Regional Brain Calcium Changes in the Rat Middle Cerebral Artery Occlusion Model of Ischemia

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Entry of Ca ions into ischemic neurons is believed to cause cell damage. Although several investigators have demonstrated changes in extracellular Ca ionic activity consistent with Ca movement into cells, direct and quantitative evidence for Ca entry into ischemic cells is lacking. We used atomic absorption spectroscopy to measure the regional distribution of tissue Ca contents of rat brains sampled at 2, 4, and 24 hours after middle cerebral artery occlusion (MCAo). At 4 hours after MCAo, Ca concentrations increased significantly (p<0.005) in the ischemic middle cerebral artery territory, i.e., the pyriform and frontoparietal cortices, but not in surrounding brain. At 24 hours, Ca concentrations in the pyriform and frontoparietal cortex were respectively 30.79 (± 2.90) and 29.19 (± 3.28) μmol/g dry tissue wt compared with 11.9 (± 1.7) μmol/g in sham-occluded rats. Tissue Ca concentration changes in the parasagittal cortex and basal ganglia adjacent to the infarct site were much smaller and did not differ significantly from controls until 24 hours. In the ischemic middle cerebral artery territory, > 1.0 μmoles of Ca entered per gram of dry tissue weight per hour during the first 4 hours after MCAo. Linear regression analysis revealed a significant correlation (r = 0.9722) between changes in tissue Ca and water, with a slope indicating that 5.88 μmoles of Ca accompanied each milliliter of water entering the lesioned hemisphere. Such massive accumulations of Ca not only confirm Ca entry into injured cells, but indicate the presence of a remarkable Ca sink which sequestered within 24 hours more than 17 times the amount of free Ca present in the tissue before MCAo.

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A steep Ca ionic gradient normally exists between intracellular and extracellular compartments of the brain. Intracellular Ca ionic activity has been estimated to be <0.1 μM compared with extracellular Ca ionic activity of >1.0 mM. Due to this large gradient, Ca ions enter injured cells. Ca entry has many potentially fatal effects on cells. First, Ca ions disrupt metabolism by uncoupling mitochondrial electron transport and enhancing anaerobic metabolism and lactic acidosis. Precipitation of Ca with inorganic phosphates also may deplete cells of substrate for ATP generation. Second, Ca ions activate phospholipases which break down membrane phospholipids and release free fatty acids, including arachidonic acid. Converted by cyclooxygenase and lipoxygenase into vasoactive prostaglandins, with generation of free radical byproducts, these fatty acids can contribute to cell damage through direct attack on membranes as well as through vascular mechanisms. Third, Ca ions activate proteinases, which degrade neurofilaments and membrane proteins. Finally, Ca entry increases membrane ionic conductance, aggravating the Na and K derangements that occur in ischemic tissues.

Thus, there are ample theoretical reasons to call Ca entry the “final common pathway” of cell death.12

The role of Ca in ischemic cell death has attracted much attention recently. Several investigators have used ion-selective microelectrodes to show that extracellular Ca ionic activity falls in ischemic brain. Unfortunately, extracellular measurements of Ca activity only indirectly reflect Ca entry into cells. We recently showed that injured spinal cords rapidly accumulate large amounts of Ca, measurable by atomic absorption spectroscopy. Combined with extracellular Ca recordings, this approach allows quantitative assessment of the amount of Ca entering cells. We therefore applied this approach to a cerebral ischemia model, measuring regional brain Ca concentrations in rat brains at 2, 4, and 24 hours after middle cerebral artery (MCA) occlusion (MCAo). Our experiments revealed that significant amounts of Ca accumulated in the ischemic MCA territory within 4 hours after MCAo. By 24 hours after occlusion, the ischemic MCA territory gained 17 times the amount of extracellular free Ca available in the tissue prior to MCAo. These findings indicate not only entry of large amounts of Ca into cells but massive shifts of Ca from surrounding tissues, cerebrospinal fluids (CSF), and blood to the infarct site. The implications of these findings will be discussed.

