Calcium Antagonist, Adenosine A<sub>1</sub>, and Muscarinic Bindings in Rat Hippocampus After Transient Ischemia

Hiroshi Onodera, MD, PhD, and Kyuya Kogure, MD, PhD

The protective roles of Ca<sup>2+</sup> channel blockers against ischemic hippocampal damage are still debated. We used autoradiography to study postischemic L-type Ca<sup>2+</sup> channels (1,4-dihydropyridine Ca<sup>2+</sup> channel blocker binding), adenosine A<sub>1</sub> receptors, and muscarinic cholinergic receptors in the rat hippocampus using [3H]PN200-110 (PN), [3H]cyclohexyladenosine (CHA), and [3H]quinuclidinyl benzilate (QNB), respectively, in 49 rats subjected to 20 minutes of forebrain ischemia. The rats were decapitated after 1 (n=7), 3 (n=7), 6 (n=8), 12 (n=7), 24 (n=6), 48 (n=6), or 168 (n=8) hours of recirculation; eight control rats were sham-operated but experienced no cerebral ischemia. Reduced receptor binding preceding the delayed death of CA1 pyramidal cells was first observed in the stratum oriens of the CA1 subfield. Significant reductions in [3H]PN, [3H]CHA, and [3H]QNB bindings of this stratum compared with control were noticed after 3 (35%, p<0.01), 12 (31%, p<0.01), and 1 (10%, p<0.05) hours of recirculation, respectively. By 168 hours after ischemia (when the populations of CA1 pyramidal cells were depleted) all strata in the CA1 subfield had lost most of their receptor sites, and [3H]PN, [3H]CHA, and [3H]QNB bindings in the stratum oriens were decreased to 23%, 30%, and 63% of control (p<0.01). Although [3H]PN binding in the CA3 subfield did not change significantly during 168 hours after ischemia, the histologically intact dentate gyrus exhibited a 31% loss of binding sites compared with control (p<0.05). These results indicate predominant localization of Ca<sup>2+</sup> antagonist binding sites on the CA1 pyramidal cells. The reduction in [3H]PN binding early during reperfusion may explain the contradictory results regarding the protective role of Ca<sup>2+</sup> channel blockers against CA1 pyramidal cell death. Postischemic administration of Ca<sup>2+</sup> antagonists may be much less effective than preischemic administration. (Stroke 1990:21:771-776)

Calcium (Ca<sup>2+</sup>) is an important component of signal transduction, but uncontrolled Ca<sup>2+</sup> influx, such as occurs during ischemia and epilepsy, can disrupt cellular homeostasis and lead to neuronal damage. Recently, three subtypes of voltage-sensitive Ca<sup>2+</sup> channels have been found in the brain, namely T-, N-, and L-types. Binding sites for 1,4-dihydropyridine drugs are associated with L-type Ca<sup>2+</sup> channels and are highly concentrated in the central nervous system, with high binding activity in the hippocampus. There has been growing interest in the application of 1,4-dihydropyridine Ca<sup>2+</sup> channel blockers as protective agents against ischemic brain damage.

Selective hippocampal pyramidal cell damage in the CA1 occurs 1–2 days after ischemia, when minimal energy crises or morphologic changes (delayed neuronal death) are noticed. Ischemic pyramidal cell death is commonly employed as a model for screening neuroprotective drugs. Although numerous studies have been designed to investigate the L-type Ca<sup>2+</sup> channel antagonists as potential therapeutic agents for ischemic neuronal injury, there has been no agreement as to their protective effect on CA1 pyramidal cell damage. However, the stroke models and drug administration schedules have been quite different among these studies. We studied the temporal profile of L-type Ca<sup>2+</sup> channels in the rat hippocampus following transient forebrain ischemia by means of quantitative autoradiography using [3H]PN200-110 (PN), a new dihydropyridine Ca<sup>2+</sup> channel blocker. We also performed adenosine A<sub>1</sub> receptor and muscarinic cholinergic receptor autoradiography for comparison with the Ca<sup>2+</sup> antagonist binding sites.

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Preliminary results of this study have been reported at the 14th International Joint Conference on Stroke and Cerebral Circulation, February 9–11, 1989, in San Antonio, Texas.

