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Accumulation of Exogenous 45Ca after Middle Cerebral Artery Occlusion in Rats
(ラットに於ける中大脳動脈閉塞後の外因性45Ca蓄積)

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Accumulation of Exogenous ^{45}Ca after Middle Cerebral Artery Occlusion in Rats

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Abstract

The distribution of exogenous ^{45}Ca in the focal ischemia rat model (middle cerebral artery occlusion) was studied using ^{45}Ca autoradiography. High ^{45}Ca accumulations were observed in the frontal cortex and caudate-putamen corresponding with morphological damage shown by HE staining. Regional ^{45}Ca concentrations were calculated from the optical density on the ^{45}Ca autoradiograms. Rapid uptake of ^{45}Ca in the ischemic brain occurred during the first 5 hours, and continued more slowly between 5 and 24 hours after ischemia. The area of ^{45}Ca accumulation was also expanded between 5 and 24 hours. An area of low ^{45}Ca concentration around the area of high accumulation developed 5 hours after ischemia, which presumably accumulated ^{45}Ca between 5 and 24 hours after ischemia. The lower concentration of ^{45}Ca in the periphery of ischemia may result from: 1) a decrease in the total amount of calcium due to narrowing of extracellular space accompanied by cytotoxic edema, and 2) delayed accumulation of exogenous ^{45}Ca due to reduced clearance of extracellular fluid.

Key words: calcium, rat, ischemia, middle cerebral artery, autoradiogram

Introduction

Recently, the importance of calcium ions to the neuronal function has been demonstrated.[9] The mechanisms that ultimately lead to ischemic cell death are unknown,[5,14] but calcium uptake by ischemic tissue is critical in the process of irreversible injury,[3,5,7,14] and abnormal calcium metabolism will initiate pathophysiological changes and contribute to neuronal injury.[9] Several studies investigating neuronal death have used the ^{45}Ca autoradiographic technique in a transient ischemic model.[5,10] Dienel[5] showed that postischemic regional ^{45}Ca accumulation is localized, time-dependent, and coincides with the extent of morphological damage, while ^{45}Ca was cleared from uninjured or reversibly injured tissue. However, the relationship between exogenous ^{45}Ca accumulation and histological changes after permanent focal ischemia is not well known. This study assessed disruption and change in neuronal calcium homeostasis caused by middle cerebral artery (MCA) occlusion in rats, using ^{45}Ca autoradiography to measure regional change in the concentration of exogenous ^{45}Ca.

Materials and Methods

Male Slc Wistar rats weighing 250-300 g were anesthetized with 1% halothane in air/oxygen 2:1 mixture with spontaneous breathing. A polyethylene catheter was inserted into the femoral vein for ^{45}Ca administration. Left subtemporal craniectomy was performed using a microdrill. After opening the dura with a fine needle, the MCA was dissected free from the pia-arachnoid. The artery was coagulated below the rhinal fissure with a bipolar radiofrequency electrical current, then divided with scissors to ensure permanent occlusion. Immediately after wound closure, 200 μCi of ^{45}Ca (Dupont/NEN Research Products, Boston, Mass., U.S.A.; specific activity 18.14 mCi/mg Ca) was injected through the catheters into the femoral vein. Seven rats (Group I) were decapitated 5 hours after ^{45}Ca administration, and another seven rats (Group II) 24 hours later, after the collection of blood samples from the jugular vein under ether anesthesia. Likewise, three rats

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which received a sham operation were decapitated at 5 hours after ^{45}Ca administration and another three rats at 24 hours to act as control groups.

After decapitation, the brains were quickly removed and frozen in isopentane previously cooled to −70°C. Serial 20 μm-thick coronal sections were cut from the frozen brain in a −20°C cryostat (Histostat Microtome; American Optical Co., U.S.A.), mounted on glass cover slips, and dried at 60°C on a hot plate. Autoradiograms were prepared by exposing a medical x-ray film (SB-5; Kodak, Rochester, N.Y., U.S.A.) to the dried section for 10–14 days in a standard x-ray cassette.