Materials and Methods

General Experimental Protocol

The left MCA was occluded in 3 groups of 8 Sprague-Dawley rats anesthetized with 40 mg/kg i.p.
pentobarbital. The surgical procedures have been described in detail previously. At 2, 4, or 24 hours after MCAo, the rats were given an overdose of pentobarbital and decapitated. The brains were removed within 90 seconds and cooled for 5 minutes at −85° C. A 4-mm thick coronal section of the brain was cut approximately 4 mm behind the frontal tips, using a double-bladed razor assembly. Four tissue cores weighing approximately 35 mg each were removed from each hemisphere of the slices, using a 3 mm i.d. metal tube.

In each rat, 4 areas were sampled at and around the MCA territory of the lesioned hemispheres. Area 1 consisted mostly of pyriform cortex. The infarct was tal and decapitated. The brains were removed within MCAo, the rats were given an overdose of pentobarbital. The surgical procedures have been described in detail previously. At 2, 4, or 24 hours after surgery, are called sham-occluded controls. Second, tissue samples were obtained from homologous sites in the hemisphere contralateral to the MCAo. These are called contralateral controls. Third, a group of 6 normal rats served as unoperated controls.

**Tissue Calcium Analysis**

Each tissue sample was placed in a ceramic crucible (Coors), weighed to a precision of 0.1 mg for the tissue wet weight (W), dried in a desiccating oven at 105° C for 24 hours, and reweighed to obtain the tissue dry weight (D). The desiccated tissue was digested in 1 ml of hot concentrated nitric acid (45° C) and then dried at 95° C and heated at 550° C for 16 hours in a muffle oven (Thermolyne, 1400 Furnace, Dubuque, Iowa). After cooling, 2 ml of a 2.5% lanthanum oxide, 0.1N hydrochloric acid (LaO-HCl) solution were added to each crucible to dissolve the ashes.

Ca concentrations of the ashed samples were determined by atomic absorption spectroscopy (Perkin-Elmer, 2280, Norwalk, Conn.), using an acetylene-air flame and a hollow cathode Ca lamp. Absorption was measured at 422.7 nm wavelength. All sample measurements were bracketed by 2 standards, 1 containing only the LaO-HCl solution and the other the LaO-HCl solution plus a known amount of Ca. Flame conditions and sample suction rates were optimized for maximal sensitivity and linearity. Tissue Ca concentrations ([Ca]d) are expressed as μmol/g D. Water concentration in the tissue samples ([H2O]d) was calculated as (W − D)/D and expressed as ml/g D.

**Data Analysis**

Means and standard deviations of [Ca]d were calculated for each area in each group. To obtain the changes in Ca concentration (∆[Ca]d), we subtracted [Ca]d measured in homologous and time-matched sites in the sham-occluded controls from [Ca]d in the lesioned hemisphere. Rates of Ca entry were calculated by subtracting ∆[Ca]d from the ∆[Ca]d of the preceding time period and dividing by the number of hours between the 2 times.

The following conventions will be followed for expressing data variability. Wherever sample means are given, standard deviations (SD) will be used. Wherever differences of sample means are referred to, the pooled standard errors of means (SEM) used to calculate values for the t test will be shown. To assess the statistical significance of mean differences between MCAo and sham-occluded or unoperated control groups, we applied the unpaired two-tailed t test. The paired t test was used to assess the differences between lesioned and contralateral hemispheres. All comparisons were time-specific and matched for homologous sites. A confidence limit of p < 0.005 served as criterion for significance. We correlated ∆[Ca]d and the change in [H2O]d (Δ[H2O]d) by linear regression, obtaining a correlation coefficient (r) and slope. Note that the slope is micromoles of Ca per milliliter of water.

