Prevention of Delayed Neuronal Death in Gerbil Hippocampus by Ion Channel Blockers

Kimiaki Izumiyama, MD, and Kyuya Kogure, MD

We used a gerbil model of cerebral ischemia to study the effects of ion channel blockers on neuronal death resulting from enhanced glutamate release and calcium ion influx. The common carotid arteries of gerbils were occluded for 5 minutes and injected intraperitoneally immediately after ischemia with an alkylene iminopropylene derivative (glutamate blocker) or a piperazinyl ethanol derivative (calcium blocker) given at high or low doses. Two vehicle groups received saline or 0.2% methyl cellulose solution. Seven days later, the gerbils were perfusion-fixed and their brains were processed for histologic study. The number of neurons per millimeter (neuronal density) of the CA1 region was calculated, and the neuronal density in each group was statistically compared using the Mann-Whitney U test. Compared with a control group not subjected to carotid ligation, neurons of the two vehicle groups and the low-dose calcium blocker group were almost nonexistent in the CA1 region. Neuronal densities of the glutamate blocker group and the high-dose calcium blocker group were similar and were found to be within normal limits by statistical analysis. Our study shows that detrimental membrane phenomena and the incidence of delayed neuronal death may be counteracted by the systemic administration of these ion channel blockers after ischemic insult. (Stroke 1988; 19:1003–1007)

A lmost all living cells retain a specific internal chemical balance that is different from the external one. The destruction of the internal homeostasis due to energy failure has been recognized as a direct cause of cell death. However, there are some strange types of neuronal necrosis, one of which is delayed neuronal death.1 This phenomenon is demonstrated with transient forebrain ischemia in rodent hippocampus.1–2 More than 90% of gerbils subjected to 5 minutes of forebrain ischemia develop uniform destruction of pyramidal cells in the CA1 region of the hippocampus 2–3 days later. The postischemic events progress slowly, finally resulting in massive cell necrosis. Ischemia-induced intracellular acidosis, together with energy failure, causes loss of homeostasis and results in an efflux of potassium from and an influx of sodium and calcium into the affected neurons.3 This phenomenon is followed by pathologic release of neurotransmitter substances.4–6 In the hippocampal region, an overabundance of the excitatory neurotransmitter glutamate7 may induce the destruction of ion channels in the postsynaptic membrane.8

With ischemia, a large amount of glutamate is released in the CA1 region of the hippocampus, which perturbs the glutamate-regulated ion gates in the postsynaptic membrane.8 Calcium ions may then pass in a disorderly fashion through these perturbed ion gates. When this shift of ions surpasses the physiologic function of ion pumps, internal chemical balance breaks down and the cells die.9 Our study suggests that, if the disruption of the ion gates can be preserved by ion channel blockers, neuron injury may be limited.

Materials and Methods
A total of 56 male Mongolian gerbils (Meriones unguiculatus) weighing 50–70 g were used. The gerbils were anesthetized with 2% halothane in a mixture of 30% O2 and 70% N2O. The carotid arteries were exposed at both sides of the neck and were separated from the surrounding tissue. The anesthesia was then discontinued, and both carotid arteries were occluded with aneurysm clips (Sugita clip, Mizuho Ikakogyo Co., Ltd., Tokyo, Japan) to produce uniform forebrain ischemia.10,11 After 5 minutes of occlusion, three groups of 10 gerbils each were treated with 40 mg/kg i.p. 4-(2-methylpropyl)-3-[3-(perhydroazepin-1-yl)propyl]-5-phenyl-1,3-oxazolidin-2-one (glutamate blocker) dissolved in saline or 20 mg/kg i.p. (low dose) or 30 mg/kg i.p. (high dose) 1-(3,4-dimethoxyphenyl)-2-(4-diphenyl methyl piperazinyl) ethanol dihydrochlo-
ride (calcium blocker) dissolved in 0.2% methyl cellulose solution (Figure 1). Nippon Chemiphar Co., Ltd. (Tokyo, Japan) supplied the two newly derived ion channel blockers. Gerbils not treated with ion channel blockers (two vehicle groups of 10 gerbils each, one injected with 0.2% methyl cellulose solution and the other with saline, and one unoperated control group of six gerbils) were also prepared. After the operation, the gerbils were kept on a warming mat (39°C) until they began to move again and then they were given food and water freely.

