Threshold of Calcium Disturbances After Focal Cerebral Ischemia in Rats
Implications of the Window of Therapeutic Opportunity

T.J. DeGraba, MD; P.T. Ostrow, MD; J.C. Grotta, MD

Background and Purpose: One explanation for inconclusive results with calcium channel blockers in human acute stroke trials may be incomplete information about the time course of calcium-mediated ischemic neuronal injury. This study explores the temporal relation between duration of focal ischemia and the functional activity of increased intracellular calcium as measured by calcium-calmodulin binding.

Methods: Calcium-calmodulin binding, determined by immunohistochemical assay of free calmodulin, was measured in 60 male spontaneously hypertensive rats after 2 minutes and after 1, 2, 4, and 24 hours of permanent tandem common carotid and middle cerebral artery occlusion, and after 1 and 2 hours of reversible middle cerebral artery occlusion followed by 1 and 22 hours of reperfusion, respectively. Light microscopic histological damage was measured after 1 hour of occlusion with 23 hours of reperfusion and after 24 hours of occlusion.

Results: Significant loss of calmodulin staining in the core of the infarction was noted by 1 hour and became maximal after 4 hours of ischemia. No reversal of calmodulin staining loss was noted after reperfusion following 1 and 2 hours of ischemia. Cortical necrosis seen by light microscopy correlated well with the area of maximal calcium-calmodulin binding. The border zone area, represented by a mild loss of calmodulin staining surrounding the central core of maximal binding, gradually decreased in size and became incorporated into the central core after 4 hours of ischemia; it may represent an area of reversible ischemia.

Conclusions: Calcium-calmodulin binding correlates with duration of focal ischemia, and histological neuronal necrosis corresponds to the cortical areas displaying a significant loss of calmodulin staining. Inasmuch as loss of calmodulin staining represents a marker for calcium-mediated activity after ischemia, it suggests a window of opportunity within 4 hours after acute stroke for therapeutic intervention with calcium antagonists. (Stroke 1993;24:1212-1217)

KEY WORDS • calcium • calmodulin • cerebral ischemia • neuroprotection • rats

Extensive experimental evidence has confirmed that the early events of ischemic neuronal damage are characterized by excessive influx of calcium. Activation of calcium-dependent enzymes such as protein kinase C, calmodulin (CaM) kinase II, calpain I, calcineurin, and phospholipase A2 is believed to play a major role in neuronal excitotoxicity. In addition, therapeutic interventions in animal models of global and focal ischemia with agents targeting either voltage- or receptor-gated ion channels have been found to have neuroprotective effects in proportion to their ability to normalize calcium homeostasis. Despite these findings, the results of clinical therapeutic trials in humans using these agents have thus far been inconclusive. Possible explanations for these results include lack of standardization of current stroke therapy, lower dose tolerance due to coexisting cardiovascular disease.

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in humans, heterogeneity in human stroke, and insensitive methods of detecting modest neuronal protection in humans.

However, another important reason for the negative results in humans may be that neuroprotective therapy has been started too late. This hypothesis is supported by the improvement in efficacy seen with preischemic vs postischemic neuroprotective treatment in animal models and is also suggested by subgroup analysis from clinical trials of calcium channel antagonists. In addition, it has been shown that maximal infarct volume, activation of calcium-dependent enzymes, and alterations in neuronal protein synthesis all occur early in the course of focal ischemia.

The purpose of recent work in our laboratory has been to explore the time course of disturbed calcium homeostasis as determined by measurements of calcium-calmodulin (Ca-CaM) binding after global and focal ischemia. By correlating these data with subsequent permanent histological damage, we wish to establish theoretical time windows of opportunity beyond which irreversible calcium-mediated damage has occurred. We have already reported that severe forebrain isch-
emia lasting more than 10 minutes leads to irreversible Ca-CaM binding and subsequent neuronal destruction in the vulnerable CA1 region. In the presence of reperfusion, initial Ca-CaM binding can be seen by 2 hours but does not become maximal until 24 hours.3