**Results**

Table 1 summarizes the mean values of [Ca]d in the unoperated, sham-occluded, and contralateral controls. [Ca]d in unoperated controls averaged 11.05 ± 0.53 μmol/g. Although [Ca]d in contralateral controls tended to be slightly higher than in the unoperated and sham-occluded controls, the differences were not significant. In the contralateral hemispheres, mean [Ca]d did not show a consistent tendency to rise or fall with time. Figure 1 shows the distribution of mean ± SD of [Ca]d in sham-occluded controls and MCAo rats. There were no systematic differences between [Ca]d in different areas or times of the sham-occluded control groups.

Table 2 lists the mean [Ca]d values in the lesioned hemisphere of rats subjected to MCAo. Values signifi-

### Table 1. Mean Calcium Concentrations in Control Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet</th>
<th>Dry</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated controls</td>
<td>2.41 ± 0.16</td>
<td>11.05 ± 0.53</td>
<td>48</td>
</tr>
<tr>
<td>Sham-occluded controls</td>
<td>2.34 ± 0.11</td>
<td>11.06 ± 0.67</td>
<td>150</td>
</tr>
<tr>
<td>Contralateral controls</td>
<td>2.39 ± 0.14</td>
<td>11.35 ± 0.55</td>
<td>96</td>
</tr>
</tbody>
</table>

Values are mean ± SD, μmol/g, regardless of site and time after surgery. Unoperated control values were averaged from 8 sites in 6 rats. Sham-occluded controls were obtained from 8 sites in 3 groups of 8 rats. Contralateral controls come from 4 sites sampled in the contralateral hemispheres in 3 groups of 8 rats subjected to middle cerebral artery occlusion (MCAo). There were no significant differences between any of the mean values listed.
The distribution of mean dry-weight concentration of calcium ([Ca]d) in rat cerebral cortex 2, 4, and 24 hours after middle cerebral artery occlusion (MCAo). Top: mean tissue [Ca]d (μmol/g dry tissue wt) in rat cerebral cortex 2, 4, and 24 hours after MCAo. The shaded area in each column represents averaged mean values, and the unshaded area gives the pooled standard errors of means (SEM) of the subtracted means. Significant Δ[Ca]d values (p < 0.005) are indicated by numbers over the bars. The right ordinate gives a scale indicating the Δ[Ca]d in percent of normal (unoperated control) [Ca]d. Bottom: rates of Δ[Ca]d, calculated by dividing the difference of Δ[Ca]d at 0–2, 2–4, and 4–24 hours after MCAo by the number of intervening hours.

**Figure 1.** The distribution of mean dry-weight concentration of calcium ([Ca]d). Top: mean tissue [Ca]d (μmol/g dry tissue wt) in rat cerebral cortex 2, 4, and 24 hours after middle cerebral artery occlusion (MCAo). Bottom: distribution of mean [Ca]d 2, 4, and 24 hours after MCAo. The shaded area in each column represents averaged values of [Ca]d measured in 8 rats. The unshaded area indicates SD. Ipsilateral refers to the operated side and contralateral refers to the other side. Areas 1–4 refer to the locations of the tissue samples, where Area 1 is the pyriform cortex, Area 2 the frontoparietal cortex, Area 3 the parasagittal cortex, and Area 4 the basal ganglia.

**Table 2.** Mean Calcium Concentrations in Rats Subjected to Middle Cerebral Artery Occlusion

<table>
<thead>
<tr>
<th>Sample site</th>
<th>2 hours</th>
<th>4 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area 1</td>
<td>14.23 ± 1.72</td>
<td>16.91 ± 2.18</td>
<td>30.79 ± 2.90</td>
</tr>
<tr>
<td>Area 2</td>
<td>13.22 ± 3.81</td>
<td>12.75 ± 1.33</td>
<td>29.19 ± 3.28</td>
</tr>
<tr>
<td>Area 3</td>
<td>11.68 ± 1.37</td>
<td>12.84 ± 2.27</td>
<td>31.76 ± 2.61</td>
</tr>
<tr>
<td>Area 4</td>
<td>10.86 ± 1.11</td>
<td>11.18 ± 1.32</td>
<td>13.90 ± 2.29</td>
</tr>
</tbody>
</table>

Values are mean ± SD, μmol/g dry tissue wt, from 8 rats.

