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RESEARCH ARTICLE

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Calcium metabolism of focal and penumbral tissues in rats subjected to transient middle cerebral artery occlusion

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Abstract The present experiments were undertaken to define changes in tissue calcium metabolism in focal and perifocal ("penumbral") tissues following 2 h of transient middle cerebral artery occlusion (MCAO) in rats, induced with an intraluminal filament occlusion technique. The extracellular calcium concentration ($[Ca^{2+}]_e$) was measured with ion-selective microelectrodes in neocortical focus and penumbra. For measurement of total tissue calcium content, tissue samples from these areas were collected and analyzed with atomic absorption spectrometry. During MCAO, $[Ca^{2+}]_e$ in a neocortical focal area fell from a normal value of about 1.2 mM to values around 0.1 mM, suggesting translocation of virtually all extracellular calcium to intracellular fluids. Recirculation was accompanied by re-extrusion of calcium within 5–7 min; however, $[Ca^{2+}]_e$ never returned to normal but stabilized at about 50% of the control value for the first 6 h, and decreased further after 24 h. In penumbral areas, $[Ca^{2+}]_e$ showed the expected transient decreases associated with spreading depression-like (or ischemic) depolarization waves. Recirculation was followed by return of $[Ca^{2+}]_e$ towards normal values. In the focus, water content increased from about 79% to about 80.4% at the end of the 2-h period of ischemia. After 2 h and 4 h of recirculation, the edema was aggravated (mean values 81.9% and 81.2%, respectively). After 6 h and 24 h, the edema was more pronounced (83.6% and 83.8%, respectively). In the penumbra, no significant edema was observed until 6 h and 24 h of recirculation. The total tissue calcium content in the focus (expressed by unit dry weight) increased at the end of the ischemia period demonstrating calcium translocation from blood to tissue. After 6 h and 24 h, the content increased two- to threefold, compared with control. Changes in the penumbra were qualitatively similar but less pronounced, and a significant increase was not observed until after 6 h of recirculation. The results suggest that 2 h of MCAO leads to a profound perturbation of cell calcium metabolism. In focal areas, cells fail to extrude the calcium that is gradually accumulated during reperfusion and show massive calcium overload after the first 4–6 h of recirculation. Penumbral tissues show a similar increase in calcium concentration after 6 h of recirculation.

Key words Extracellular calcium concentration · Total tissue calcium content · Middle cerebral artery occlusion · Reperfusion · Rat

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Introduction

Dense or complete ischemia is known to lead to influx of calcium from extra- to intracellular fluids (Nicholson et al. 1977; Hansen and Zeuthen 1981; Harris et al. 1981; for reviews, see Hansen 1985; Erecinska and Silver 1994; Kristián and Siesjö 1996a). In forebrain ischemia in rats, this has been documented both as a decrease in extracellular calcium concentration ($[Ca^{2+}]_e$), and as a rise in $[Ca^{2+}]_i$, the intracellular calcium concentration (Silver and Erecinska 1990, 1992). However, since the total calcium concentration remains unchanged for at least 24 h following a 10-min ischemic transient, the initial calcium load to which cells are exposed is moderate, since it is confined to the amount of calcium contained in the extracellular fluid (Dienel 1984; Deshpande et al. 1987; Martins et al. 1988; for data on gerbils see Kato et al. 1987).

In the core of a focal ischemic lesion, changes in $[Ca^{2+}]_e$ are similar, suggesting that virtually all of the extracellular calcium is translocated to intracellular fluids. In such "focal" areas ischemia is accompanied by a d.c. potential shift, by a marked rise in $[K^+]_e$, and by a de-

crease in $[Ca^{2+}]_e$ (see results by Kristián et al. 1995; Gidö et al. 1997). In these areas, loss of calcium homeostasis and the accompanying d.c. potential shift are as extensive as in complete or near-complete global ischemia. In the perifocal ("penumbral") areas, though, where cerebral blood flow (CBF) is borderline for maintenance of cell viability, similar ionic changes are observed. However, they occur irregularly as repeated spreading depressions (SD) or ischemic depolarizations (Gill et al. 1992; Iijima et al. 1992; Nedergaard and Hansen 1993; Back et al. 1994).

