Calcium/Calmodulin–Dependent Protein Kinase II Activity in Focal Ischemia With Reperfusion in Rats

S.K. Hanson, MD; J.C. Grotta, MD; M.N. Waxham, PhD; J. Aronowski, PhD; P. Ostrow, MD

Disruption of calcium homeostasis has long been known to play a key role in cell death resulting from a variety of toxic injuries. More recently, the phenomenon of calcium overload has been theorized to be important in the process of neuronal death in the excitotoxin hypothesis of ischemic injury. According to this theory, the stimulation of excitatory amino acid receptors (both N-methyl-D-aspartate [NMDA] and non-NMDA receptors) initially causes a movement of ions across cell membranes, resulting in neuronal swelling. The second phase in this process is a delayed cell injury, which may be mediated in part by an influx of calcium into the cell. The elevation of intracellular calcium occurs via agonist-associated calcium channels, voltage-operated channels, and release of intracellular calcium stores. After calcium entry, a complex cascade of events results in cell injury. The details of this process, not yet clearly defined, probably involve the following: alterations of cell energy metabolism induced by calcium overload, activation of catabolic enzymes with degradation of structural proteins, activation of lipases and phospholipases, and the generation of superoxide radicals. The possible role of changes in activity of calcium-activated protein kinases, such as calcium/calmodulin–dependent protein kinase II (CaM-KII) and protein kinase C (PKC), during ischemia has recently received the attention of a number of investigators.

CaM-KII is a calcium/calmodulin–dependent protein kinase found in high concentration in the central nervous system, particularly in dendritic arborizations where it constitutes 15% to 50% of the protein in postsynaptic densities. There is evidence implicating CaM-KII in a variety of cell functions including regulation of gluconeogenesis and glycogenolysis, regulation of lipid and amino acid metabolism, neurotransmitter synthesis and release, cytoskeletal functions, modulation of ion channels, and the induction of long-term potentiation. The notion that it may be involved in the process of excitotoxic injury derives from its dependence on calcium for activation, as well as other indirect evidence closely linking the changes in calmodulin and CaM-KII with the process of cellular injury. The extreme sensitivity of calcium/calmodulin–dependent protein kinase II to focal ischemia and the parallel temporal and regional changes in its activity to those of more delayed cell injury point to a potential role for this enzyme in the process of excitotoxic injury. 

Results: Calcium/calmodulin–dependent protein kinase II activity was evaluated in a rat model of focal ischemia after 5 minutes, 30 minutes, and 1 hour of tandem middle cerebral artery and common carotid artery occlusion both with and without reperfusion.

Methods: Change in calcium/calmodulin–dependent protein kinase II activity was calculated in sham-operated control animals, and in both ischemic core and border-zone regions. Deposition of activity occurred in a regionally graded fashion, with the most severe decrease in infarct core and progressively smaller decreases in samples moving out from the center, corresponding to the severity of histological injury later detected in infarct core and border-zone regions. There were only minor differences between the three durations of ischemia in the degree of enzyme depression noted in the more peripheral regions, indicating that the initial decrease in calcium/calmodulin–dependent protein kinase II activity is an early, sensitive marker for an ischemic insult. After reperfusion, the differences between the 5-minute group and longer periods of ischemia widened because of an increase toward baseline in the 5-minute group and a trend toward further decrease in the 30- and 60-minute groups.

Conclusions: The extreme sensitivity of calcium/calmodulin–dependent protein kinase II to focal ischemia and the parallel temporal and regional changes in its activity to those of more delayed cell injury point to a potential role for this enzyme in the process of excitotoxic injury. (Stroke. 1994;25:466-473.)
ischemia and reperfusion. However, it has been suggested that focal ischemia may harbor a condition particularly prone to the delayed form of excitotoxic injury. In a review by Siesjo and Bengtsson, the penumbral region, unique to focal ischemia, was described as a partially ischemic zone where energy charge may be low enough to trigger excitotoxicity but high enough to maintain calcium-dependent glutamate release. In addition, pH is high enough to maintain NMDA receptor function. For this reason we have chosen to extend our evaluation of CaM-KII changes to the focal model of ischemia, concentrating our efforts on distinguishing the changes occurring in the infarct core from those occurring in the border zone of the infarction.