The present study evaluated the effect of focal ischemia on Ca-CaM binding. After variable durations of ischemia, a central core of ischemic tissue (represented by severe loss of CaM immunostaining) and a border zone region (represented by tissue with milder decreased staining surrounding the central core) were compared with the area of neuronal necrosis seen by light microscopy after 24 hours. A secondary objective was to explore the effect of reperfusion on reversing the disruption of calcium homeostasis. Based on the results of this study, we hypothesize that the inability of the reestablishment of blood flow to correct the effects of early Ca2+ influx indicates that it will ultimately be necessary to use neuroprotective agents in combination with reperfusion therapy (ie, thrombolysis) to achieve maximal neuroprotection.

Materials and Methods

Male spontaneously hypertensive rats (n=80) weighing 250 to 300 g were subjected to fasting overnight, with free access to water; they were anesthetized with chloral hydrate intraperitoneally before surgery. The femoral artery was cannulated with a polyethylene catheter for blood pressure monitoring and serial blood gas sampling. A microprobe was used to monitor skull temperature, which was maintained with a heating lamp and warming blanket at 37±0.5°C (as was the rectal temperature). The right common carotid artery (CCA) was isolated through a midline ventral cervical incision and tagged with 3-0 silk. An incision was made perpendicularly to a line between the external auditory canal and the lateral canthus of the right eye through the temporalis muscle, which was retracted with 4-0 silk. Under direct visualization with a Zeiss surgical microscope, the right middle cerebral artery (MCA) was exposed through a 1×3-mm burr hole drilled 2 to 3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. A continuous 0.9% saline drip was maintained over the drilling site to prevent thermal injury to the underlying brain tissue. Once the MCA was visible through the transparent bone layer, a jeweler’s forceps was used to remove the remaining bone. A 23-gauge hypodermic needle was used to pierce and open the dura along the entire length of the burr hole.

The method for producing reversible ischemia was a modification of the procedure described by Kaplan et al12 and Brint et al.14 A 10-0, BV130-5 Ethicon Neurosurgical suture was placed under the MCA rostral to the rhinal fissure, proximal to the major bifurcation of the MCA but distal to the lenticulostriate arteries. The MCA was lifted approximately 1 mm, and the suture was rotated clockwise so that one end pointed toward the frontal bone, forming a right angle to the long axis of the burr hole. Verification of the absence of blood flow was made visually with the surgical microscope. Immediately after MCA occlusion, the right CCA was occluded with twoatraumatic Heifetz aneurism clips. Reperfusion was achieved by turning the suture counterclockwise and removing it. Aneurysm clips were removed, and flow in both the CCA and MCA was confirmed visually with the operating microscope. In rats exposed to 24 hours of focal ischemia, permanent ligation of the right CCA and MCA was performed. Again, absence of blood flow was verified visually. This technique produces a nearly exclusive cortical infarction, sparing the deep white matter and basal ganglia.

CaM immunohistochemical staining was carried out as previously described.15 After being anesthetized with ethyl ether (purified grade), rats were hand-perfused with 4% paraformaldehyde, then killed. The brain slice preparations (40-µm thickness) were incubated in sheep-induced CaM antibody, which binds specifically to free CaM (ie, CaM not bound to Ca2+ and target protein). The sections were incubated with rabbit anti-sheep peroxidase-conjugated secondary antibody, which binds and stains only CaM-antibody complex. Thus, only normal cells with free CaM will stain, and ischemic cells with Ca2+-activated CaM-target protein complex do not stain. Sets of normal and posts ischemic brains were processed simultaneously to ensure comparable staining.

The specificity of the CaM antibody to free CaM was demonstrated by in vitro immunohistochemical dot staining of CaM incubated in ethylene glycol tetraacetic acid, Ca2+, and Ca2+ with brain homogenate. Staining occurred only in the absence of Ca2+ and target protein.15 Overlays of sodium dodecyl sulfate–polyacrylamide gel electrophoresis confirmed the persistent presence of target protein and CaM in ischemic brain homogenate after up to 4 hours of focal ischemia.