*Different from unoperated controls, p < 0.005, unpaired t test.

†Different from homologous area in contralateral hemisphere, p < 0.005, paired t test.

‡Different from homologous time-matched sham-occluded controls, p < 0.005, unpaired t test.

reach significance at p < 0.005 and also was much smaller than the mean Δ[Ca]d in Areas 1 and 2. By 24 hours, [Ca]d in Areas 1 and 2 was, respectively, 30.79 ± 2.90 and 29.19 ± 3.28 μmol/g, yielding Δ[Ca]d values of 19.4 and 19.6 μmol/g. These are > 170% greater than in normal controls.

The mean Δ[Ca]d reflects the cumulative increase of Ca from the time of occlusion to the sample time. Figure 2 (bottom) illustrates the mean rates of Δ[Ca]d. The highest rates of Ca entry occurred in Areas 1 and 2 between 0 and 2 hours after MCAo. In Area 1, Ca entered the tissues at rates of 1.12 and 1.15 μmol/g/hr during 0–2 and 2–4 hours after MCAo. Between 4 and 24 hours, Ca accumulated at a slightly slower rate of 0.74 μmol/g/hr. In Areas 2 and 3, Ca initially entered the tissue at a relatively high rate at 0–2 hours, slowed down at 2–4 hours, and increased again.

Linear regression analysis of the relation between Δ[Ca]d and Δ[H₂O]d indicated a significant correlation (r = 0.9722, p < 0.001) between Ca and water entry. Both Δ[Ca]d and Δ[H₂O]d were expressed relative to sham-occluded controls. The slope of this line has units of micromoles of Ca per milliliters of water entry. The value of the slope was 5.88 μmol Ca/ml water. This slope is significantly greater than the value of 1.2 μmol Ca/ml water expected if Ca ions simply moved passively into the tissue with water.
Discussion

The results indicate large Ca accumulations in the ischemic MCA territory at 2–24 hours after arterial occlusion. Although the mean increase in total tissue Ca were 170% of unoperated control [Ca]d levels, the amounts of Ca entering the tissue were actually many times the amount of available Ca ions in the tissue prior to MCAo. Normally, extracellular Ca ionic activity is about 1.2 mM. If we assume that the extracellular volume fraction is 20% and that intracellular amounts of Ca ions entering the tissue were actually ischemic MCA territory at 2–24 hours after arterial occlusion, Ca in tissue should be about 0.24 μmol/g W. Since the tissue D/W ratio is 21.5%, we estimated that uninjured brain tissues contain about 1.1 μmol free Ca/g D. Note that unoperated rats have 11.05 μmol Ca/g D, suggesting that >90% of the Ca in the tissue is normally bound.

Within 2 hours after MCAo, Area 1 gained 2.2 μmol Ca/g D. The rate of Ca entry into Area 1 between 0 and 4 hours is approximately 1.1 μmol/g D/hr (Figure 2, bottom), suggesting that enough Ca entered the tissue hourly to replace the amount of free Ca present in the tissue prior to injury. By 24 hours after injury, the gains in Ca in Areas 1 and 2 were both >19 μmol/g D, equivalent to 17 times the amount of free Ca in the tissue prior to MCAo. From this perspective, the amounts of Ca that enter ischemic tissues are very large indeed. The large amounts of Ca accumulating in the ischemic tissue raise several intriguing questions. Where is all this Ca coming from? Where is it sequestered in the tissue? What can bind so much Ca? We will address each of these questions sequentially below.