**Materials and Methods**

**Surgery**

Adult spontaneously hypertensive rats (weight, 224 to 288 g) were subjected to focal ischemia using an adaption of a tandem middle cerebral artery (MCA) and common carotid artery (CCA) occlusion model allowing for reperfusion. The rats were fasted overnight with free access to water and then anesthetized with intraperitoneal chloral hydrate before surgery. The femoral artery was cannulated with a polyethylene catheter for blood pressure monitoring. A microprobe thermometer (Yellow Springs Instrument Co, Yellow Springs, Ohio) was used to monitor skull temperature. Head temperature and rectal temperature were maintained at 36.6±0.5°C and 36.6±0.6°C, respectively. The left CCA was isolated through a midline incision and tagged with 3.0 silk suture. An incision was made through the temporalis muscle perpendicular to a line drawn between the external auditory ear canal and the lateral canthus of the right eye, and then the muscle was retracted with 4.0 silk suture. Under direct visualization with a surgical microscope (Zeiss, Germany) the left MCA was exposed through a 1×3-mm rectangular burr hole situated 2 to 3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. The beveled edge of a 23-gauge hypodermic needle was used to pierce and open the dura along the entire length of the burr hole. The needle from a 10-0 BV130-5 Ethicon neurosurgical suture was placed under the MCA in a position rostral to the rhinal fissure, proximal to the major bifurcation of the MCA, and distal to the lenticulostrate arteries. Then the needle was rotated clockwise so that one end pointed toward the vertex forming a right angle to the burr hole, thus lifting the MCA from the brain surface and occluding MCA blood flow. Immediately after MCA occlusion, the left CCA was occluded using twoatraumatic Heifetz aneurysm clips. Reperfusion was achieved by turning the needle under the MCA counterclockwise and removing it and then removing the aneurysm clips from the ipsilateral CCA. Reinstatement of blood flow was visually confirmed with the operating microscope. This technique produces a cortical infarction, almost completely sparing the deep white matter and basal ganglia.

Rats were subjected to one of three time intervals of ischemia (5 minutes, 30 minutes, and 1 hour) and were killed either immediately after ischemia or after 24 hours of reperfusion (n=4 at each of the six time points). There were two sham-operated groups killed either immediately after ischemia or 24 hours after the operation (n=4 for each group). Sham-operated animals underwent anesthesia, isolation of the CCA, drilling of the burr hole, and opening of the dura but were not subjected to any manipulation of the MCA.

The rats were killed by decapitation under chloral hydrate anesthesia. The brain was quickly removed, and a 3-mm-thick coronal slice was taken 7 mm from the frontal pole and immediately placed in ice-cold phosphate-buffered saline (PBS). The posterior face of the slice was confirmed to be in the region of the dorsal hippocampus. The time elapsed from decapitation to immersion of the cortical slice in cold PBS was 60 seconds. Five 2×2×3-mm consecutive sections of cortex were dissected from the left (ischemic) portion of this slice beginning at the center of the region of ischemia and moving to the vertex (Fig 1A). This included regions from the core of the infarct (L1 and L2), border-zone region (L3 and L4), and unaffected vertex (L5) as defined histologically in the ischemic hemisphere. A sample was also taken from the contralateral-nonischemic cortex in a region comparable to the infarct core or region L1 (labeled "region R" in text but not pictured here). Panel b, Photograph of the same histological preparation depicting the infarct core (large arrow), which was used in the calculation of infarct volume, and the border-zone region (small arrow), where there was incomplete cell loss.

![Fig 1. Panel a, Photograph of histological preparation of an animal subjected to 1 hour of ischemia with 23 hours of reperfusion with a diagram showing the location of the five 2×2×3-mm sections that were dissected from the ischemic cortex for enzyme assay. The dissection included samples from the core of the infarct (L1 and L2), border-zone region (L3 and L4), and unaffected vertex (L5) as defined histologically in the ischemic hemisphere. A sample was also taken from the contralateral-nonischemic cortex in a region comparable to the infarct core or region L1 (labeled "region R" in text but not pictured here). Panel b, Photograph of the same histological preparation depicting the infarct core (large arrow), which was used in the calculation of infarct volume, and the border-zone region (small arrow), where there was incomplete cell loss.](http://stroke.ahajournals.org/figs/1a)
tic acid (EDTA), 0.5 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/L leupeptin, and 50 mg/L soybean trypsin inhibitor) with a Teflon/glass homogenizer using 30 strokes. Aliquots of homogenates were stored at −80°C. Protein concentration of homogenates was determined with a modification of the Lowry method using bovine serum albumin as a standard.