CaM staining in the ischemic region of each animal exposed to focal ischemia was graded on a scale of 0 to 15, with 0 representing normal and 15 the largest area of severe loss of CaM staining (see Table). CaM studies were carried out in groups of 7 to 11 animals exposed to either 2 minutes, 1 hour, 2 hours, 4 hours, or 24 hours of

<table>
<thead>
<tr>
<th>Calmodulin Staining Grade</th>
<th>Size of core</th>
<th>Loss of stain in core (severity)</th>
<th>Size of border zone</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>Mild</td>
<td>Small</td>
<td>Large</td>
<td>1</td>
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<tr>
<td>Moderate</td>
<td>Small</td>
<td>Large</td>
<td>2</td>
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</tr>
<tr>
<td>Severe</td>
<td>Small</td>
<td>Large</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>Mild</td>
<td>Small</td>
<td>Large</td>
<td>7</td>
</tr>
<tr>
<td>Moderate</td>
<td>Small</td>
<td>Large</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>Small</td>
<td>Large</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>Mild-moderate</td>
<td>None</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Moderate-severe</td>
<td>None</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No staining</td>
<td>None</td>
<td>15</td>
<td></td>
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</table>

Grade of calmodulin staining in each ischemic rat brain was assigned on a scale of 0 to 15. Score is based on size of central core with substantial staining abnormalities, severity of staining loss in the core, and size of border zone with mild staining abnormalities.
ischemia; 1 hour of ischemia with 1 hour of reperfusion; or 2 hours of ischemia with 22 hours of reperfusion.

For measurement of infarct volume, groups of 10 animals were exposed to 1 hour of occlusion with 23 hours of reperfusion or to 24 hours of permanent occlusion and then, under ether anesthesia, were perfused with phosphate-buffered 10% formalin, and killed. Their brains were removed and examined to verify the presence or absence of MCA occlusion. After the whole brains were embedded in paraffin, they were serially sectioned at 5 μm. Sections were stained at 0.5-mm intervals with luxol fast blue/hematoxylin and eosin; they were then digitized on an Amiga computer, and infarct volumes were calculated. The area of necrosis was identified by inspection of the image analysis and then compared to the area of maximal loss of CaM immunostaining.

CaM stain grading and histological grading were carried out by an investigator blinded to the duration of ischemia. All data were analyzed by comparison of group means using the Kruskal-Wallis test for nonparametric data.

**Results**

Minor differences in physiological variables (including weight, blood pressure, PO2, PCO2, pH, and temperature) were noted among comparable ischemic groups. Animals in the 1-hour occlusion/1-hour reperfusion group were slightly lighter (251 g, P = .05). Those in the 2-hour occlusion group had slightly higher mean arterial blood pressure (157 mm Hg, P = .05). Those in the 2-hour occlusion/22-hour reperfusion group had lower mean arterial blood pressure (127 mm Hg, P = .05) and higher PO2 and PCO2 (48 and 120 mm Hg, respectively; P = .05). Finally, those in the 24-hour group had slightly higher rectal temperature (36.9°C, P = .05).

After variable durations of focal MCA occlusion, significant Ca-CaM binding represented by loss of CaM staining (increased CaM staining “grade”) was noted by 1 hour of ischemia and was maximal by 4 hours (Fig 1). Ca-CaM binding was seen in a time-dependent pattern, with progressive decrease in staining between immediate vs 1-hour occlusion (1.00 ± 0.28 vs 8.63 ± 1.16, P < .005), 1-hour vs 2-hour occlusion (8.63 ± 1.16 vs 11.11 ± 0.48, P < .01), and 2-hour vs 4-hour occlusion (11.11 ± 0.48 vs 13.8 ± 0.39, P < .005). No difference was seen when comparing 4-hour vs 24-hour occlusion (13.8 ± 0.39 vs 12.83 ± 0.50, P > .10). In general, with increasing duration of ischemia, the pattern seen was an enlargement of a central core of minimal CaM staining in the neuronal soma surrounded by a shrinking border zone of intermediate staining.