Seven days after ischemia, all treated gerbils and the six unoperated control gerbils were anesthetized with 50 mg/kg i.p. pentobarbital (Nembutal, Abbott, North Chicago, Illinois) and transcardially fixed with 300 ml of 3.5% formaldehyde at 120 cm H2O at room temperature for 1 hour. The whole brains were then removed and immersed in the same fixative for 24 hours. Each brain was then dissected into 7-mm coronal sections that contained the hippocampal area and were further fixed for 24 additional hours. After fixation, brain sections were dehydrated with graded ethanol, passed through chloroform, and embedded in wax.

Sections 5 μm thick that included the dorsal hippocampi located 1.5 mm posterior to the bregma were prepared using a microtome and stained with cresyl violet. The pyramidal cells of each CA1 region were counted under a light microscope at ×400 magnification. Each section of the CA1 region was photographed at ×40 magnification, the CA1 regions of both vehicle-treated groups were similar to those of the vehicle-treated groups (data not shown). However, when the gerbils were treated with the high-dose calcium blocker, only tiny morphologic changes were observed, and most of the cells in the CA1 region were preserved within normal limits (Figure 2, bottom). The morphologic findings of the glutamate blocker group were similar to those of the high-dose calcium blocker group (data not shown).

Neuronal densities of the high-dose calcium blocker and the glutamate blocker groups were found to be within normal limits by statistical analysis, while neurons of the low-dose calcium blocker and the two vehicle groups were almost completely nonexistent (Table 1).

**Discussion**

The incidence of delayed neuronal death was markedly reduced by systemic administration of two ion channel blockers immediately after ischemia. The glutamate blocker we used is known to cross the blood–brain barrier (BBB) and to powerfully block the glutamate response in rat cortical neurons and crayfish neuromuscular junctions. This agent acts as an open channel blocker, but it demonstrates no specificity to glutamate receptor subtypes. In rat ischemia, the use of a glutamate blocker to protect the hippocampal neurons is reported with 2-amino-7-phosphonoheptanoic acid (AP7), an antagonist of the N-methyl-D-aspartate–preferring receptor. However, AP7 was applied by focal microinfusion before ischemia. Other reports indicate that AP7 is effective by systemic administration before or after 20 minutes of ischemia in gerbils. However, since those gerbils were prepared under methoxyflurane anesthesia, a transynaptic and neuroprotective effect of the anesthetic modifies the morphologic outcome.

The calcium blocker, which is a newly derived and liposoluble agent, has a structure and actions closely resembling those of flunarizine. The calcium blocker has strong calcium antagonistic action on arteries and neurons (unpublished data) and also crosses the BBB. However, low-dose application could not prevent hippocampal cell necrosis. Alphs et al reported the cytoprotective activity of a small amount of nicardipine (a calcium antagonist) on the same model as ours, but their agent was injected before ischemia and was repeated twice daily for 72 hours. The method of postischemia treatment of delayed cell death is still limited.
FIGURE 2. Top: Coronal section of dorsal hippocampus in unoperated control gerbil. CA1 pyramidal cell layer (between arrows) is well-stained as are other CA regions. Bar=500 μm. Inset: All CA1 neurons are well-preserved under higher magnification. Bar=100 μm. Middle: Coronal section of dorsal hippocampus in saline vehicle-treated gerbil. Almost all pyramidal cells of CA1 region (between arrows) are less receptive to staining. Inset: Most CA1 neurons disappear and are replaced by many glial cells. Bottom: Coronal section of dorsal hippocampus in calcium blocker-treated gerbil. CA1 region (between arrows) is very receptive to staining, as receptive as that of unoperated control gerbil. Inset: Most CA1 neurons are preserved within normal limits.
The glutamate blocker and the calcium blocker we used are potent and have no depressant effects on the central nervous system or the behavior of gerbils.