**CaM-KII Assay**

A synthetic peptide substrate (MHRQETVD; NTP) was used to determine CaM-KII activity in the homogenates from each of the regions dissected. To initiate phosphorylation reactions, 3 μg (10 μL) of protein from homogenate was added to a 40-μL mixture, resulting in the following concentrations of reagents: 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.4 mmol/L dithiothreitol, 20 μmol/L NTP, 1 μmol/L calmodulin, 2.0 mmol/L CaCl₂, 15 μmol/L ATP, 5 mmol/L MgCl₂, and 3 μCi of γ-[³²P]ATP (3000 Ci/mmol). Each sample was also analyzed in reactions excluding calcium and calmodulin and containing 2 mmol/L EGTA (final concentration) as a measure of calcium/calmodulin-independent activity. Reactions were incubated at 30°C for 1 minute, and then 20 μL of the mixture was spotted on P-81 phosphocellulose filters. Filters were then washed in 75 mmol/L phosphoric acid, and the remaining radioactivity was quantitated as the amount of this protein in arbitrary units of area under a curve. The infarct volume was performed by a blinded investigator. Data from the animals before reperfusion and CaM-KII activity data after reperfusion were subsequently each analyzed separately using a two-factor ANOVA (repeated-measures of region by time). Post hoc analysis was performed using Duncan’s multiple range test to compare individual regions at separate time points of ischemia.

**Histology**

Ten rats exposed to 1 hour of occlusion with 23 hours of reperfusion and five rats exposed to 5 minutes of occlusion with 23.9 hours of reperfusion were analyzed for infarct volume in a manner previously described. In preparation, the animals were first perfused with phosphate-buffered 10% formalin. After perfusion with formalin, whole brains were dehydrated, cleared, and embedded in paraffin. Serial sections were cut at 5 μm in the coronal plane, and every 10th section (0.5-mm interval) was stained with a combination of hematoxylin and eosin and Luxol fast blue. This stain provides sharp demarcation of infarcted areas at low magnification and allows automated recognition by a color video-imaging system. Images of the sections were digitized with a DCTV (Digital Creations) frame grabber and analyzed with an Amiga 2000 computer (Commodore Business Machines). The infarcted area on each section was measured by counting recognized pixels, and the volume was calculated by representing each slice area mathematically as a circle and connecting their perimeters to form a solid model. The image analysis for volume of infarct was performed by a blinded investigator.

**Results**

**Physiological Parameters**

There were no differences between sham-operated and ischemic groups with respect to physiological parameters including weight, blood pressure after chloral hydrate anesthesia, head temperature, and rectal temperature. Mean arterial blood pressure measured before anesthesia was significantly lower in animals subjected to 30 minutes (133±4 mm Hg) and to 1 hour of ischemia (122±5 mm Hg) compared with sham-operated animals (155±14 mm Hg) (P<.05 for both groups). The differences in blood pressure were lost after the administration of anesthesia, making it unlikely that there was a significant influence on enzyme measurements or specific conclusions drawn in this study. In all other groups there was no difference between sham and ischemic animals with respect to preanesthesia blood pressure.

**Histology**

Rats subjected to 1 hour of ischemia with 23 hours of reperfusion had a mean infarct volume of 47.4±6.2 mm³. All these animals had a clearly defined infarct core where virtually all cortical cells were lost. This was the region measured on consecutive coronal slices for the calculation of infarct volume. At the level of the dorsal hippocampus (the region used for enzyme sampling), the...
CAM-KII Activity in Focal Ischemia With Reperfusion

Hanson et al

CAM-KII activity was measured to assess its role in ischemia and reperfusion. The activity was compared across different regions within the zone of ischemia: L1, L2, L3, and L4. The activity in these regions was significantly lower than in the sham-operated animals.

**CAM-KII Assay**

The repeated-measures ANOVA tests showed a significant effect for duration of ischemia and the interaction of reperfusion and duration. However, the effect of reperfusion and the interaction of reperfusion and duration did not reach significance.