There was no significant change in overall score in animals allowed reperfusion, evidenced by comparing 1-hour occlusion vs 1-hour occlusion with 1 hour of reperfusion (8.63 ± 1.16 vs 8.13 ± 1.3, P > .90), and 2-hour occlusion vs 2-hour occlusion with 22 hours of reperfusion (11.18 ± 0.48 vs 12.25 ± 0.74, P > .10). An apparent threshold of calcium influx was seen after 1 hour of ischemia, demonstrated by examining individual scores (Fig 1). After 2 minutes of focal ischemia, the cortical tissue was found to have little-to-no loss of CaM staining. By 2 hours or more of focal ischemia, significant loss of CaM staining was seen in the central core of all rats. A variable response to ischemia, however, was seen in the groups of rats exposed to 1 hour of occlusion and 1 hour of occlusion with 1 hour of reperfusion, suggesting that a threshold of up to 1 hour of focal ischemia exists in this model before extensive Ca-CaM binding occurs. A similar phenomenon was noted after severe global ischemia, although after a much shorter duration of ischemia (10 minutes).3

The area of infarction measured by hematoxylin and eosin staining correlated very closely with the area of the “central core” of extensive CaM staining loss after 24 hours of ischemia (Fig 2, A and B). Areas of milder loss of CaM staining (considered border zone areas) did not show up as infarct in comparable slices by hematoxylin and eosin staining after 1 hour of occlusion and 23 hours of reperfusion (Fig 2, C and D). Histological necrosis occurred only in areas that also showed significant loss of CaM staining.

**Discussion**

The observation that neuronal damage seen by light microscopy does not become maximal for up to 72 hours or more after 10 to 30 minutes of global ischemia has led to the perception that calcium-mediated events triggered by such brief but complete ischemic insults may take many hours to become maximal and therefore may
be potentially reversible during this interval. Although our data do not refute that possibility, they do indicate that after the initiation of both global and focal ischemia there is substantial Ca-CaM target protein activation by 2 hours even in the presence of reperfusion. In the global model, we found that more than 10 minutes of ischemia followed by reperfusion results in irreversible Ca-CaM target protein activation and the appearance of subsequent cellular damage in vulnerable CA1 regions.

In the present study, focal MCA ischemia lasting more than 1 hour was associated with abnormal Ca-CaM binding in the entire cortical region supplied by the occluded artery. Maximum Ca-CaM binding in the central core was found beyond 2 hours of MCA occlusion, and maximal binding in the entire MCA region occurred by 4 hours of occlusion. Of particular interest is the finding that reperfusion after only 1 hour of occlusion did not reverse Ca-CaM binding, indicating that reperfusion therapies might have to be coupled with neuroprotective therapies to provide optimal reduction of calcium-mediated ischemic injury. The inability of reperfusion alone to rectify severely abnormal calcium homeostasis and the complementary effect of reperfusion and neuronal protective therapies have recently been demonstrated by Uematsu et al and Zivin and Mazzarella.

The regions of maximal Ca-CaM binding found in our study correlated almost exactly with the extent of cortical necrosis seen by light microscopy after 24 hours. Less than 2 hours of ischemia was associated with a border zone of limited Ca-CaM binding surrounding a central core of maximal binding. The border zone region was not included in the zone of necrosis seen by light microscopy at 24 hours. Longer periods of MCA occlusion, even if followed by reperfusion, resulted in maximal Ca-CaM binding and necrosis in the entire MCA territory at 24 hours. Therefore, reperfusion occurring by 2 hours can arrest further Ca-CaM binding and limit histological damage in threatened border zone regions but has no effect on the more extensive Ca-CaM binding occurring in the central core. Although in this study we have not directly evaluated the ability of calcium antagonist drugs to reverse this process after focal ischemia, other investigators have found that progressively longer delays before initiation of neuroprotective therapy result in decreased efficacy. Our data do not preclude the possibility that other "rescue therapies," such as growth factors that target later events in the ischemic cascade, might be effective if started beyond 2 hours after ischemia. Furthermore, our method of measuring Ca-CaM binding is only semi-quantitative, and changes in Ca^{2+} influx/efflux and Ca-CaM binding may occur after 2 hours of focal ischemia that may be remediable but are not detectable by our immunostaining technique.