Where is the Ca coming from? When total tissue Ca increased at the sampled sites, it must have come from blood, cerebrospinal fluid (CSF), or surrounding brain. Since the amount of Ca in extracellular fluids of brain tissues is about 1.1 μmol/g D as argued above, even if all available extracellular Ca ions in adjacent brain moved into the infarct site, it would not account for the >19 μmol/g D of Ca accumulated by 24 hours in Areas 1 and 2. Note that the rates of Δ[Ca]d in Areas 2 and 3 were negative at 2–4 hours, consistent with depletion of Ca ions from these areas. Mean [Ca]d values at all sampled sites in the MCAo and contralateral hemispheres exceeded unoperated control levels. Therefore, Ca lost from the surrounding tissues must have been replenished from external sources, i.e., blood or CSF.

Where is the Ca being sequestered? At 24 hours after MCAo, if we assume that the extracellular space enlarged to occupy 80% of the tissue volume due to cell death and that extracellular Ca activity is 0.6 mM, the amount of free Ca in the tissue should be about 0.5 μmol/g W or 2.5 μmol/g D. Since tissue Ca concentration at 24 hours was 30 μmol/g D, >90% of the Ca must be bound at the infarct site 24 hours after MCAo. It is likely that the vast majority of the Ca ions entering the lesion site was bound by some substance in the tissue. In Areas 1 and 2, this represents binding of about 20 μmol Ca/g D.

Two other observations support our interpretation that the Ca ions entering the infarct site precipitated in the ischemic tissue. First, if the tissue simply equilibrated with blood, i.e., the [Ca]w in brain and blood became equal, the maximum amount of Ca that could enter the tissue would be limited to about 5 μmol/g D, one-fourth the observed Δ[Ca]d. Second, the linear regression analysis of Δ[Ca]d vs. Δ[H2O]d indicated 5.88 μmol Ca entered the tissue per milliliter of water. If Ca ions diffused passively into the tissue and did not precipitate, we would expect a slope close to 1.2 μmol/ml, the Ca ionic activity in plasma. Instead, nearly 5 times as much Ca entered the tissue.

What can bind so much Ca? Mitochondria sequester Ca but require energy to do so. In fact, ischemic and injured mitochondria release Ca into the cytosol.17 While a variety of organic substances bind Ca, they either do not bind well under the acid conditions18 expected in ischemic tissues or are not available in sufficient quantity to bind such large amounts of Ca. One class of substances, however, can precipitate Ca in the quantities observed and will bind Ca under acid conditions. Inorganic phosphate binds avidly to Ca with an association constant of 10^-7 -19 As pointed out by Kretzinger,30 normal intracellular levels of 5 mM inorganic phosphate ions will effectively buffer Ca ions to <0.1 μM.

Continued precipitation of Ca by inorganic phosphate should cause prolonged depressions of extracellular Ca activity. Note that elevated intracellular Ca activity will activate phospholipases and thereby release phosphates from cellular organic substrates. Total tissue phosphates have been estimated to be 50–140 mM in brain21 and therefore constitute a very large reservoir of phosphate available to bind Ca ions. Our data suggest that Ca continued to accumulate at the infarct site for as long as 24 hours after MCAo. For example, between 4 and 24 hours after MCAo, Ca entered Areas 1 and 2 at rates of 0.74 and 0.89 μmol/g/hr.

The possible role of Ca in ischemic injury has prompted a series of recent therapeutic studies centered on the role of calcium channel blockers (CCBs) in animal models22–25 and clinical studies.26–28 In complete ischemic anoxia, CCBs given prior to the onset of injury improved postischemic perfusion23,24 and neurologic recovery.22,23 In a focal cerebral ischemia model, Harris et al28 found no consistent effect of nimodipine, a CCB, on the magnitude and time course of extracellular Ca activity changes. Although several investigators have proposed that CCBs act on an as yet unidentified intracellular site,29,30 the issue of whether or not CCBs prevent Ca entry into ischemic cells remains controversial. Measurements of total tissue Ca in cerebral ischemia models treated with CCB may help resolve the issue.

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References


KEY WORDS • atomic absorption spectroscopy • brain • calcium • edema • infarct • ischemia • middle cerebral artery • stroke
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