**Fig 2.** Bar graph shows calcium/calmodulin-dependent protein kinase II (CaM-KII) activity in the cortex of animals subjected to varying periods of ischemia followed by immediate death. The length of ischemia interval and severity of the change were related to the severity of the change.

**Fig 3.** Bar graph shows calcium/calmodulin-dependent protein kinase II (CaM-KII) activity in the cortex of animals subjected to varying periods of ischemia followed by reperfusion. The activity showed recovery in animals subjected to 5 minutes of ischemia after reperfusion.

This region of completely infarcted tissue roughly corresponded to the location of sampling regions L1 and L2 for enzyme assays. However, in many of the animals a border zone could be detected that stained lighter than normal cortex, microscopically showing some normal neurons, but with some cell loss as well (Fig 1b). Again at the level of the dorsal hippocampus, this region of incompletely infarcted tissue corresponded to sampling regions L3 and L4 for the enzyme assays. Animals with only 5 minutes of ischemia followed by 23.9 hours of reperfusion had no measurable infarct by these methods and little histologically detectable cell loss.

**CaM-KII Assay**

The repeated-measures ANOVA tests for effects of region by duration of ischemia and presence or absence of reperfusion demonstrated a significant effect for duration of ischemia (F=21.32, P=0.0001) (Fig 2). The effect of reperfusion and the interaction of reperfusion and duration did not reach significance (F=2.42, P=0.13 and F=1.35, P=0.28, respectively). One possible explanation for the absence of significance with the effect of reperfusion may have been that the changes in CaM-KII activity with reperfusion in different ischemic duration groups were divergent, so that small changes were cancelled out. CaM-KII activity in those animals subjected to 5 minutes of ischemia followed by 24 hours of reperfusion was higher than the activity in those animals subjected to the same period of ischemia without reperfusion (Fig 3). In contrast, CaM-KII activity decreased further in the infarct zone in those animals subjected to 30 minutes and 60 minutes of ischemia followed by reperfusion when compared with animals subjected to these two durations of ischemia without reperfusion (Fig 3). This divergent change would also dilute the immediate effect of ischemia in the 5-minute group reperfusion phase.
ischemia group when assessing animals with and without reperfusion as a single group. Therefore, we felt justified in a separate analysis of the two groups (ischemia without reperfusion and ischemia with reperfusion) using a two-factor ANOVA. A discussion of this analysis follows.

**CaM-KII Assay: No Reperfusion**

The repeated-measures ANOVA for effects of region by duration of ischemia for CaM-KII activity in the groups of animals without reperfusion demonstrated a significant effect for duration of ischemia, as expected ($f=7.59$, $P=.0042$). There was a significant effect when considering region ($f=94.41$, $P=.0001$) and an interaction between the variables of duration of ischemia and region affected ($f=14.78$, $P=.0001$). The analyses of individual regions assessing the variable of ischemic duration showed a significant effect for regions in the ischemic core (L1 and L2) as well as the outer regions (L3 and L4) (Table 1). Using Duncan’s multiple range test, a significant decrease in activity compared with sham-operated animals was seen for all three durations of ischemia compared with sham-operated rats. The activity in L2 and L3 after 5 or 30 minutes of ischemia, although significantly decreased when compared with the sham-operated group, was not as depressed as that seen after 60 minutes of ischemia ($P<.05$). Region L4, the ischemic region farthest from the core, only showed a significant depression in activity in the animals subjected to 60 minutes of ischemia ($P<.05$). There was no significant decrease in activity in samples from the opposite hemisphere (region R, $f=0.60$ and $P=.63$) or from the nonischemic ipsilateral ventricle (region L5, $f=0.46$ and $P=.71$). The regional differences correspond closely with previously published blood flow data in a similar model of focal ischemia in spontaneously hypertensive rats.32 In a location similar to where our samples are chosen for CaM-KII activity analysis, the study by Brint et al32 demonstrated cerebral blood flow of 22±4 mL/100 g per minute for infarct core (L1 and L2), 34±3 mL/100 g per minute for border zone (L3 and L4), and 87±12 mL/100 g per minute at the vertex (L5).