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Received March 28, 1989; accepted December 8, 1989.
Materials and Methods

We used 57 male Wistar rats weighing 220–280 g and induced transient forebrain ischemia in 49 of them using a slightly modified version of the method of Pulsinelli and Brierley\(^1\) as described previously. All rats were anesthetized with 50 mg/kg i.p. pentobarbital, and both vertebral arteries of each rat were electrocauterized. The next day, the common carotid arteries were exposed under 2% halothane anesthesia. In the 49 ischemic rats, 3 minutes after the halothane was discontinued both common carotid arteries were clamped for 20 minutes. The eight control rats were prepared and handled similarly, except their common carotid arteries were not clamped. The criteria used to judge the occurrence of ischemia are described elsewhere.\(^2\) After 1 (n=7), 3 (n=7), 6 (n=8), 12 (n=7), 24 (n=6), 48 (n=6), or 168 (n=8) hours of recirculation the rats were decapitated, and the brains were removed quickly and frozen in powdered dry ice. Coronal sections 12 \(\mu\)m thick were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Adjacent sections were used for histopathology.

L-type Ca\(^{++}\) channels were localized autoradiographically using \([\text{H}]\)PN as described by Cortes et al.\(^3\) Sections were incubated with 0.1 nM \([\text{H}]\)PN in 170 mM Tris-HCl buffer (pH 7.7) at room temperature for 90 minutes. The slides were then washed in the buffer at 4°C for 20 minutes. Nonspecific binding was determined using 1 \(\mu\)M nitrendipine.

Adenosine A\(_1\) receptors were visualized using \([\text{H}]\)cyclohexyladenosine (CHA, New England Nuclear, Boston, Massachusetts).\(^4\) Sections were incubated with 5 nM \([\text{H}]\)CHA and 2 units/ml adenosine deaminase (Boehringer-Mannheim) in 50 mM Tris-HCl buffer (pH 7.4) at room temperature for 90 minutes. The slides were then washed in the buffer at 4°C for 1 minute. Nonspecific binding was determined using 10 \(\mu\)M L-phenylisopropyladenosine (Boehringer-Mannheim).

Muscarinic cholinergic receptors were quantified using the radiolabeled antagonist \([\text{H}]\)quinuclidinyl benzilate (\([\text{H}]\)QNB, New England Nuclear) as reported previously.\(^5\) Sections were incubated with 1 nM \([\text{H}]\)QNB in phosphate buffer (pH 7.4) at room temperature for 90 minutes. The slides were then washed in the buffer at 4°C for 5 minutes and dried under a stream of cold air. Nonspecific binding was determined using 1 \(\mu\)M atropine.

The slides were dried under a stream of cold air and apposed to LKB Ultrasfilm (Gaithersburg, Maryland) for 2–4 weeks. The optical density of the regions of interest was measured using an image analyzer system (MICD system, Imaging Research, Inc., St. Catharines, Canada). The relation between optical density and radioactivity was calculated using third-order polynomials with reference to tritium standards exposed along with the sections. Preliminarily, brain mash sections containing known amounts of radioactivity were apposed to Ultrasfilm along with a tritium microscale (Amersham, Arlington Heights, Illinois), and the grain density correlation between the brain mash sections and the microscale was obtained. This microscale was used in our routine studies. Optical densities of the brain regions measured were within the range in which the optical density and the tritium microscale radioactivity showed a near-linear relation. No significant differences in nonspecific binding activity were observed between the control and ischemic rats (data not shown). As discussed in our previous study,\(^6\) we did not correct for quenching after hippocampal lesioning due to ischemia.

The three binding assays were performed in duplicate. The groups were compared using the Mann-Whitney U test.

Results

CA1 pyramidal cells exhibited delayed neuronal death\(^9,12–14\) after ischemia. They appeared intact by light microscopy 24 hours after ischemia, but by 48 hours some pyramidal cells were damaged. Most pyramidal cells were necrotic 72 hours after ischemia, and the populations were completely depleted by 168 hours (data not shown). CA3 pyramidal cells and dentate granule cells showed no visible damage by light microscopy at any time (data not shown).