The frontal cortex and lateral caudate nucleus of the anterior forebrain remaining after cutting the sections was used to measure the ^{45}Ca concentration (nCi/g wet weight) in each structure. The brain samples placed in vials were weighed (Direct Reading Balance NL-200P; Shimadzu, Tokyo), dissolved in 1.5 ml of Protosol (DuPont) in plastic scintillation vials, incubated at 55°C for 24 hours, and the radioactivity measured in 10 ml of Clear-Sol I (Nacalai Tesque Inc., Kyoto) with a liquid scintillation system (LS9000; Beckman, Cal., U.S.A.). The radioactivity of each tissue and jugular blood sample was calculated from the percentage efficiency counted by an internal standard method. The figure was then corrected for the half life of ^{45}Ca (164 days). The true radioactivity of the brain tissue was calculated by subtracting the radioactivity of blood (in this study, the value of 55.6 μl/g brain tissue for cerebral blood volume was used[6]) from the measured radioactivity of the brain at decapitation.

The optical density of the autoradiograms was measured with an image processing system (Unigraphy UHG101; Unique Medical Co., Tokyo). A standard curve based on radioactivities of tissues and optical density on the ^{45}Ca autoradiogram of each tissue was made and used to calculate the regional ^{45}Ca concentration from the optical density. To compare areas of high accumulation in Groups I and II, area measurements in the coronal section of the autoradiogram at the level of the caudate nucleus were made using an image analyzer (MOP-AM03; Kontron, München, Germany). The high accumulation area was then expressed as a percentage of the area of the ipsilateral cerebral hemisphere.

Coronal sections adjacent to those used for the autoradiograms were stained with HE and the histology was examined simultaneously.

Values are expressed as mean ± SD. Single comparisons between the two groups were made by Student's t-test.

Results

I. ^{45}Ca radioactivities of brain tissue

Table 1 shows the mean accumulated ^{45}Ca radioactivities in the frontal cortex and caudate nucleus of rats after MCA occlusion. The affected frontal cortex contained 102.9 ± 24.1 nCi/g wet weight in Group I, and 122.6 ± 35.8 nCi/g wet weight in Group II, while the affected caudate nucleus contained 95.9 ± 35.7 and 118.2 ± 43.2 nCi/g wet weight, respectively. The values in Group II were slightly greater than in Group I, but the difference was not statistically significant. The regional ^{45}Ca accumulation in the ischemic area increased more during the first 5 hours than from 5 to 24 hours.

II. ^{45}Ca autoradiograms

Both Groups I and II demonstrated a high ^{45}Ca accumulation in the frontal cortex and caudate nucleus on the affected side. The area of the ^{45}Ca accumulation at the level of caudate nucleus in the affected hemisphere was 47.1 ± 8.4% in Group I and 66.9 ± 3.0% in Group II ($p < 0.001$). Therefore, the area of ^{45}Ca accumulation expanded significantly from 5 to 24 hours after MCA occlusion. The sham-operated groups demonstrated no abnormal accumulation except at the operation site.

The regional ^{45}Ca concentrations for various brain structures calculated from the optical density of the autoradiograms are shown in Table 2. The regional

Table 1 ^{45}Ca radioactivities of brain tissue

	Group I (n = 7)		Group II (n = 7)	
	Left	Right	Left	Right
Cerebral cortex	102.9 ± 24.1	32.7 ± 6.6	122.6 ± 35.8	35.1 ± 7.5
Caudate nucleus	95.9 ± 35.7	45.2 ± 7.7	118.2 ± 43.2	32.0 ± 5.2

Group I and II rats were decapitated 5 and 24 hours after ^{45}Ca administration, respectively. Total ^{45}Ca radioactivities (nCi/g wet weight) are reported as mean ± SD. Differences between the two groups were not significant.

Table 2 Regional ^{45}Ca concentrations obtained from autoradiograms

	Group I (n = 7)		Group II (n = 7)	
	Left	Right	Left	Right
Cerebral cortex				
paracentral cortex	53.1 ± 12.5	54.7 ± 7.1	38.2 ± 15.0	37.8 ± 15.3
accumulation area (A)	87.7 ± 30.0	33.7 ± 10.0	130.0 ± 51.8	43.4 ± 15.7
dorsal adjacent area (B)	25.4 ± 8.2[a]	38.5 ± 6.8[a]	42.3 ± 16.6	42.7 ± 14.9
Caudate nucleus				
accumulation area (C)	70.4 ± 12.8[b]	41.8 ± 13.9	153.8 ± 44.1[b]	47.8 ± 12.7
medial adjacent area (D)	29.4 ± 17.3[a]	50.0 ± 16.9[a]	43.5 ± 18.5	42.9 ± 14.4