**CaM-KII Assay: Reperfusion**

The repeated-measures ANOVA for effects of region by duration of ischemia for CaM-KII activity in the groups of animals with reperfusion demonstrated a significant effect for duration of ischemia ($f=16.03$, $P=.0002$). There was a significant effect when considering region ($f=43.34$, $P=.0001$) and an interaction between the variables of duration of ischemia and region affected ($f=11.77$, $P=.0001$). The analyses of individual regions assessing the variable of ischemic duration showed a significant effect for regions in the ischemic core (L1 and L2) as well as the border of the ischemic area (L3 and L4) (Table 2). Using Duncan’s multiple range test, a significant decrease in activity compared with sham-operated animals was seen for all three

### Table 1. Calcium/Calmodulin-Dependent Protein Kinase II Activity After Three Different Periods of Ischemia and in Sham-Operated Rats Followed by Immediate Death

<table>
<thead>
<tr>
<th>Region</th>
<th>Time</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>(n=4)</td>
<td>0.542±0.11</td>
<td>0.542±0.10</td>
<td>0.525±0.048</td>
<td>0.605±0.048</td>
<td>0.544±0.10</td>
<td>0.533±0.062</td>
</tr>
<tr>
<td>5 min</td>
<td>(n=4)</td>
<td>0.186±0.13</td>
<td>0.211±0.077</td>
<td>0.246±0.11</td>
<td>0.573±0.21</td>
<td>0.519±0.058</td>
<td>0.631±0.16</td>
</tr>
<tr>
<td>30 min</td>
<td>(n=4)</td>
<td>0.101±0.056</td>
<td>0.139±0.083</td>
<td>0.310±0.21</td>
<td>0.468±0.090</td>
<td>0.506±0.084</td>
<td>0.579±0.10</td>
</tr>
<tr>
<td>60 min</td>
<td>(n=4)</td>
<td>0.074±0.024</td>
<td>0.062±0.028</td>
<td>0.110±0.037</td>
<td>0.400±0.046</td>
<td>0.472±0.097</td>
<td>0.549±0.088</td>
</tr>
</tbody>
</table>

Values are mean±SD. See "Materials and Methods" for definition of regions.

*Significantly less than a similar region in the sham group.
†Significantly less than a similar region in the sham and 5-minute time groups.

### Table 2. Calcium/Calmodulin-Dependent Protein Kinase II Activity After Three Different Periods of Ischemia and in Sham-Operated Rats Followed by 24 Hours of Reperfusion

<table>
<thead>
<tr>
<th>Region</th>
<th>Time</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>(n=4)</td>
<td>0.573±0.12</td>
<td>0.511±0.10</td>
<td>0.524±0.087</td>
<td>0.505±0.11</td>
<td>0.455±0.16</td>
<td>0.512±0.081</td>
</tr>
<tr>
<td>5 min</td>
<td>(n=4)</td>
<td>0.309±0.13*</td>
<td>0.344±0.077*</td>
<td>0.497±0.078</td>
<td>0.472±0.10</td>
<td>0.462±0.10</td>
<td>0.536±0.10</td>
</tr>
<tr>
<td>30 min</td>
<td>(n=4)</td>
<td>0.069±0.054†</td>
<td>0.058±0.042†</td>
<td>0.190±0.10†</td>
<td>0.313±0.13*</td>
<td>0.355±0.11</td>
<td>0.452±0.084</td>
</tr>
<tr>
<td>60 min</td>
<td>(n=4)</td>
<td>0.003±0.003†</td>
<td>0.002±0.001†</td>
<td>0.059±0.11†</td>
<td>0.248±0.11†</td>
<td>0.421±0.078</td>
<td>0.533±0.037</td>
</tr>
</tbody>
</table>

Values are mean±SD. See "Materials and Methods" for definition of regions.

*Significantly less than a similar region in the sham group.
†Significantly less than a similar region in the sham and 5-minute time groups.
durations of ischemia in the ischemic core (L1 and L2, P < 0.05) and for both the 30- and 60-minute groups in the border zone (L3 and L4, P < 0.05) (Fig 3). The changes with reperfusion among the different time points of ischemia were divergent, with a trend toward an increase in activity back to baseline in the 5-minute group and a trend toward further decrease in activity in the 30- and 60-minute groups with reperfusion. This resulted in a significant difference in activity in regions L1, L2, and L3 between the 5-minute group and the 30-minute group and in regions L1, L2, L3, and L4 between the 5-minute group and the 60-minute group. That is, there appeared to be a threshold for recovery of enzyme activity with reperfusion somewhere between 5 and 30 minutes of ischemia. Once again, no significant decreases in activity were noted in the opposite hemisphere (region R, f = 0.93 and P = 0.45) or in the nonischemic ipsilateral vertex (region L5, f = 0.73 and P = 0.55).