Neuronal damage following brief periods of forebrain ischemia is conspicuously delayed, particularly in the CA1 sector of the hippocampus (Kirino et al. 1992; Pulsinelli et al. 1992). It has been proposed that this is due to a gradual rise in $[Ca^{2+}]_i$, with eventual mitochondrial calcium overload (Deshpande et al. 1987; Kristián and Siesjö 1996b). Recent data, demonstrating that cyclosporin A (CsA) markedly ameliorates the CA1 damage after ischemic periods of 7 min or 10 min (Uchino et al. 1995) suggests that a mitochondrial permeability transition (MPT) is involved in the delayed cell death (see Kristián and Siesjö 1996b; Siesjö and Siesjö 1996). Thus, CsA is a specific inhibitor of the MPT that is observed in isolated mitochondria which are calcium-loaded or subjected to oxidative stress (Crompton et al. 1988; Duchen et al. 1993; Zoratti and Szabó 1995; Bernardi and Petronilli 1996).

The question arises of whether the more rapidly maturing neuronal and tissue damage after transient focal ischemia is triggered by mitochondrial calcium overload, and if the latter is accelerated by mass transfer of Ca^{2+} across the blood-brain barrier (BBB). A net increase in tissue calcium content has been recorded by atomic absorption spectroscopy or assumed to occur from ^{45}Ca fluxes (Young et al. 1986; Rappaport et al. 1987; Shirotani et al. 1994). However, no data exist on changes in $[Ca^{2+}]_e$ and total calcium content during the 1st hours of recirculation. In the present experiments, we induced ischemia of 2 h duration, allowing recirculation for 2, 4, 6, or 24 h, recorded $[Ca^{2+}]_e$ in focal and penumbral areas, and measured total tissue calcium content to explore whether translocation of Ca^{2+} from blood to tissue, e.g., driven by a persistent reduction of $[Ca^{2+}]_e$, sets the stage for rapidly developing cellular calcium overload.

Materials and methods

Male Wistar rats (305–335 g) of a specific pathogen-free strain (Mollegaard's Breeding Center, Copenhagen, Denmark), fasted overnight with free access to tap water, were used in the present experiments. The animals were anesthetized with 3.5% halothane and artificially ventilated on 1.5% halothane in N_2O:O_2 (70:30) during operation. A tail artery catheter was inserted for arterial blood sampling and measurement of mean arterial blood pressure (MABP). The body temperature of animals was controlled with the use of a thermistor-guided heating blanket, with the thermistor probe placed into the rectum, and kept at 37°C during middle cerebral artery (MCA) occlusion. After MCA occlusion, the animals were allowed to wake up.

The right MCA was occluded by the intraluminal filament technique of Koizumi et al. (1986; see also Longa et al. 1989), as described by Memezawa et al. (1992; and by Zhao et al. 1994). A surgical incision was made to expose the right common, internal, and external carotid arteries. Thereafter, the external carotid artery was ligated and 0.1 ml of heparin (150 IU·ml^{-1}) was given. A small incision was made in the common carotid artery 1 mm proximal to the carotid bifurcation, and the occluder was inserted into the internal carotid artery. The filament was further gently advanced 19 mm from the bifurcation to occlude the origin of the MCA. After occlusion of the MCA, the animals were allowed to wake up, while freely moving in a cooling box to prevent a rise in temperature (Zhao et al. 1994).

The animals were divided into several groups, in which $[Ca^{2+}]_e$ was recorded during the 2 h of MCA occlusion, or in the recirculation periods 0–2 h, 2–4 h, 4–6 h, 6–8 h, and 19–21 h. The experimental setup did not allow continuous recording of $[Ca^{2+}]_e$ before, during, and after the ischemic period, since manipulation of the carotid artery required that the animal was turned. Furthermore, the total tissue calcium content was measured by atomic absorption spectrophotometry at the end of the above-mentioned reperfusion periods. Since ischemia could be expected to be accompanied by edema, the water content of the tissue samples was measured prior to calcium determination. The neurologic status of each animal that had been used for $[Ca^{2+}]_e$ recording and for total calcium measurement was evaluated according to the neurologic examination grading system described by Bederson et al. (1986). Animals which did not show the required neurologic deficit during ischemia were excluded from the experimental series. All procedures followed the guidelines of the National Institutes of Health (*Guide for the care and use of laboratory animals*), and were approved by the Lund University animal ethical committee.

