration of glutamate during global cerebral ischemia when compared with the normothermic control group. This effect was also seen in the early reperfusion periods. Repeated ischemia (twice) appeared to have no cumulative effect on the extracellular concentrations of glutamate.

This study used a well-characterized animal model of transient global cerebral ischemia. The effects of various durations of ischemia (5, 10, and 15 minutes)\(^\text{17}\) and changes in temperature (37°C vs 29°C)\(^\text{16}\) on extracellular hippocampal excitatory amino acid concentrations in this model have been described. In the current experiment, the magnitude of the increases in glutamate and glycine was comparable to what had been previously observed.\(^\text{16,17}\)

In the present study, the concentrations of glutamate and glycine in the microdialysate were corrected by using the in vitro recovery rate for dextrose. The relative recovery for glutamate correlates well with that for dextrose. Although some authors question the use of in vitro recovery factors to estimate extracellular concentrations, our purpose was simply to reduce the variability that may arise from differences in the condition of probe membranes.

Extracellular concentrations of glutamate in the brain depend on the balance between release and uptake. In the normal situation, glutamate released from nerve terminals is promptly taken up by nerve terminals and glia cells, resulting in low extracellular concentrations of glutamate. During ischemia, however, the extracellular concentration of glutamate may rise to a neurotoxic level.\(^\text{20}\) Increased glutamate release and a dysfunction of the uptake systems has been demonstrated.\(^\text{3-8}\) After the onset of ischemia, glutamate may be released from a specific transmitter pool in the nerve terminals by a calcium-dependent mechanism after an increase in cytosolic free calcium.\(^\text{3,5,8}\) However, this release wanes within a few minutes because it is energy dependent.\(^\text{3,5,8}\)

As the ischemic duration increases, increased extracellular concentrations of potassium may disturb the function of the Na⁺-/cotransport uptake system, resulting in reduced uptake of glutamate by glia cells (probably also by nerve terminals) and/or glutamate release from the cytoplasm of both nerve terminals and glia cells by reversal of the uptake system.\(^\text{3-8}\) This release of glutamate is calcium independent and increases with time.\(^\text{3-7}\)

Therefore, as the ischemic duration increases, the calcium-dependent release of glutamate, which could potentially be antagonized by VSCC antagonists, accounts for a decreasing fraction of the total increase in the concentration of extracellular glutamate. Our laboratory has demonstrated that in the rabbit global ischemia model 5 minutes of ischemia did not increase the extracellular hippocampal concentration of glutamate, whereas 10 minutes of ischemia increased the glutamate concentration by fivefold to eightfold.\(^\text{17}\) Because we selected an intermediate but still brief duration of global ischemia (7.5 minutes), it is likely that this allowed the inhibitory effects of the VSCC antagonists (S-emopamil) on the concentrations of glutamate to be evident. Globus et al\(^\text{21}\) demonstrated that preischemic administration (intraperitoneal) of 5 mg · kg⁻¹ of S-emopamil attenuated the increased concentration of dopamine, but not of glutamate in the peri-ischemic period in rats subjected to 20 minutes of two-vessel occlusion with hypotension. Although we also administered a total dose of 5 mg · kg⁻¹ of S-emopamil before ischemia, we administered it intravenously instead of intraperitoneally. Thus, the brain tissue concentrations of S-emopamil in the present study might have been higher than that in Globus’ study. Furthermore, 20 minutes of ischemia might simply be too severe of an ischemic insult to rat hippocampal neurons compared with 7.5 minutes of total global ischemia in rabbits. It may be difficult to observe the effects of VSCC antagonists on glutamate release in models using severe ischemia, because the calcium-independent release of glutamate is predominant in that situation.

It is appropriate to wonder why S-emopamil but not nimodipine attenuated the increased concentration of glutamate during ischemia. Although the failure to demonstrate the inhibitory effect of nimodipine might simply represent an inadequate dose of nimodipine, the dose used was thought to be almost maximal from a cardiovascular standpoint as evidenced by the need to infuse phenylephrine before ischemia. An in vitro study using rat cerebellar slices suggested that endogenous glutamate release evoked by high concentration of potassium was calcium dependent and that potassium-evoked glutamate release was reduced by a phenylalkylamine-like compound, verapamil, but not by a dihydro- pyridine-like compound, nifedipine.\(^\text{22}\) S-emopamil is a phenylalkylamine, whereas nimodipine is a dihydropyridine. In rat sympathetic neurons, dihydropyridine-like compounds have been found not to affect norepinephrine release.\(^\text{23}\) It is likely that S-emopamil but not nimodipine decreases the energy-dependent release of glutamate. Excellent permeability to the blood-brain barrier of S-emopamil might also help to explain the difference.\(^\text{24}\)

Our results agree with the histological results of complete cerebral ischemia in a cat ventricular fibrillation model.\(^\text{25}\) Fleischer et al\(^\text{25}\) demonstrated that preischemic administration of S-emopamil attenuated hippocampal CA1 damage in cats subjected to 14 minutes of ventricular fibrillation. However, postischemic administration of S-emopamil did not ameliorate ischemic damage as judged by both neurofunctional and CA1 histology.\(^\text{25}\) It is possible that preischemic administration of S-emopamil in that model attenuated increases in hippocampal glutamate concentrations during ischemia (probably via the calcium-dependent mechanism), resulting in the attenuation of CA1 damage. When S-emopamil was administered after 14 minutes of global ischemia, it is likely that the calcium-dependent mechanisms of glutamate release were already inoperative, and thus no protection was seen.