**Protein Kinase A Assay**

Protein kinase A activity was found to be significantly decreased in the ischemic zone (L1, P = 0.003; L2, P = 0.02; L3, P = 0.02; and L4, P = 0.004). The univariate tests demonstrated a significant effect for region, but the effect of time was similar for all regions. This may be an indication that the changes in CaM-KII activity are relatively more specific for the ischemic insult compared with the changes seen in PKA activity (changes in CaM-KII are proportional to the severity and duration of the ischemia).

**CaM-KII 50-kD Protein**

No regional differences in CaM-KII 50-kD protein were discovered in animals subjected to 5 minutes or 1 hour of ischemia. The quantity of 50-kD protein detected in representative animals was expressed as a percentage of the nonoperated hemisphere. This was found to be 106% (L1) in infarct core and 103% (L3) and 105% (L4) in border-zone regions in an animal subjected to 5 minutes of ischemia without reperfusion. Similar measurements in an animal subjected to 1 hour of ischemia were 104% (L1) in infarct core and 101% (L3) and 92% (L4) in border-zone regions.

In animals with 5 minutes or 1 hour of ischemia followed by 24 hours of reperfusion, there was only a small decrease in 50-kD protein in ischemic regions compared with the nonischemic hemisphere (5 minutes of ischemia followed by 24 hours of reperfusion: L1, 81%, L3, 103%, and L4, 103%; 1 hour of ischemia followed by 24 hours of reperfusion: L1, 86%, L3, 76%, and L4, 87%).

**Discussion**

Our data demonstrate the extreme sensitivity of CaM-KII to ischemia, closely linking a decrease in its activity to the onset of ischemic injury. The change is immediate and dramatic, detected after only 5 minutes of a focal ischemic insult that does not produce historically recognizable injury. The magnitude of the decrease in activity immediately after ischemia also correlated with increasing duration of ischemic insult. This has also been demonstrated by others in both global and focal ischemia models. In a rabbit spinal cord model of ischemic injury, Ziven et al described significant decreases in PKC activity after 10 minutes of ischemia and in CaM-KII after 60 minutes of ischemia. Dramatic decreases in CaM-KII activity have been seen in two separate models of global ischemia after only 5 minutes of occlusion in the gerbil and in the Wistar rat.

The level of CaM-KII activity continued to decrease during the reperfusion phase in those animals subjected to a period of ischemia sufficient in length to produce eventual infarction (30 minutes or longer). The changes in activity, which correlated with the pattern of infarction seen histologically after 24 hours of reperfusion, occurred despite relative absence of significant change in the quantity of enzyme present after reperfusion, indicating that reduction in CaM-KII activity was not merely due to destruction of the enzyme in concert with cell destruction, although this possibility cannot be completely excluded. We have observed similar reperfusion-associated decline in CaM-KII activity in recent experiments after 2 hours of ischemia and after 2 hours of ischemia with 2 hours of reperfusion, supporting the notion that there is an actual reduction in CaM-KII activity during reperfusion that occurs before cell destruction. Ideally, the current study should have been designed to sample a point after reperfusion before maturation of the cortical infarct. The continued decline of activity may implicate CaM-KII downregulation as an important factor in a delayed injury process after reperfusion, but these data cannot confirm this because the degree of maturation of infarction present at 24 hours. Further studies of enzyme activity at different time points after reperfusion may clarify the course of these changes in relation to reperfusion, the process of cell death, and the maturation of the infarction. Further definition of this relation may have implications for the optimal use of neuroprotective agents aimed at maintaining calcium homeostasis after human stroke; they may be used to best advantage if started before reperfusion.

The changes in CaM-KII activity appear to be distinctive, in that other enzymes are either unaltered by the insult or require a longer ischemic insult to show any change in activity. We found only limited changes in PKA activity with short periods and onset of modest decrease after 1 hour of focal ischemia with no reperfusion. Our findings were similar in the global model, showing a less dramatic decrease in PKC but no significant decrease in PKA activity after 5, 10, or 20 minutes of ischemia. Similarly, Ziven et al showed significant decreases in both PKC and CaM-KII in the rabbit spinal cord, but changes in PKA did not reach significance.