Recording of $[Ca^{2+}]_e$

When the surgical procedures had been completed and the occluder filament inserted, the halothane concentration in the gas mixture was decreased to 0.3–0.5%, and an infusion of 1.2 ml·h^{-1} of the muscle relaxant vecuronium bromide (Norcuron) was started.

The $[Ca^{2+}]_e$ was measured by means of ion-selective glass microelectrodes, according to Kristián et al. (1993). The glass microelectrodes were double-barreled, where one barrel was filled with 150 mM NaCl for recording the changes in d.c. potential and the ion-selective one, with calcium ionophore I (Cocktail A 21048, Fluka, Switzerland) and 100 mM $CaCl_2$. Each barrel was connected to a high-input resistance amplifier via Ag-AgCl wires. The signals representing the d.c. potential and ion concentration changes were recorded and stored by a Macintosh computer using the MacLab data acquisition system. The animals were grounded via a glass tube, filled with 3% agar in 150 mM NaCl, placed subcutaneously in the neck. The ion-selective electrodes were calibrated before and after the experiments in solutions containing 5, 1, 0.5, and 0.1 mM $CaCl_2$ in 3 mM KCl, 130 mM NaCl, and 10 mM 3-(*N*-morpholino)propane sulfonic acid, sodium salt (MOPS; Sigma, St. Louis, USA) buffer, pH 7.3. The microelectrodes were considered acceptable when they responded with a 26- to 30-mV change to a tenfold change in calcium concentration.

The animals were placed in a head holder, and a craniotomy (outer diameter 1.5–2 mm) was made over the right cortex at coordinates suitable for recording in the ischemic focus (bregma anterior 0 mm, lateral 5.5 mm, and ventral 2 mm), or for recording in penumbral areas (bregma anterior 0 mm, lateral 3.5 mm, ventral 1 mm; see Fig. 1). In the animals in which $[Ca^{2+}]_e$ was recorded during the occlusion, the head temperature was maintained at 37°C by a heating box (see Ekholm and Siesjö 1992). After a small incision in the dura had been made, the electrodes were lowered into the parietal cortex by a motor-driven micro-manipulator to the desired depth.

The $[Ca^{2+}]_e$ recording was then started and continued for 2 h. Physiological parameters were kept within normal ranges during the experiment. Following 2 h of recording, the animals were allowed to regain consciousness. They were then housed in cages with access to tap water and pellet food.

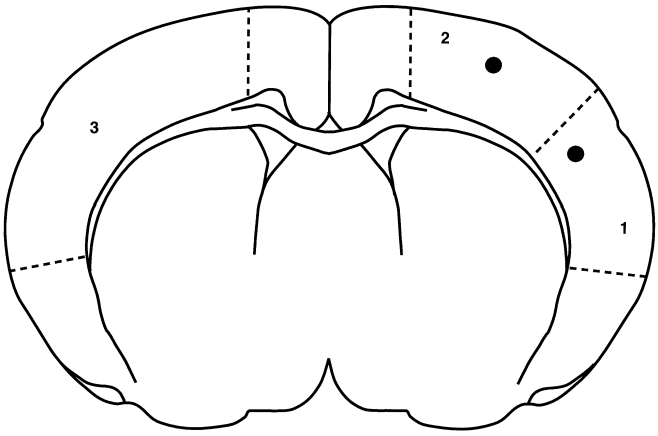

Fig. 1 Schematic drawing showing the recording places (*black dots*) and the three brain regions dissected for total calcium measurement. Region *1* represent the cortical focus, region *2* the penumbral area, and region *3* the contralateral control cortex

Evaluation of infarcted area

After 48 h of recovery, the animals were anesthetized with 3.5% halothane and killed by decapitation. Their brains were quickly removed and chilled in ice-cold saline for 10 min. One-millimeter coronal slices were cut in a tissue slicer, and the slices were immersed in a saline solution containing 1.0% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical, St. Louis, Missouri, USA) at 37°C for 20 min (Bederson et al. 1986).