Group I and II rats were decapitated 5 and 24 hours after ^{45}Ca administration, respectively. Values are nCi/g wet weight (means ± SD). [a]Significantly different from contralateral homologous area ($p < 0.05$). [b]Significantly different between Groups I and II ($p < 0.001$). A-D denote the areas in Fig. 1.

concentration of ^{45}Ca in the ischemic area of Group II was higher than that in Group I, but ^{45}Ca accumulation was more rapid up to 5 hours after ischemia than between 5 and 24 hours. The ^{45}Ca concentrations in the areas adjacent to regions of increased accumulation were lower than that in the contralateral normal area in Group I (Fig. 1). The regional ^{45}Ca concentration was 25.4 ± 8.2 nCi/g (38.5 ± 6.8 nCi/g on the contralateral side) in the cerebral cortex and 29.4 ± 17.3 nCi/g (50.0 ± 16.9 nCi/g on the contralateral side) in the caudate-putamen, showing significant differences ($p < 0.05$) between the lesioned and contralateral sides. In Group II, such a lower ^{45}Ca concentration on the lesioned side was not observed in the boundary region (Fig. 2).

III. Histological examination

HE staining showed less staining in regions corresponding to the high ^{45}Ca accumulation area in the frontal cortex and caudate-putamen of Group I (Fig. 1). The periphery of the ischemic area with low ^{45}Ca concentration revealed no histological changes. In Group II, regions corresponding to areas of high ^{45}Ca accumulation were also less stained by HE (Fig. 2).

Discussion

Ischemic insults that lead to irreversible damage are associated with calcium accumulation in the tissue.[2,5,8,12,15] Regional calcium concentration (determined by atomic absorption spectroscopy) increases significantly in the ischemic area 4 hours after MCA occlusion in rats.[12] Postischemic accumulation of calcium in the region of the cerebral cortex supplied by the MCA correlates with the extent of infarction.[2] In our study, the distribution of ^{45}Ca accumulation coincided with morphological damage. Our analysis of Dienel's work suggested that the increase in total calcium nearly corresponded with the increase in ^{45}Ca. Therefore, the regional ^{45}Ca accumulation measured by autoradiography may indicate the movement of free calcium.

Calcium accumulation may represent damaging calcium-mediated processes activated before irreversible cell injury occurs or a secondary event due to membrane breakdown.[1] Net accumulation of calcium in the superior region of the hippocampus precedes marked necrosis of CA1 pyramidal cells, following reversible ischemia,[4] while ^{45}Ca accumulation in the dentate hilus occurs before irreversible cell damage after ischemia.[1] These results suggest that increased calcium accumulation is the primary event that leads to irreversible neuronal damage.[4,11]

We found that ^{45}Ca accumulation was greatest in the first 5 hours after ischemia and slower between 5 and 24 hours. However, the area of ^{45}Ca accumulation in the affected cortex extended during the 5- to 24-hour interval. These results indicate, as previously suggested,[5] that rapid calcium accumulation occurs during cell death, and is slower in dead cells.

Conventional autoradiography using exogenous ^{45}Ca does not discriminate between intra- and extracellular calcium accumulation after cerebral ischemia. However, intracellular calcium influx is more likely because a 90% reduction in brain extracellular calcium concentration occurs during cerebral ischemia.[13] The increase in calcium concentration in the ischemic area, 24 hours after injury, is equivalent to 17 times the amount of free calcium in the tissues prior to MCA occlusion, suggesting that calcium binds to intracellular inorganic phosphorus and this massive accumulation of calcium indicates

