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

Background and Purpose Evidence linking changes in calcium/calmodulin–dependent protein kinase II activity with ischemic cell death has been reported in animal models of global ischemia. The purpose of this study was to delineate the course of these changes after focal ischemia and to clarify the relation of changes in activity of calcium/calmodulin–dependent protein kinase II to the process of ischemic cell death.

Methods Change in 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.

Results Calcium/calmodulin–dependent protein kinase II activity was significantly decreased after all three durations of ischemia followed by immediate decapitation compared with sham-operated animals, in both ischemic core and border-zone regions (P<.05 for all groups). Depression 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.)

Key Words • calcium • cerebral ischemia, focal • excitotoxins • protein kinases • rats
CaM-KII Activity in Focal Ischemia With Reperfusion

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 temporals 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 lenticulostriate 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 two atrumatic 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. Reinstitution 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. The 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.

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 penumbra 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.

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 temporals 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 lenticulostriate 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 two atrumatic 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. Reinstitution 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. The 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 an animal subjected to 1 hour of ischemia with 23 hours of reperfusion. A similar section was taken from the right, 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.

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 an animal subjected to 1 hour of ischemia with 23 hours of reperfusion. A similar section was taken from the right, 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.
tic acid (EDTA), 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 50 mg/mL 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 mM dithiothreitol, 40 μM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) (pH 7.4), 0.4 mM dithiothreitol, 20 μmol/L NTP, 1 μmol/L calmodulin, 2.0 mM/L CaCl₂, 15 μmol/L ATP, 5 mM/L MgCl₂, and 3 μCi of γ-[32P]ATP (3000 Ci/mmol). Every sample was also analyzed in reactions excluding calcium and calmodulin and containing 2 mM/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 mM/L phosphoric acid, and the remaining radioactivity was quantitated in a scintillation counter. This assay was previously described with a 20-μmol/L NTP concentration, and the reaction has been shown to be linear for up to 1.5 minutes with as much as 12 μg of sample protein. Greater than 80% of NTP phosphorylation could be blocked by the addition of a specific peptide inhibitor of CaM-KII (CaM-KIIi; MHRQEVDCLLKFKNARKK-LKGA), indicating that most of the activity measured represented phosphorylation by CaM-KII.

**CaM-KII 50-kD Subunit**

The amount of enzyme in homogenate samples was evaluated in the animals with 5 minutes and with 1 hour of ischemia with no reperfusion to determine whether initial changes in enzyme activity before cell loss were due to degradation of the enzyme. Measurements were also carried out after 24 hours of reperfusion. This was semiquantitatively determined using a Western blot technique described previously. Thirty-five micrograms of protein from each sample was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then was electrophoretically transferred onto nitrocellulose. The nitrocellulose paper was incubated first with a monoclonal antibody (2D5; 1:2000 dilution) specific to the 50-kD subunit of CaM-KII and subsequently with anti-mouse alkaline phosphatase-conjugated antibody. A colorimetric developer was added to a 40-μL mixture, resulting in the following concentrations of reagents: 10 mM dithiothreitol, 20 μmol/L NTP, 1 μmol/L calmodulin, 2.0 mM/L CaCl₂, 15 μmol/L ATP, 5 mM/L MgCl₂, and 3 μCi of γ-[32P]ATP (3000 Ci/mmol). Every sample was also analyzed in reactions excluding calcium and calmodulin and containing 2 mM/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 mM/L phosphoric acid, and the remaining radioactivity was quantitated in a scintillation counter. This assay was previously described with a 20-μmol/L NTP concentration, and the reaction has been shown to be linear for up to 1.5 minutes with as much as 12 μg of sample protein. Greater than 80% of NTP phosphorylation could be blocked by the addition of a specific peptide inhibitor of CaM-KII (CaM-KII; MHRQEVDCLLKFKNARKK-LKGA), indicating that most of the activity measured represented phosphorylation by CaM-KII.

**Protein Kinase A Assay**

Using the same homogenates used for CaM-KII assay, reactions of 50-μL volume and consisting of 10 mM/L HEPES (pH 7.4), 0.4 mM dithiothreitol, 10 μmol/L cyclic AMP, 2 μmol/L EGTA, 30 μmol/L kemptamide (LRRASLG; Peninsula Lab, Inc), 5 μmol/L MgCl₂, 15 μmol/L ATP, and 3 μCi of γ-[32P]ATP (3000 Ci/mmol) were performed at 30°C. They were terminated after 1 minute by pipetting 20 μL onto P-81 phosphocellulose filters. The filters were rinsed, and the remaining activity was counted as described above. Approximately 90% of the 32P incorporated into kemptamide by protein kinase A (PKA) was inhibited by the peptide inhibitor of PKA, suggesting that other kinases contributed approximately 10% of the total phosphorylation in these reactions.