After staining, the slice at the bregma level was manually depicted to verify the infarct size at the recording place. Animals with no neuronal necrosis at the recording site were discarded from the analysis.

Measurement of total calcium content

After 2 h of MCA occlusion and 2, 4, 6, and 24 h of reperfusion, the rat brains were frozen in situ, removed, and stored at –80°C, as described by Pontén et al. (1973). For analysis of tissue calcium content, areas of frontoparietal cortex corresponding to the ischemic focus and penumbra (see Fig. 1) were dissected at –22°C and weighed. The areas corresponded to those previously sampled to characterize tissue energy state and mitochondrial respiratory activity following transient MCAO (Folbergrová et al. 1995; Kuroda et al. 1996). Cortex samples from the contralateral hemisphere were used as controls. The Ca content was also measured in cortical samples from unoperated control rats. The samples were dried at 105°C for 48 h, weighed again, and then extracted for 24 h with 70% HNO_3 at 1 ml·100 mg^{-1} tissue (see Bradbury et al. 1968). The suspension was centrifuged at 15000 *g* for 10 min; the supernatant fluid was then diluted 20-fold and used for analysis. The final acid concentration was 7%, and the calcium was determined in the presence of 1% lanthanum nitrate. Subsequently, the samples were measured on an atomic absorption spectrophotometer (Perkin-Elmer 306).

Statistical analysis

The data presented were analyzed by analysis of variance (ANOVA). At the same time points, comparisons between groups were performed with one-factor ANOVA followed by Scheffe's test. Statistical significance was calculated with two-factor ANOVA followed by Scheffe's test between the groups with different time points. All values are expressed as mean ± SD.

Results

The physiological parameters were kept within normal ranges in all experimental groups. The animal's body temperature was kept at about 37°C, plasma glucose concentration was between 4 and 5.8 mM, blood PCO_2 was kept within 36 and 39 mmHg, and PO_2 levels were about 120 mmHg. There were no significant differences between different experimental groups.

Changes in $[Ca^{2+}]_e$ during and after transient MCA occlusion

In the group of animals were changes in $[Ca^{2+}]_e$ were measured during ischemia, recording of $[Ca^{2+}]_e$ started about 15–20 min after the MCA occlusion and continued for the next 2 h. In the other groups, the recording started at 2, 4, 6, or 19 h of recirculation after 2 h MCA occlusion and continued for 2 h. From the recordings, $[Ca^{2+}]_e$ was calculated every second, the values so obtained being used to calculate the mean from a 2-h recording period.

In the ischemic core, the $[Ca^{2+}]_e$ usually remained stable at the 0.1–0.2 mM level (Fig. 2). However, two of five rats showed one transient increase in $[Ca^{2+}]_e$ (data not shown). Immediately upon reperfusion, the $[Ca^{2+}]_e$ started to increase. However, the recovery of $[Ca^{2+}]_e$ was not complete, since the values stabilized at about 0.6 mM (Fig. 2, upper panel). This subnormal $[Ca^{2+}]_e$ level persisted during the first 6–8 h of reperfusion. However, after 19–21 h a secondary decrease was observed (Fig. 3).

Recording from penumbral areas showed the typical pattern of irregularly occurring spreading depression-like waves (Fig. 2, lower panel). Similarly, as in the ischemic focus, the $[Ca^{2+}]_e$ during cell depolarization decreased to the 0.1 mM level. The mean depolarization time was 7.2 ± 9.3 (n = 17) min. Reperfusion gave rise to a normalization of $[Ca^{2+}]_e$, which reached values of 0.89 mM ± 0.14 mM (n = 7). During the first 6 h of reperfusion, $[Ca^{2+}]_e$ was stable both in the penumbral and in the core areas. Thus, no spontaneous decreases in $[Ca^{2+}]_e$ of the SD-type were observed. As remarked, though, after about 20 h of reperfusion, $[Ca^{2+}]_e$ in the ischemic focus was further reduced to about 0.3 mM (see Fig. 3).