There was a very high density of silver grains (\([\text{H}]\)PN binding) in the stratum molecular of the dentate gyrus in control rats (Figure 1, Table 1); the CA3 subfield also showed a high grain density. Early during recirculation, density in the CA1 subfield decreased when there were no obvious morphologic abnormalities of the CA1 pyramidal cells. In the stratum oriens of the CA1 subfield \([\text{H}]\)PN binding was significantly lower by 3 hours (\(p<0.01\)) and remained approximately 30% lower than control at 6, 12 (\(p<0.01\)), and 24 hours after ischemia. By 48 hours after ischemia, when a few damaged neurons were noticed in the stratum oriens of the CA1 subfield, there was a 46% reduction in \([\text{H}]\)PN binding (\(p<0.05\)). A 77% reduction at this site at 168 hours (\(p<0.01\)) was concomitant with a 74% decrease in the stratum radiatum (\(p<0.01\)). At this time, the CA3 subfield exhibited a 33% reduction (not significant) in \([\text{H}]\)PN binding. The stratum molecular of the dentate gyrus lost 31% of its \([\text{H}]\)PN binding by 168 hours after ischemia (\(p<0.05\)).

The CA1 subfield had many \([\text{H}]\)CHA binding sites in control rats (Table 2),\(^7\) with the highest densities in the strata oriens and radiatum. The dendritic fields of the CA3 subfield and the stratum molecular of the dentate gyrus also showed high densities. Analogous to the time course shown by \([\text{H}]\)PN autoradiography, reduced \([\text{H}]\)CHA receptor density in the CA1 subfield preceded delayed histopathologic damage to the CA1 pyramidal cells. In the stratum radiatum of the CA1 subfield, \([\text{H}]\)CHA binding decreased by 12% by 6 hours after ischemia (not significant), while the stratum oriens lost 31% of its
Figure 1. \[^{3}H\]PN200-110 autoradiograms of hippocampal formation from control rat (A) and rat killed 168 hours after 20 minutes of transient forebrain ischemia (B). Grain density in CA1 subfield of ischemic rat was markedly reduced (arrows) in accordance with delayed death of CA1 pyramidal cells. Scale bar=0.5 mm. DG, dentate gyrus; Im, stratum lacunosum-moleculare; o, stratum oriens; r, stratum radiatum.

Table 1. Time Course of \[^{3}H\]PN200-110 Binding in Rat Hippocampus After 20 Minutes of Transient Forebrain Ischemia (Four-Vessel Occlusion Model)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>n</th>
<th>CA1 subfield</th>
<th>CA3 subfield (average)</th>
<th>Dentate gyrus (stratum moleculare)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stratum oriens</td>
<td>Stratum radiatum</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>14.1±1.2</td>
<td>12.5±1.2</td>
<td>30.7±3.7</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>10.7±0.9</td>
<td>10.5±1.2</td>
<td>20.5±2.9</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>9.1±0.8*</td>
<td>9.3±0.9</td>
<td>20.1±2.9</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>10.1±1.4</td>
<td>10.5±0.9</td>
<td>23.0±2.2</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>9.4±0.6*</td>
<td>11.5±1.2</td>
<td>24.5±1.0</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>10.2±1.4</td>
<td>10.3±1.2</td>
<td>25.7±1.4</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>7.6±1.6†</td>
<td>10.5±1.6</td>
<td>25.9±1.6</td>
</tr>
<tr>
<td>168</td>
<td>8</td>
<td>3.2±0.7*</td>
<td>3.3±0.4*</td>
<td>20.5±2.9</td>
</tr>
</tbody>
</table>

Data are mean±SEM fmol/cm². *p<0.01, 0.05, respectively, different from control by Mann-Whitney U test.
were reduced by 11% after 12 hours of recirculation. The stratum oriens failed to show significant changes in binding activity. The dentate gyrus exhibited a reduction in binding (approximately 10%) 24, 48, and 168 hours after ischemia.