Mild hypothermia (32°C) significantly attenuated the increase in hippocampal concentrations of glutamate in the current study. This is a well-known effect of hypothermia.\(^\text{26}\) Baker et al\(^\text{16}\) demonstrated that moderate hypothermia (29°C) significantly attenuated increases of
hippocampal glutamate concentrations in the same model and that neurological function assessed 48 hours after ischemia was significantly better in the hypothermic group compared with the control group. Although assessments of neither neurological function nor neuropathology were done in the present study, the results suggest that the institution of preischemic mild hypothermia (32°C) may have a protective effect in the setting of global ischemia.

Baker et al48 also demonstrated that moderate hypothermia (29°C) prevents increases in the concentration of glycine during ischemia. In the present study, no difference was found between the groups in the concentrations of extracellular glycine. This might be due to the differences in the brain temperatures achieved in the two studies (32°C vs 29°C). Indeed, examination of Fig 2 suggests that glycine concentrations in the hypothermia group appeared lower than those in the control group at many time points and that the only possible trend for differences between groups regarding glycine concentrations would be that between control and hypothermia (P=.151). It is possible that, had more animals been studied, this difference might have achieved statistical significance.

Tomida et al22 first described the repeated ischemia model. However, the duration of ischemia was too long for the study of delayed neuronal death.25 Kato et al8 developed the repeated ischemia model and showed that sublethal short ischemic insults have a cumulative effect on ischemic brain damage if they are repeated at given intervals. Short ischemic insults of 3-minute duration repeated three times at 1-hour intervals revealed significantly more CA1 damage compared with the damage after a single 9-minute ischemic episode in rats.11 In the present study, repeated ischemia did not have a cumulative effect on the peak hippocampal concentrations of glutamate. These results agree with the results of other investigators. For example, Lin et al20 demonstrated that three 5-minute ischemic insults, repeated at 1-hour intervals, had no cumulative effect on the peak concentration of glutamate in the rat thalamus and the striatum. Nakata et al29 also demonstrated that pretreatment with sublethal ischemia (2 minutes) 1 hour before a second ischemic episode did not have any cumulative effect on the hippocampal extracellular concentration of glutamate during the second ischemic episode in gerbils. These studies suggest that the mechanism of enhanced damage after repeated ischemia does not appear to be related to increased extracellular concentrations of excitatory amino acids per se. Other studies examining different aspects of excitatory amino acid (EAA) physiology in the setting of repeated ischemia such as EAA receptor affinity will be necessary to determine the ultimate role of EAs in provoking injury during repeated ischemic insults.

Fig 1 also suggests that glutamate concentrations were also very high and increasing at the latter stages of the experiment, ie, 240 minutes and 300 minutes in the control group. This did not achieve statistical significance and was not observed in all animals; rather, it was due to very high levels in only two of the animals. Examination of the physiological data of these animals did not provide us with a hypothesis as to why these two animals demonstrated these high glutamate concentrations. Both animals were stable with regard to blood pressure, pulse, and blood gases. There was no evidence of epileptiform activity.

In conclusion, we demonstrated that preischemic administration of S-emetapil, but not nimodipine, attenuated the increase in the hippocampal concentration of glutamate in response to repeated episodes of global ischemia in rabbits. The magnitude of this effect approximated that produced by a mild degree of hypothermia (32°C). Given the known neurotoxicity of glutamate, these results suggest decreased levels of EAAs as a possible mechanism for the observed neuroprotective properties of some VSCCs antagonists such as S-emetapil.

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References

Interneuronal communication is an important function in the human brain. Under physiological conditions, release of neurotransmitters by presynaptic neurons is the primary mechanism by which transfer of information is accomplished. During periods of ischemia, excessive release of excitatory neurotransmitters occurs and has deleterious effects on survival of postsynaptic neurons. Thus, it has been suggested that reducing the excessive release of excitatory neurotransmitters would augment neuronal survival in transient ischemia.

Dr. Matsumoto's laboratory has been investigating the effect of glutamate release and other parameters as studied neuroprotection in ischemia. The laboratory has an extensive publication record investigating excessive neurotransmitter release and neuronal pathology. This laboratory and others have shown protective effects of reducing the ischemia-induced neurotransmitter release by hypothermia. Expanding on this observation by deciphering the mechanisms of ischemia-induced neurotransmitter release will provide vital insight into neuronal necrosis after ischemia.

Increased intracellular calcium is a primary candidate for neuronal death. Neurons use transient increases in intracellular calcium for multiple functions, including neurotransmitter release and transduction of the neurotransmitter signal. Thus, reducing the ischemia-induced increase in intracellular calcium, both presynaptically and postsynaptically, will be an important mechanism in neuronal protection in ischemia.

However, since no single mechanism responsible for the excessive neurotransmitter release has been shown, it is important to understand the pharmacology of ischemia-induced neurotransmitter release. This will be accomplished only by systematic determination of processes that are involved in excessive neurotransmitter release.

Work performed in this laboratory and others will provide the groundwork for such investigations.

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EDITORIAL COMMENT

Interneuronal communication is an important function in the human brain. Under physiological conditions, release of neurotransmitters by presynaptic neurons is the primary mechanism by which transfer of information is accomplished. During periods of ischemia, excessive release of excitatory neurotransmitters occurs and has deleterious effects on survival of postsynaptic neurons. Thus, it has been suggested that reducing the excessive release of excitatory neurotransmitters would augment neuronal survival in transient ischemia.

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