Calcium-Calmodulin Binding in Ischemic Rat Neurons After Calcium Channel Blocker Therapy

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Calcium channel blockers such as nicardipine improve outcome after global cerebral ischemia and may attenuate ischemic neuronal injury by preventing calcium influx and binding to calmodulin. We followed the temporal and regional sequence of neuronal calcium-calmodulin binding in normal rats (n=6), untreated ischemic rats (n=15), and ischemic rats treated with 0.05 mg/kg/hr s.c. nicardipine (n=13). After 30 minutes of four-vessel occlusion, 40-μm brain sections were incubated in an anti-calmodulin antibody specific for calmodulin not bound to calcium and brain protein. Light-microscopic sections were examined immediately after ischemia and after 2 and 24 hours of reperfusion. Extensive staining of unbound calmodulin was seen in all hippocampal regions and in the cortex in normal rats. In untreated ischemic control rats, staining was lost, indicating calcium-calmodulin binding immediately after ischemia in all regions. However, after 24 hours, staining returned to normal in the cortex and dentate, and minimal staining returned in CA1 and CA3. Nicardipine-treated animals had significantly less calcium-calmodulin binding in CA1 and in the dentate after 2 hours of reperfusion. This study demonstrates that in clinically relevant doses nicardipine has a limited effect on calcium-calmodulin binding in selectively vulnerable regions after severe ischemia. (Stroke 1990;21:948-952)

This study describes the effect of a dihydropyridine calcium channel blocker on intraneuronal calcium-calmodulin (Ca²⁺-CaM) binding in a commonly used in vivo model of global cerebral ischemia. It is now commonly accepted that calcium entry into neurons and release of stored intracellular calcium are pivotal events leading to irreversible cellular damage during the reperfusion phase after an ischemic insult. Ionic calcium binds with intracellular calmodulin; this binding activates target enzymes such as kinases, proteases, and phospholipases. In a previous study, we found persistent Ca²⁺-CaM binding in neurons that eventually exhibited ischemic cellular injury; this finding supports the concept that elevated intracellular calcium acting through calmodulin plays a causative role in ischemic neuronal damage.¹

Therapeutic strategies for cellular protection after cerebral ischemia are presently directed at preventing calcium flux into neurons. Our immunohistochemical assay of intracellular calcium monitored by calmodulin binding might be of use in assessing the mechanism of action of pharmacotherapeutic agents, such as dihydropyridine calcium channel blockers and N-methyl-D-aspartate receptor antagonists, which are under evaluation in animal stroke models.

Nicardipine (Syntex Research, Palo Alto, California) was chosen for this study because of our findings in previous studies.²⁻⁴ These studies have shown that nicardipine is extracted into brain and increases cerebral blood flow. When nicardipine was used at the same dose as in the present study, it was shown to improve functional outcome as measured by somatosensory-evoked potentials and learning ability. However, we were unable to detect any histologic protection against ischemic neuronal damage when sections stained with hematoxylin and eosin were graded by light microscopy.²⁻⁴ Nicardipine is currently undergoing clinical evaluation in stroke patients, but the exact mechanism of action of therapeutic doses of this and other
dihydropyridine calcium channel blockers is not known; of particular interest is their efficacy in limiting calcium entry into ischemic neurons.

Materials and Methods

Our preliminary studies demonstrated that immunohistochemical staining of brain sections by a sheep anti-calmodulin antibody represented only calmodulin bound to Ca\(^{2+}\) or target protein and that postischemic decreases in this staining must be due to Ca\(^{2+}\)-CaM binding and not to changes in the quantity of calmodulin or calmodulin target proteins that were not depleted in brain homogenates after ischemia.

For the present study, rats were divided into three groups: normal rats (n=6), untreated ischemic rats (n=15), and ischemic rats treated with 0.05 mg/kg/hr s.c. nicardipine for 48 hours (n=13). For the nicardipine-treated group, infusion was begun 24 hours before ischemia; higher doses of nicardipine were not used since they were found in previous studies to cause significant hypotension. For the two ischemic groups, ischemia was produced for 30 minutes without anesthesia by a modification of the four-vessel occlusion model, and the rats were killed immediately after ischemia or after 2 or 24 hours of reperfusion. Blood pressure, blood gases, glucose, hematocrit, and head and rectal temperature were monitored throughout the ischemic period. Brains were hand-perfused and fixed with 4% paraformaldehyde in situ. Forty-micron sections through the hippocampus were incubated in sheep anti-calmodulin antibody. A dianinobenzidine-labeled anti-sheep secondary antibody was added to stain the sheep anti-calmodulin antibody. Staining in the endal limb of the dentate, dorsal CA1, lateral CA3, and parietal cortex was graded by a blinded examiner on a 4-point scale (0, no staining; 1, minimal staining; 2, some staining; 3, extensive staining but no distinguishable soma; 4, extensive staining of neuronal soma). Statistical analysis was performed by the Kruskal-Wallis one-way analysis of variance (ANOVA) for evaluating treatment group differences across all three time intervals for each region and across all four regions for each time interval. Subsequent analyses of individual group pairs were performed to identify the specific regional and temporal location of differences between treatment groups.