**Data Analysis**

Initially a multivariate ANOVA test was applied to all CaM-KII activity data, but the divergent changes with reperfusion (increase after 5 minutes of occlusion further increased after 24 hours of reperfusion after 30 to 60 minutes of occlusion) confounded interpretation of early changes in CaM-KII activity and the significance of the effect of reperfusion. For this reason it seemed inappropriate to only analyze the two groups (reperfusion and no reperfusion) together. Therefore, the PKA activity and CaM-KII activity 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. 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 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),
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=.0001) (Fig 2). The effect of reperfusion and the interaction of reperfusion and duration did not reach significance (f=2.42, P=.13 and f=1.35, P=.26, 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
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 and in sham-operated rats followed by immediate death ($P<.05$). 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 vertex (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 durations 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, $f=0.50$ and $P=.63$) or from the nonischemic ipsilateral vertex (region L5, $f=0.71$ and $P=.46$). The regional differences correspond closely with previously published blood flow data in a similar model of focal ischemia in spontaneously hypertensive rats.

### 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 (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>
<td></td>
</tr>
<tr>
<td>5 min (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>
<td></td>
</tr>
<tr>
<td>30 min (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>
<td></td>
</tr>
<tr>
<td>60 min (n=4)</td>
<td>0.074±0.024</td>
<td>0.0621±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>
<td></td>
</tr>
</tbody>
</table>

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

### 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 (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>
<td></td>
</tr>
<tr>
<td>5 min (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>
<td></td>
</tr>
<tr>
<td>30 min (n=4)</td>
<td>0.068±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>
<td></td>
</tr>
<tr>
<td>60 min (n=4)</td>
<td>0.003±0.003†</td>
<td>0.002±0.001†</td>
<td>0.059±0.011</td>
<td>0.248±0.011</td>
<td>0.412±0.078</td>
<td>0.533±0.037</td>
<td></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<.05) and for both the 30- and 60-minute groups in the 
border zone (L3 and L4, P<.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 hemi-
sphere (region R, f=0.93 and P=.45) or in the nonisch-
emic ipsilateral vertex (region L5, f=.73 and P=.55).

Protein Kinase A Assay

Protein kinase A activity was found to be significantly 
decreased in the ischemic zone (L1, P=.003; L2, P=.02; 
L3, P=.02; and L4, P=.004). The univariate tests demon-
strated 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 spe-
cific for the ischemic insult compared with the changes 
seen in PKA activity (changes in CaM-KII are propor-
tional to the severity and duration of the ischemia).

CaM-KII 50-kd Protein

No regional differences in CaM-KII 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 
sampled at 5 minutes of ischemia without reperfusion. 
Similar measurements in an animal sampled at 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 histo-
logically 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.17,18 
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 destruc-
tion, 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 reperfu-
sion 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 reperfu-
sion 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 distinc-
tive, 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 shorter periods and only a modest 
decrease after 1 hour of focal ischemia with no reperfu-
sion. Our findings were similar in the global model, 
showing a less dramatic decrease in PKC but no signifi-
cant decrease in PKA activity after 5, 10, or 20 minutes of 
ischemia.17 Similarly, Ziven et al19 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 isch-
emic 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 corre-
sponds closely to the degree of depression in blood flow 
that was demonstrated in a similar model of focal 
ischemia.32 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 un-
clear, but after global ischemia we have demonstrated 
translocation of enzyme in neurons from the cytosolic 
into the particulate fraction where CaM-KII is no longer
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\(^7\) and change in affinity to ATP and anti-CaM-KII specific antibody.\(^8,9\) 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,\(^4,10\) 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.\(^11\) 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

This study was supported by the National Institutes of Health, National Institute of Neurological Disorders and Stroke grants NS-23979 (Dr Grotta) and NS-26806 (Dr Waxham). The authors wish to acknowledge Rosa Earls and Roger Strong for their excellent technical support.

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.

Robert J. DeLorenzo, MD, PhD, MPH, Guest Editor
Departments of Neurology, Pharmacology and Toxicology, Biochemistry, and Molecular Biophysics
Molecular Neuroscience Research Facility
Medical College of Virginia
Virginia Commonwealth University
Richmond
Calcium/calmodulin-dependent protein kinase II activity in focal ischemia with reperfusion in rats.
S K Hanson, J C Grotta, M N Waxham, J Aronowski and P Ostrow

Stroke. 1994;25:466-473
doi: 10.1161/01.STR.25.2.466

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/25/2/466

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/