From the data presented, we feel that Ca-CaM binding is a valid marker of Ca^{2+}-mediated events within the cell during and after ischemic insults and that retention of CaM staining indicates a limitation of toxicity due to calcium excess and thus a return of calcium homeostasis. This is supported by studies of calcium-dependent enzyme systems during ischemic injury. We have carried out parallel studies of CaM kinase II activity after global ischemia and have demonstrated that this calcium-activated enzyme system is profoundly disturbed very early after the onset of ischemia; these disturbances correlate very closely to eventual histological damage.

Our data may help explain why calcium and glutamate antagonists are apparently more effective in focal than in global ischemic models. After only 10 to 20 minutes of global ischemia, there is development of maximal Ca-CaM binding and neuronal destruction in CA1, where histological damage is usually measured. However, up to 4 hours of MCA occlusion is required to produce maximal Ca-CaM binding and neuronal destruction in the entire cortex supplied by the occluded MCA. Although cerebral blood flow measurements were not carried out in this study, Brint et al using the same model we used in this study, have shown that
except in a central core of cortex supplied by a permanently occluded MCA, cerebral blood flow does not fall in collaterally supplied border zones to levels as low as those occurring after four-vessel occlusion. Thus, it is likely that damage in less severely ischemic border zone regions after MCA occlusion is due to partial (and therefore presumably more therapeutically reversible) calcium disturbances.

We have demonstrated that CaM and its target proteins remain present for up to 4 hours after focal ischemia. Therefore, loss of CaM binding is most likely due to loss of antigenicity when Ca-CaM is bound to target protein. It must be considered, however, that other cell processes resulting from ischemia could lead to decreased availability of CaM. For example, it has been shown that P73, a CaM-binding protein within the family of heat-shock proteins, is not only preferentially spared but is selectively produced hours after ischemic injury and could be responsible in part for the lack of return of CaM binding. Because evidence suggests that activation of these proteins is Ca-CaM dependent, one hypothesis could be that this is a protective mechanism against the effects of excess Ca-CaM activity.

The infarct volumes recorded in the present study are smaller than those reported by other investigators using a similar model of ischemia. We believe that this is predominantly a function of our use of the paraffin-embedding technique (rather than frozen sections), which eliminates much of the water content from the brain tissue. Also, our method of computer analysis of volume is based on a conal estimation as opposed to the traditional square section volume averaging. As can be seen in Fig 2, the percent area of infarction is in fact comparable to that in previously published reports.

In conclusion, to the extent that our animal model reflects conditions of focal ischemia in humans, our data indicate that if left untreated, irreversible calcium-mediated damage occurs after 1 hour of MCA occlusion even if perfusion is reestablished. After MCA occlusion, maximal calcium-mediated damage occurs by 4 hours. These data provide the biological basis for establishing a theoretical window of opportunity for therapeutic intervention aimed at limiting early events activated by Ca-CaM binding.

Acknowledgments

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References


Editorial Comment

There is substantial evidence that excess calcium influx is an important event in ischemic cerebral damage, probably because many vital enzyme systems are calcium dependent. Dr DeGraba and colleagues have been investigating this process by measuring calcium-calmodulin binding. Calmodulin is one of the calcium-activated enzymes that appears to play a role in cell damage. The authors are not claiming that calmodulin is the critical step, although that is possible, but simply that calcium binding to this enzyme is a good marker of injured cells.

Evidence supporting this contention includes loss of binding in the core of the ischemic area in this rat model of focal ischemia. Irreversible loss of binding begins within 1 hour and becomes maximal within 4 hours. It is clear from many other investigations that permanent
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T J DeGraba, P T Óstrow and J C Grotta

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