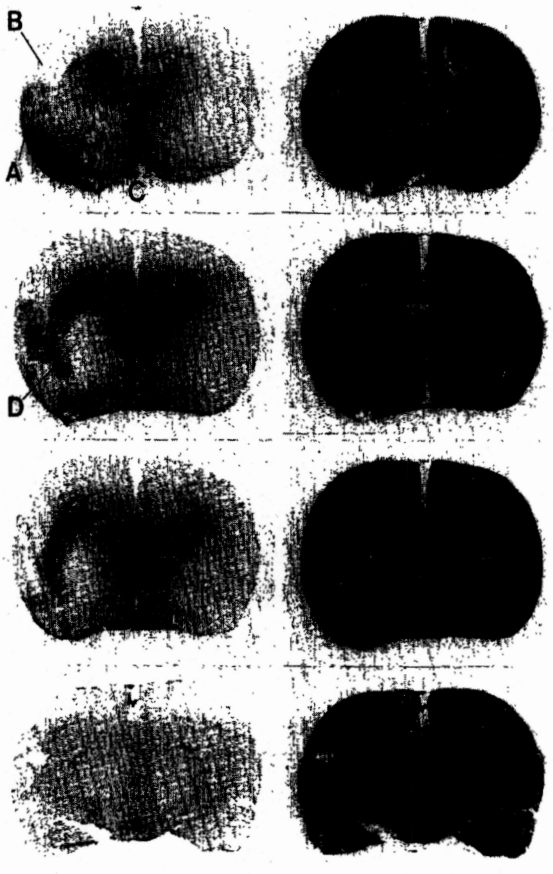

Fig. 1 Group I. *left*: ^{45}Ca autoradiograms, showing ^{45}Ca accumulation in the frontal cortex (A) and caudate-putamen (C), and lower ^{45}Ca concentrations in the areas adjacent dorsally (B) and medially (D) to the high accumulations than those in the contralateral hemisphere. *right*: HE staining of adjacent frozen sections, showing less staining in the regions corresponding to the high ^{45}Ca accumulation areas. No histological changes were noted in the low ^{45}Ca concentration areas.

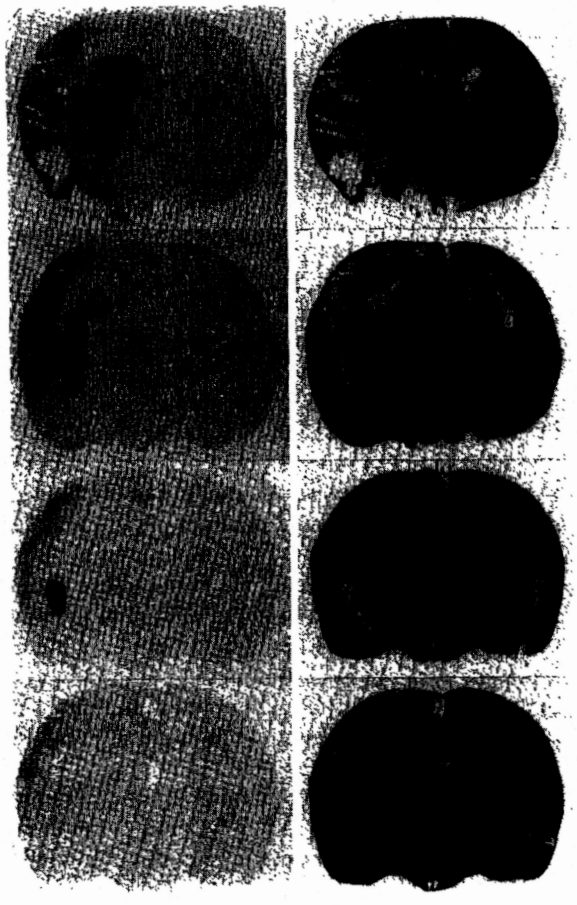

Fig. 2 Group II. *left*: ^{45}Ca autoradiograms, showing ^{45}Ca accumulation in the frontal cortex and caudate-putamen. The ^{45}Ca accumulation area in the cortex had expanded. *right*: HE staining of adjacent frozen sections, showing less staining in the regions corresponding to areas of high ^{45}Ca accumulation.

not only passive influx across a leaky plasma membrane but also an active accumulation mechanism.[12] ^{45}Ca accumulation may also represent an increased turnover of calcium before irreversible cell damage occurs, not a passive influx of calcium.[1]

The areas of low ^{45}Ca concentration around the high accumulation areas occurring 5 hours after ischemia are interesting. These areas were more distinct in the cortex than in the caudate-putamen and showed no definite morphological changes on HE staining. These areas would probably accumulate more ^{45}Ca between 5 and 24 hours after MCA occlusion because the high accumulation area was extended. Two mechanisms are possible for the low accumulation: 1) a decrease in total calcium due to narrowing of the extracellular space accompanied by cytotoxic edema, and 2) delayed accumulation of exogenous ^{45}Ca due to reduced clearance of extracellular fluid.

The expansion of ^{45}Ca accumulation between 5 and 24 hours, and the low concentration areas around the high accumulation 5 hours after ischemia are new features in the focal ischemia rat model. Further investigations are needed to clarify the mechanism responsible for the low concentration of calcium in the peripheral zone of ischemia and the relationship between this zone and the ischemic penumbra.

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