Changes in total tissue calcium content

In order to assess whether the subnormal $[Ca^{2+}]_e$ level in the ischemic focus during reperfusion triggered translocation of calcium from blood to tissue, we measured the total tissue calcium content following different reperfusion periods.

The tissue water content in control samples was about 79%. After 2 h of MCAO, there was a significant increase

Table 1 Changes in tissue water content after 2 h of middle cerebral artery occlusion at different reperfusion periods (values are mean±SDS percentage of wet weight)

Sample site	0 (n=6)	2 (n=5)	4 (n=5)	6 (n=6)	24 (n=5)
Control	78.9±0.6	79.4±0.3	79.1±0.5	78.9±0.6	78.9±0.7
Penumbra	79.5±0.6	80.0±0.6*[6]	80.0±0.7*[6]	82.6±1.7*[1, 3]	83.5±1.4*[2, 5]
Ischemic focus	80.4±0.7*[1]	81.9±0.5*[2]	81.2±0.4*[2]	83.6±2.3*[2, 4]	83.8±2.0*[2, 5]

Significantly different from controls:*[1] $P<0.05$; *[2] $P<0.01$ (one-factor ANOVA followed by Scheffe's test)
Significantly different from 0-h reperfusion group: *[3] $P<0.05$; *[4] $P<0.01$ (two-factor ANOVA followed by Scheffe's test)
Significantly different from 0-, 2-, and 4-h reperfusion groups: *[5] $P<0.01$ (two-factor ANOVA followed by Scheffe's test)
Significantly different from ischemic focus: *[6] $P>0.05$ (one-factor ANOVA followed by Scheffe's test)

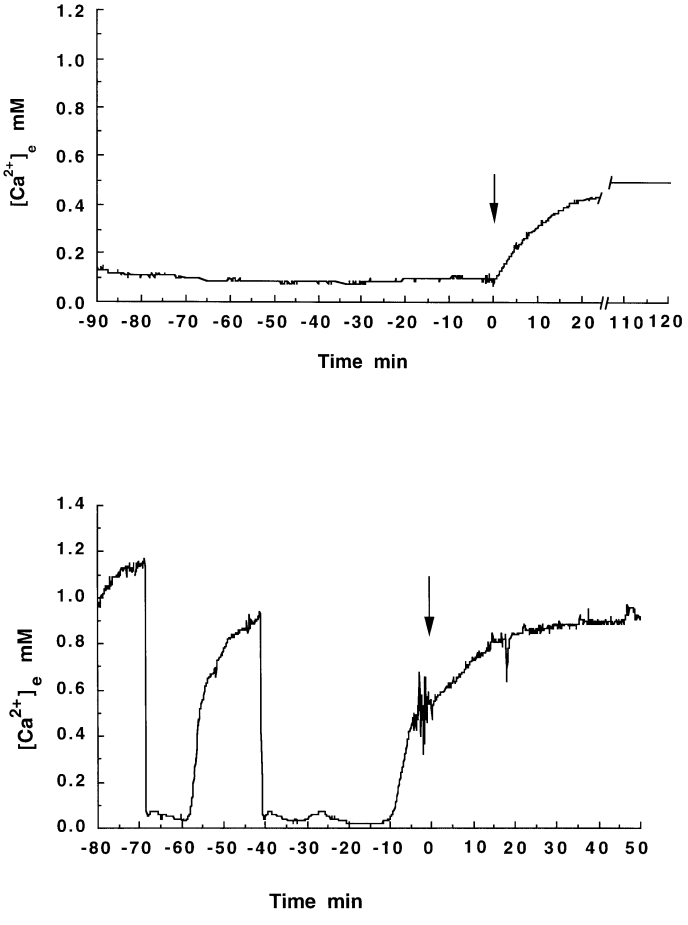

Fig. 2 Typical changes in $[Ca^{2+}]_e$ during middle cerebral artery occlusion in cortical focus (*upper panel*) and penumbral areas (*lower panel*). Zero time represents the onset of reperfusion (*arrow*)