**TABLE 3. Time Course of [3H]Quinuclidinyl Bendlate Binding in Rat Hippocampus After 20 Minutes of Transient Forebrain Ischemia**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>12</td>
<td>24</td>
<td>48</td>
<td>168</td>
</tr>
<tr>
<td>Stratum oriens</td>
<td>268±9</td>
<td>246±11</td>
<td>243±10</td>
<td>235±13</td>
<td>186±11</td>
<td>230±11</td>
<td>184±14</td>
<td>80±11</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>312±5</td>
<td>310±14</td>
<td>297±12</td>
<td>274±12</td>
<td>256±18</td>
<td>276±11</td>
<td>231±10</td>
<td>121±18</td>
</tr>
<tr>
<td>Stratum lacunosum-moleculare</td>
<td>162±10</td>
<td>170±7</td>
<td>152±7</td>
<td>153±12</td>
<td>122±8</td>
<td>158±9</td>
<td>131±5</td>
<td>72±8</td>
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<tr>
<td>Stratum oriens</td>
<td>173±9</td>
<td>182±4</td>
<td>167±10</td>
<td>161±10</td>
<td>127±16</td>
<td>171±8</td>
<td>143±4</td>
<td>130±11</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>236±9</td>
<td>240±8</td>
<td>222±14</td>
<td>200±9</td>
<td>176±19</td>
<td>212±15</td>
<td>181±6</td>
<td>160±16</td>
</tr>
<tr>
<td>Dentate gyrus (stratum moleculare)</td>
<td>236±5</td>
<td>227±7</td>
<td>224±7</td>
<td>220±9</td>
<td>179±11</td>
<td>208±9</td>
<td>195±7</td>
<td>168±9</td>
</tr>
</tbody>
</table>

Data are mean±SEM fmol/cm². *p<0.01, 0.05, respectively, different from control by Mann-Whitney U test.

[3H]CHA binding by 12 hours (p<0.01). The density of [3H]CHA binding sites in the CA1 subfield decreased to approximately 38% of control by 168 hours after ischemia. In the CA3 subfield silver grain density decreased gradually during recirculation, and the stratum radiatum lost 15% of its binding by 6 hours after ischemia (not significant). The strata oriens and radiatum of the CA1 subfield showed binding activity losses of 25% (p<0.05) and 32% (p<0.01), respectively, by 168 hours. The stratum moleculare of the dentate gyrus exhibited a significant reduction in binding of 24% after 12 hours of recirculation (p<0.05). However, further decreases in binding activity were not observed in this area.

We found the highest density of [3H]QNB receptors in the CA1 subfield (Table 3); the CA3 subfield and the stratum moleculare of the dentate gyrus had moderate numbers of [3H]QNB binding sites. We noted significant posts ischemic changes in [3H]QNB binding after only 1 hour of recirculation in the stratum oriens of the CA1 subfield. In the stratum radiatum of the CA1 subfield, [3H]QNB receptors were reduced by 11% after 12 hours of recirculation (p<0.05). At 168 hours, [3H]QNB binding in the strata oriens and radiatum of the CA1 subfield decreased to 63% (p<0.01) and 76% (p<0.01) of control, respectively. In the CA3 subfield, a significant decrease (approximately 10%, p<0.01) in [3H]QNB binding was observed in the stratum radiatum 24, 48, and 168 hours after ischemia, although the stratum oriens failed to show significant changes in binding activity. The dentate gyrus exhibited a reduction in binding (approximately 10%) 24, 48, and 168 hours after ischemia.

**Discussion**

We demonstrate that a decrease in [3H]PN binding precedes delayed neuronal death of CA1 pyramidal cells. We did not determine whether the loss of [3H]PN silver grain density after ischemia indicates a reduction in the number of binding sites (Bmax) or a reduction in binding affinity (Kd). In the case of [3H]CHA, we have reported that the decrease in [3H]CHA binding represents a reduction in Bmax with no alteration in Kd in the rat hippocampus after kainic acid lesioning.15 In the stratum oriens of the CA1 subfield, [3H]PN binding decreased significantly after 3 hours of recirculation. Thus, neuronal activity of the CA1 pyramidal cells may be altered despite minimal morphologic damage early during recirculation. By 168 hours after ischemia, when the populations of CA1 pyramidal cells were depleted, approx-

**Table 3. Time Course of [3H]Quinuclidinyl Benzilate Binding in Rat Hippocampus After 20 Minutes of Transient Forebrain Ischemia**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>12</td>
<td>24</td>
<td>48</td>
<td>168</td>
</tr>
<tr>
<td>Stratum oriens</td>
<td>444±6</td>
<td>404±11</td>
<td>404±11</td>
<td>415±9</td>
<td>390±11</td>
<td>373±16</td>
<td>357±25</td>
<td>280±17</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>458±7</td>
<td>442±6</td>
<td>442±6</td>
<td>442±7</td>
<td>407±14</td>
<td>385±15</td>
<td>401±15</td>
<td>350±13</td>
</tr>
<tr>
<td>Stratum lacunosum-moleculare</td>
<td>319±6</td>
<td>328±8</td>
<td>328±8</td>
<td>333±10</td>
<td>296±9</td>
<td>283±24</td>
<td>279±25</td>
<td>240±17</td>
</tr>
<tr>
<td>Stratum oriens</td>
<td>351±8</td>
<td>347±14</td>
<td>347±14</td>
<td>336±8</td>
<td>326±14</td>
<td>312±14</td>
<td>324±28</td>
<td>327±11</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>382±6</td>
<td>360±14</td>
<td>360±14</td>
<td>370±6</td>
<td>352±15</td>
<td>325±14</td>
<td>327±11</td>
<td>341±10</td>
</tr>
<tr>
<td>Dentate gyrus (stratum moleculare)</td>
<td>436±4</td>
<td>427±6</td>
<td>428±6</td>
<td>430±9</td>
<td>399±14</td>
<td>394±11</td>
<td>388±24</td>
<td>400±10</td>
</tr>
</tbody>
</table>