Results

Normal rats had grade 4 staining, which indicated a preponderance of unbound calmodulin in all four brain regions examined (Figure 1, top panel). Untreated ischemic rats demonstrated loss of staining (grade 0–1) in CA1 and the dorsal aspect of CA3 immediately, 2 hours, and 24 hours after ischemia, indicating persistent binding of calmodulin with Ca\(^{2+}\) and target proteins in these regions (Figure 1, lower panel). Staining only decreased to grade 2–3 in the dentate and cortex up to 24 hours after ischemia. These changes correlate with the distribution of morphologic damage recognizable by hematoxylin and eosin staining on light microscopy in this model. Ca\(^{2+}\).CaM binding clearly precedes morphologic changes that are not found in CA1 after 24 hours of reperfusion but are prominent by 72 hours.

Calmodulin staining was significantly greater in the nicardipine-treated ischemic rats than in untreated ischemic rats in the CA1 region across all three time periods (F=7.1, p<0.05, Figure 2) and after 2 hours of reperfusion across all four brain regions (F=7.3, p<0.05) when tested by ANOVA. Individual treatment group pairs were significantly different only in CA1 and dentate after 2 hours of reperfusion (Figure 3). No significant differences were found at other time periods or in CA3 or the cortex, although in the cortex all nicardipine-treated rats had grade 3–4 staining immediately and 2 hours after ischemia (Figure 3).

Discussion

In a previous study, we described a method for detecting Ca\(^{2+}\).CaM binding applicable to in vivo stroke models. We found that, although most brain regions demonstrate increased Ca\(^{2+}\).CaM binding immediately after ischemia, reflecting an increase in intracellular calcium ion in neurons, this binding is only moderate in brain regions destined to recover, such as the cortex and dentate. By contrast, this binding is very severe and persists beyond 24 hours in hippocampal regions, such as the selectively vulnerable CA1 and its partially vulnerable CA3 afferent connections, which are destined to undergo irreversible damage. In this study, the CA3 region where Ca\(^{2+}\).CaM binding was assessed was at the junction of the vulnerable dorsal aspect and the more resistant ventral aspect.

In the present study, we found evidence that nicardipine transiently prevented Ca\(^{2+}\).CaM binding in CA1 and, to a lesser extent, in the dentate and cortex. The drug was given throughout 24 hours of reperfusion, but its greatest effect on Ca\(^{2+}\).CaM binding was seen after 2 hours of reperfusion. Very little effect was noted after 24 hours, but this minimal effect may have been due to the small number of rats in each group. The use of a larger number of rats might have produced significant differences at 24 hours; however, increasing the number to achieve a statistically significant result amounts to a "demonstration" rather than an experiment. Since we had previously found no reduction of ischemic damage by light microscopy in CA1 after nicardipine treatment, we hypothesized that nicardipine did not prevent Ca\(^{2+}\).CaM binding during the critical first 24 hours of reperfusion after ischemia. Using a nonparametric method for grading Ca\(^{2+}\).CaM binding, we found it difficult to estimate how many rats we needed to evaluate this hypothesis. In our studies in ischemic control rats, we observed that less damaged regions, such as the dentate and cortex, primarily demonstrated grade 2–4 staining throughout the 24-hour postischemic period, whereas vulnerable regions, such as CA1, demonstrated grade 0–1 stain-
ing. Therefore, we designed the study with four or five rats in each group and anticipated that, if treatment were effective in a particular region, grade 2–4 staining for the entire 24 hours would result. As seen in Figures 2 and 3, this occurred in CA1 only 2 hours after ischemia; by 24 hours, staining was again grade 0–1. This contrasts with results obtained after treatment with a competitive N-methyl-D-aspartate receptor antagonist, CGS 19755. As determined by both light microscopy and grade 2–4 Ca²⁺-CaM staining in groups of similar size, this treatment resulted in histologic protection, which was significantly different from that of ischemic control rats over the entire 24 hours of reperfusion in CA1.⁷

In a study measuring brain calcium after calcium channel blocker therapy, Hossmann et al⁸ found that flunarizine had no effect on increased total brain calcium after ischemia, but Hadani et al⁹ recently reported less ionic Ca²⁺ after nicardipine therapy in ischemic hemispheres. Both of these studies looked at total brain calcium rather than evidence of intraneuronal calcium as measured in our experiments. Studies adding dihydropyridines directly to tissue slices have shown reduction of Ca²⁺ flux through the L-type calcium channel, one of three voltage-sensitive Ca²⁺ channel subtypes found in neuronal membranes,¹⁰ and

Uematsu et al¹¹ found less histologic damage and cytosolic free calcium by using a fluorescent intracellular Ca²⁺ indicator in ischemic cortical neurons after high-dose intravenous nimodipine therapy. Although Ca²⁺ flux through voltage operated L-type calcium channels in neurons may be blocked by dihydropyridines, intraneuronal Ca²⁺ can increase after ischemia by many other mechanisms. These alternate pathways include receptor-operated channels typified by the N-methyl-D-aspartate receptor,
nonspecific membrane channels, and release of intracellular calcium stores after activation of the inositol second messenger pathway by a variety of stimuli. It is possible that the ischemic insult we produced was so severe in CA1 and CA3 that calcium flooded into neurons through these alternate pathways and caused the persistent Ca\(^{2+}\)-CaM binding that was found in this study after 24 hours of reperfusion and also caused the lack of histologic protection in the CA1 and CA3 regions that was found in our previous studies.\(^2,4\)

In conclusion, although it is likely that nicardipine transiently blocks Ca\(^{2+}\) entry through L-type calcium channels, in light of persistent Ca\(^{2+}\)-CaM binding found after 24 hours of reperfusion and our previous negative histologic studies,\(^2,4\) it is unlikely that in clinically relevant doses nicardipine sufficiently prevents Ca\(^{2+}\) entry into severely ischemic hippocampal neurons to provide histologic protection.

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References


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