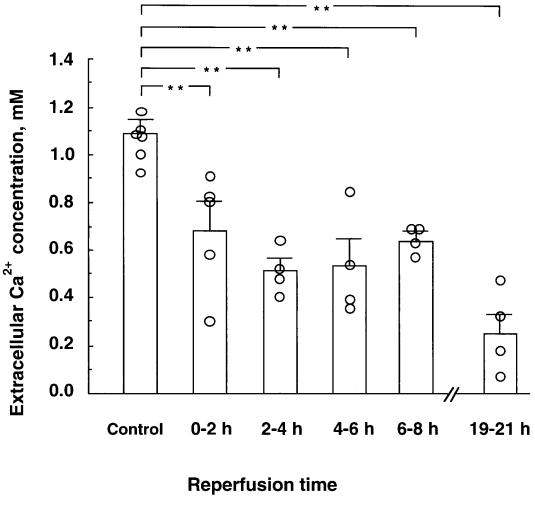

Fig. 3 Individual values showing the temporal profile of $[Ca^{2+}]_e$ at different reperfusion periods. Control values represents the normal level of $[Ca^{2+}]_e$ in control animals. **Significantly different when compared with control, $P < 0.01$ (ANOVA followed by Dunnett's test)

in water content in the ischemic core. A further rise in water content was observed following 6 h of reperfusion (see Table 1). In penumbral areas, the water content increased significantly after 6 h of reperfusion.

Tissue calcium content in the contralateral cortex was 5.77 ± 0.22 mmol·kg^{-1} dry weight (mean value from all control groups). The values in unoperated animals were slightly lower (5.52 ± 0.20, n=6) . At the end of ischemia (0 h reperfusion) the mean value increased by about 20% in the focus. After 6 h of recovery, calcium content was about two-fold higher than in control samples (see Fig. 4). In penumbral areas, tissue calcium accumulation was observed by 6 h of recirculation, and there was a further increase in the total calcium content in both core and penumbral tissues after 24 h, the values being about three-fold higher than in control samples (see Fig. 4).

Discussion

Changes in calcium metabolism accompanying ischemic insults, particularly in models of forebrain ischemia, have been extensively documented in the literature. This applies to changes in $[Ca^{2+}]_e$ (Hansen and Zeuthen 1981; Harris et al. 1981; Siemkowicz and Hansen 1981; Silver and Erecinska 1990, 1992; Kristián et al. 1994; Ekholm et al. 1995), in total calcium content (Dienel 1984; Deshpande et al. 1987; Martins et al. 1988), and in mitochondrial calcium content (Simon et al. 1984; Dux et al. 1987; Zaidan and Sims 1994).

In focal ischemia, as for example the one induced by MCA occlusion, there is usually a densely ischemic focus, supplied by the occluded artery, and perifocal "penumbral" areas with less dense ischemia (Siesjö et al. 1990;