Data are mean±SEM fmol/cm². *p<0.05, 0.01, respectively, different from control by Mann-Whitney U test.
immediately 75% of the [3H]PN binding was lost in the CA1 subfield. Thus, Ca2+ antagonist binding sites in the CA1 subfield are predominantly located on the CA1 pyramidal cells. Residual [3H]PN binding in the CA1 subfield suggests that other structures (e.g., γ-aminobutyric acid [GABA]ergic interneurons, presynaptic components, glial cells, and blood vessels) may also have Ca2+ antagonist binding sites. As observed in [3H]PN autoradiography, changes in adenosine A1 and muscarinic receptors in the hippocampus preceded the death of CA1 pyramidal cells. We have also reported a decrease in [3H]CHA and [3H]QNB binding preceding CA1 pyramidal cell necrosis in gerbils after forebrain ischemia. Interestingly, a significant reduction in [3H]PN and [3H]QNB binding was first observed in the stratum oriens of the CA1 subfield after 3 and 1 hours of recirculation, respectively (Figure 2). In contrast, a significant decrease in [3H]PN binding in the stratum radiatum of the same subfield was not observed until 168 hours after ischemia. The decrease in [3H]PN binding in the dentate gyrus, not significant until 168 hours after ischemia, suggests the alteration of neuronal activity in the dentate granule cells because that is where L-type Ca2+ channels in the dentate gyrus are located. [3H]CHA and [3H]QNB binding sites in the stratum moleculare of the dentate gyrus also decreased significantly during recirculation.

We cannot conclude that [3H]PN binding in the CA1 subfield lost early during recirculation are L-type Ca2+ channel molecules on CA1 pyramidal cell membranes since a considerable number of Ca2+ antagonist binding sites have no direct association with Ca2+ channels. Although the contribution of reduced binding early during recirculation to delayed neuronal death is not clear, it may reflect damage to dihydropyridine-sensitive voltage sensors.

Postischemic Ca2+ homeostasis is markedly modulated immediately after recirculation. Thus, many studies have been designed to estimate the effect of Ca2+-antagonists on ischemic neuronal death, assuming that these drugs can modify undesirable biochemical processes mediated by Ca2+. However, the protective role of L-type Ca2+ channel blockers is not specific but rather subsidiary. Ca2+ antagonists have little effect on transmitter release. Ca2+ antagonists administered both before and during ischemia have been shown to protect against CA1 pyramidal cell death. In contrast, their administration after ischemia fails to prevent CA1 pyramidal cell death. Our data should help reconcile these contradictory results. Since [3H]PN binding was reduced after only 3 hours of recirculation in the CA1 subfield, postischemic administration of Ca2+ antagonists may be much less effective than preischemic administration. Similarly, the decrease in [3H]CHA binding early during recirculation also suggests that preischemic administration of adenosine agonists, which have been reported to prevent CA1 pyramidal cell death, may be more effective in protecting against CA1 damage. We need well-controlled trials that pay particular attention to maintaining sufficient drug concentrations in target tissues during the early stages of recirculation, at which time the fate of CA1 pyramidal cells may be determined.

Acknowledgments

The authors wish to thank Drs. Araki H. and Okuyama S. for performing densitometric analyses of the autoradiograms and Araki T. for technical assistance.

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**KEY WORDS**
- hippocampus
- autoradiography
- calcium channel blockers
- rats
Calcium antagonist, adenosine A1, and muscarinic bindings in rat hippocampus after transient ischemia.
H Onodera and K Kogure

doi: 10.1161/01.STR.21.5.771

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