The close relation of CaM-KII alterations and ischemic injury is also implied by the pattern of decline of CaM-KII activity in the regions of cortex that were sampled. The most profound decrease was seen in the core of the infarct, with progressively less change in samples moving out from the core to border-zone regions. This graded change in CaM-KII activity corresponds closely to the degree of depression in blood flow that was demonstrated in a similar model of focal ischemia. In like manner, the pattern of histological damage seen on coronal sections, at the same level as that used for sampling CaM-KII activity, is also similar to the pattern of depression in enzyme activity.

The mechanism of CaM-KII downregulation is unclear, but after global ischemia we have demonstrated translocation of enzyme in neurons from the cytosolic to the particulate fraction where CaM-KII is no longer...
active. Our own work as well as the work of others suggests that CaM-KII during the initial minutes of ischemia undergoes posttranslational modification characterized by aberrant phosphorylation and change in affinity to ATP and anti-CaM-KII specific antibody.

We have shown by immunostaining that binding of calcium to calmodulin and brain protein occurs in the same brain regions as downregulation of CaM-KII in a similar graded relation to ischemic duration and distance from the ischemic core, and that calcium-calmodulin binding and downregulation of CaM-KII activity can be prevented by dextrorphan, an antagonist of the glutamate ligand gated calcium channel. It appears, therefore, that CaM-KII downregulation results from calcium influx into neurons. Its downregulation probably results in loss of some critical (but as yet still unidentified) CaM-KII-related function necessary for cell survival.

In summary, the extreme sensitivity of this enzyme to focal ischemia with rapid decline in activity after short periods of ischemia and the further decline in activity during reperfusion (following a threshold duration of ischemia) may implicate changes in CaM-KII-mediated events in the process of delayed cell death or in so-called reperfusion injury. Although this does not constitute direct evidence, these data add to the increasing indirect evidence closely linking changes in CaM-KII to the process of ischemic cell death. Whether these changes are an integral part of the process, and the exact mechanism by which this downregulation might result in cell injury, are yet to be determined.

Acknowledgments

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References

Calcium has been implicated as a major second messenger system involved in delayed neuronal cell death in numerous brain injury models. A major thrust of stroke research is to understand the mechanisms that mediate some of the effects of brain injury on calcium-regulated systems at the biochemical and molecular levels. Calcium/calmodulin–dependent protein kinase II (CaM-KII) is a major calcium-regulated enzyme system and has been implicated in mediating many of the effects of calcium on neuronal function. Thus, regulation of this enzyme has numerous effects on cell structure and function. Recent research has indicated that CaM-KII activity is especially sensitive to ischemic injury. It is important to establish whether the effect of ischemia or brain injury on CaM-KII activity is important in mediating the effect of ischemia on cell function producing delayed neuronal cell death.

The accompanying article by Hanson et al is an important report from the stroke and neuroscience research group at the University of Texas that provides significant insight into the role of this major enzyme system in brain injury. This article demonstrates that CaM-KII activity is downregulated in a focal ischemia model in rat that can be carefully studied anatomically to correlate the degree and distribution of neuronal injury with the level of CaM-KII activity. The importance of this article is that it provides stronger evidence that there is a cause and effect link between the effect of ischemia on CaM-KII and the development of delayed cell death. This report was able to demonstrate both temporal and anatomic correlations between brain injury and changes in CaM-KII activity. These results further substantiate the importance of this major calcium second messenger system in the pathophysiology of brain injury.

The current research in this field suggests that there is a posttranscriptional modification of CaM-KII almost immediately after ischemic injury in brain tissue. This modification in the activity of this major calcium-calmodulin second messenger system may produce profound effects on cell function that then set in motion the process of delayed neuronal cell death. Although further research is needed to clearly prove that this enzyme is involved in the cause of cell death rather than the result of cell death, this article by the University of Texas research team provides a major step toward making this association. Further research on the effects of ischemia and brain injury on CaM-KII may provide an important insight into a molecular understanding of the mechanisms involved in mediating brain injury. The effect of decreased CaM-KII activity on cell function is another important area that warrants further investigation. Understanding these basic mechanisms may provide new insights into developing specific therapeutic interventions to prevent or minimize the resultant brain injury during the time that precedes delayed neuronal cell death.

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