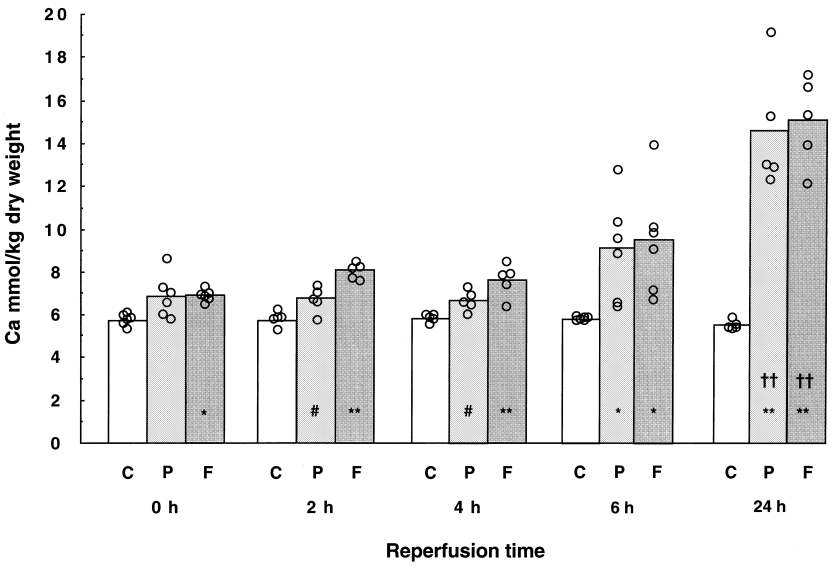

Fig. 4 Changes in total calcium content after 2 h of middle cerebral artery occlusion and different reperfusion periods in focal and penumbral areas. Significantly different when compared with control: $^{*}P < 0.05$; $^{**}P < 0.01$ (one-factor ANOVA followed by Scheffe's test). Significantly different when compared with 0, 2, and 4 h of reperfusion: $^{\dagger\dagger}P < 0.01$ (two-factor ANOVA followed by Scheffe's test). Significantly different when compared with focus: $^{\#}P < 0.05$ (one-factor ANOVA followed by Scheffe's test)

Pulsinelli 1992; Siesjö 1992a, b; Hossmann 1994). Changes in $[Ca^{2+}]_e$ during focal ischemia were reported by Gill et al. (1992). The authors studied the effect of the NMDA antagonist dizocilpine maleate (MK-801) on $[Ca^{2+}]_e$ transients in penumbral areas during permanent MCA occlusion in the rat. Similar data were reported by Iijima et al. (1992). In this study only changes in d.c potential were recorded in perifocal areas together with blood flow changes, and the effect of MK-801 on spreading depression-like waves and the infarct volume was defined. Finally Nedergaard and Hansen (1993) showed that the spontaneous depolarizations evoked by focal ischemia could be classified as SD-like or as ischemic depolarization waves, the latter probably being caused by a transient decrease in the local blood flow.

To the best of our knowledge there are no data describing changes in calcium homeostasis after transient focal ischemia. This means that no information is available on recovery of $[Ca^{2+}]_e$, or postischemic Ca^{2+} transients, if any.

Recent results have demonstrated that recirculation after 2 h of MCA occlusion leads to an initial recovery of the bioenergetic state of tissues coresponding to core ("ischemic focus") and penumbral tissues ("penumbra"), but that this is followed by secondary metabolic deterioration after about 4 h (Folbergrová et al. 1995). Analyses of mitochondrial respiration in tissue homogenates suggest that, at least in part, this reflects secondary failure of mitochondrial function (Kuroda et al. 1995, 1996). The transient recovery of the bioenergetic state is accompanied by a rapid recovery of extracellular potassium concentration ($[K^+]_e$) upon reperfusion, which is then followed by a delayed increase in $[K^+]_e$ at about 6 h of recirculation, suggesting cell depolarizaton and K^+ release (Gidö et al. 1997).

The present results demonstrate that reperfusion leads to recovery of $[Ca^{2+}]_e$; however, full recovery of $[Ca^{2+}]_e$ was observed only in penumbral areas. In core tissues $[Ca^{2+}]_e$ stabilized at about 50% of the normal values. Thus, about half of the Ca^{2+} that entered the cells during ischemia remained in intracellular compartments during reperfusion. Intracellularly, most of the calcium ions are bound or sequestrated into subcellular organelles. After forebrain ischemia the calcium taken up by the cells is extruded upon recirculation, and $[Ca^{2+}]_e$ is recovered in about 20 min (Silver and Erecinska 1992; Ekholm et al. 1995). Transient focal ischemia induced by 2 h of MCA occlusion obviously gives rise to more severe disturbances in cell calcium homeostasis, since it leads to subnormal $[Ca^{2+}]_e$ even if the cells repolarize immediately after onset of reperfusion, as judged by the reversal of d.c. potential shifts and recovery of $[K^+]_e$ (see Gidö et al. 1997). Repolarization of cells suggests that there is enough ATP produced to fuel plasma membrane pumps for K^+ and Na^+ and that membrane integrity is maintained. However, the sustained reduction of $[Ca^{2+}]_e$ demonstrates that cell calcium metabolism remains perturbed.