The excitatory feed-forward afferent fibers in the hippocampus are glutamatergic. When these fibers are stimulated within physiologic limits, glutamate is released from the presynaptic terminals and the calcium ion gate opens at a postsynaptic site. The physiologic activities are thought to be triggered by calcium ion influx following this gate opening. In the case of ischemia, the extracellular concentration of the neurotransmitter glutamate rises to an excessive level,\(^8,21\) which induces a large influx of calcium ions into neurons, perturbs ionic homeostasis of the postsynaptic site, and leads to cell death.\(^22\) This phenomenon is the reason for administering antiglutamatergic agents or calcium channel blockers.

The hippocampus is known for its susceptibility to anoxia and ischemia.\(^2\) The cellular and vascular constructions of this region have little singularity, and changes in hippocampal blood flow,\(^9,11\) energy state (i.e., ATP level), and pH during ischemia recover within 2 hours.\(^23,24\) Therefore, these alterations cannot be the cause of the cell necrosis that occurs 2–3 days after ischemia. However, enhanced neuronal activity of the CA1 neurons is observed to last for approximately 24 hours after 5 minutes of ischemia\(^25\) and is probably caused by the perturbed ion gates in the postsynaptic membrane. This accelerated glucose metabolism takes place in the perikarya of the pyramidal cells and in the distal dendrites of the stratum radiatum of the CA1 region.\(^26\) These two rapidly metabolizing areas can also support accelerated transsynaptic stimulation and neuronal activity.\(^27\) The suppression of amino acid incorporation and protein synthesis is also recognized to be caused by this energy failure.\(^28,29\) The main excitatory extrinsic pathway to the CA1 neurons, which originates in the entorhinal cortex, consists of three distinct neuronal steps. When these neuronal steps, such as CA3 neurons, dentate granular cells, and the entorhinal area, are destroyed by kainate injection,\(^30\) colchicine injection,\(^31\) and abrasion of the entorhinal area,\(^32\) respectively, prior to ischemia, the CA1 neurons of each model are preserved. That is, one recognizes that delayed ischemic cell necrosis is mediated through some transsynaptic mechanism.

The transsynaptic overactivity and associated enhanced influx of calcium are the important factors of delayed neuronal death. Noninvasive pharmacologic treatment with ion channel blockers and antagonists of excitatory neurotransmitters after the ischemic episode is a valuable therapy.

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**References**

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**Table 1. Neuronal Densities in CA1 Region of Gerbils 7 Days After 5 Minutes of Ischemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Neuronal density (no./mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate blocker</td>
<td>10</td>
<td>45.69 ± 5.75*</td>
</tr>
<tr>
<td>High-dose calcium blocker</td>
<td>10</td>
<td>47.16 ± 4.31*</td>
</tr>
<tr>
<td>Low-dose calcium blocker</td>
<td>10</td>
<td>9.13 ± 3.26</td>
</tr>
<tr>
<td><strong>Not treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unoperated control</td>
<td>6</td>
<td>66.03 ± 7.37</td>
</tr>
<tr>
<td>Saline vehicle</td>
<td>10</td>
<td>3.50 ± 4.47</td>
</tr>
<tr>
<td>0.2% methylcellulose vehicle</td>
<td>10</td>
<td>7.51 ± 2.52</td>
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Values are mean ± SEM. *p<0.01 compared with both vehicles (Mann-Whitney U test).

KEY WORDS • calcium channel blockers • hippocampus • gerbils • cerebral ischemia
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