As already remarked, $[Ca^{2+}]_e$ is normally 10 000-fold higher than $[Ca^{2+}]_i$. It is generally held that the level of $[Ca^{2+}]_i$ is set by the affinity of calcium translocation mechanisms for Ca^{2+} at their cytosolic sites (Carafoli 1987; Miller 1991). These mechanisms either transport Ca^{2+} out of the cell or into intracellular organelles such as the endoplasmic reticulum or the mitochondrion. However, little is known about the mechanisms that regulate $[Ca^{2+}]_e$ (see Katzman and Pappius 1973; Murphy and Rapoport 1988). In theory, $[Ca^{2+}]_e$ is set by the balance between Ca^{2+} transport across the BBB and the calcium fluxes via the plasma membrane, which are controlled by cell membrane channels and transporters as well as by intracellular binding/sequestration of the calcium. Since the $[Ca^{2+}]_e$ did not recover completely upon recirculation, some part of the calcium from extracellular fluids was either irreversibly sequestred or bound intracellularly, or removed from the tissue via the BBB. The latter is not very likely, since the BBB tightly controls ion movement between the brain and plasma. Another possibility is that in spite of recovery of d.c. potential and $[K^+]_e$, the cell plasma membranes have an increased per-

meability for calcium ions, the pumps being unable to keep the physiological, high-concentration gradient for Ca^{2+}. This could lead to enhanced influx of Ca^{2+} accross the plasma membrane and set the stage for cell calcium overload.

Changes in total tissue calcium content due to ischemic insults have been estimated by using autoradiography (Dienel 1984; Nagasawa and Kogure 1990; Shirotani et al. 1994). In focal ischemic models, ^{45}Ca was administrated at different time periods after onset of permanent MCA occlusion and then the distribution of radioactive calcium was evaluated 5 h after the radioisotope injection (Shirotani et al. 1994). Significant ^{45}Ca accumulation was detected in the cortex normally supplied by the occluded MCA after 4 h and 24 h of MCA occlusion.

In one study, recirculation was allowed, i.e., the MCA occlusion was transient, and ^{45}Ca was injected after the start of reperfusion (Nagasawa and Kogure 1990). One hour or 90 min of transient focal ischemia followed by 6 h of recirculation resulted in a massive ^{45}Ca accumulation in the cortex and lateral segment of the caudate putamen, areas corresponding to ischemic focus. These data suggest that the brain tissue that is subjected to prolonged (1 h and more) dense ischemia has an increased rate of ^{45}Ca uptake.

Since an enhanced influx of ^{45}Ca could, at least in part, reflect an increased BBB permeability, it does not quantitatively reflect net transfer of Ca^{2+} from plasma to tissue. However, data reported by Rappaport et al. (1987) on total calcium content following permanent MCA occlusion demonstrated that there was a marked increase in total tissue calcium content in areas with dense ischemia 4 h after MCA occlusion and massive accumulation of calcium after 24 h of MCA occlusion.

As Fig. 4 shows, ischemia of 2 h duration led to a 20% increase in total cell calcium content. The values obtained after 2 h and 4 h of recirculation were slightly higher. However, the 0-, 2-, and 4-h recirculation data suggest that there was relatively little change in the total cell calcium content during the first 4 h of recirculation. This means that $[Ca^{2+}]_e$ was reduced in spite of only slightly increased total cell calcium contents. It seems difficult to argue, therefore, that there was a constant flux of calcium from plasma to extracellular fluid during the first 4 h of reperfusion, since this would require a gradual rise in the total tissue calcium content.

The results in Fig. 4 demonstrate that at 6 h and 24 h of reperfusion the total tissue calcium content increased to excessive levels in focal as well as penumbral tissues. This increase may reflect the irreversible phase which leads to tissue infarction. It remains to be shown in what cells and in what organelles these excessive amounts of calcium are sequestered.

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