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Calcium in the mitochondria following brief ischemia of gerbil brain

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Calcium was localized in neurons and glial cells of the CA_1 region of hippocampus and in neurons of parietal cortex with the oxalate–pyroantimonate electron cytochemical method following recovery from 5-min ischemia of gerbil brain. During the early postischemic recirculation phase a maximum amount of calcium deposits was detected in mitochondria of all investigated cells. A second rise in mitochondrial calcium deposits was observed in parallel with the onset of ultrastructural degeneration of mitochondria and the loss of cellular integrity beginning 6 h postischemia.

Recently the calcium metabolism of the central nervous system was recognized to play an important role in the modulation of normal cell function via protein phosphorylation, proteolysis and lipolysis [13, 19]. During ischemia or in the early postischemic recirculation period, increased calcium fluxes may trigger these reactions leading to calcium overload of mitochondria and uncontrolled degradation of cellular membrane structures [21]. In many tissues the influx of calcium across the damaged plasma membrane through series of metabolic alterations represents the final common pathway in the toxic cellular death [3, 17, 18]. Based on the observation of decreased extracellular calcium ions and of increased calcium deposits visualized by electron microscopy, this pathomechanism was considered to be responsible for cell injury during anoxia and epileptic seizures [5–7, 15, 16, 19].

For neuronal cell damage developing after recovery from transient ischemia it is characteristic that some neurons immediately undergo cellular necrosis but others obviously survive for a certain period of time before the onset of irreversible neuronal necrosis becomes manifest. The delayed neuronal death is typical for neurons of the

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hippocampal CA_1 subfield after short-term global ischemia of gerbil brain [9]. The aim of the present study was to verify semiquantitatively the postischemic time course of calcium deposits in selective vulnerable CA_1 neurons in comparison to more resistant cortical neurons and CA_1 glial cells.

Adult Mongolian gerbils (*Meriones unguiculatus*) of either sex (50–60 g) were anesthetized with halothane. Both common carotid arteries were freed from connective tissue and occluded with Yasargil aneurysm microclips (Aesculap, F.R.G.) for 5 min under light halothane anesthesia. After the occlusion recirculation times were 5, 15, 30 min, 1, 2, 6, 24 and 48 h. Altogether 64 gerbils were operated and 8 sham-operated animals served as controls. For the electron cytochemical visualization of calcium the combined oxalate–pyroantimonate method of Borgers et al. [1, 22] was employed. The CA_1 subfield of hippocampus and pieces from the IIIrd and Vth layers of the parietal cortex were dissected from Araldite-embedded Vibratome sections. Semithin and ultrathin sections were prepared with a Reichert OmU 2 ultramicrotome. Semi-thin sections were stained with Methylene blue, the ultrathin ones with 0.5% uranyl acetate and 0.4% lead citrate. The specificity of cytochemical reaction was tested by treating the specimens from each tissue block with 5 mM EGTA for 1 h at 60°C [2]. This treatment was controlled by incubation of sections for 1 h at 60°C in distilled water. The sections were examined in a Zeiss EM 9 and in a JEOL 100 B electron microscope. The X-ray microprobe analysis of unstained ultrathin sections was carried out using a JEOL ASID-I scanning unit and an EDAX Si/Li detector attached to the JEOL 100 B electron microscope as described in detail earlier [20].

The calcium deposits visible in the mitochondria of investigated cells (pyramidal cells from the hippocampal CA_1 region and from the IIIrd and Vth layers of parietal cortex as well as glial cells from CA_1 subfield) were analysed semiquantitatively. From each group 40 micrographs were taken at a magnification of 20,000. Mitochondria were classified similarly to the work of Hossmann et al. [8]; 0: no calcium deposits, 1: few dust-like deposits, 2: several grains, 3: numerous massive calcium deposits associated with ultrastructural alteration. Mean mitochondrial calcium uptake index of neurons or glial cells was calculated by averaging individual gradings. Differences between groups were tested for statistical significance using the Wilcoxon (Mann–Whitney) test.

Fig. 1a shows the semi-thin section of intact hippocampal CA_1 pyramidal cells from a control gerbil. As early as 6 h of recirculation after common carotid arteries occlusion, a few pyramidal cells revealed 'dark cell type' of degeneration (Fig. 1b). The number and degree of degeneration of cells became more expressed during later periods of postischemic recirculation. In the parietal cortex no histopathological changes were noticed by light microscopic examination.

In control animals, dense precipitates corresponding to calcium-related deposits were observed in every investigated brain region in the myelin lamellae of axons, in synaptic vesicles, in a few perikaryonal lysosomes and in threshold amounts in the mitochondria. No remarkable alteration in the number and localization of precipitates in the myelin lamellae, synaptic vesicles and lysosomes were noticed after recovery from the ischemic insult. However, numerous dust-like calcium precipitates

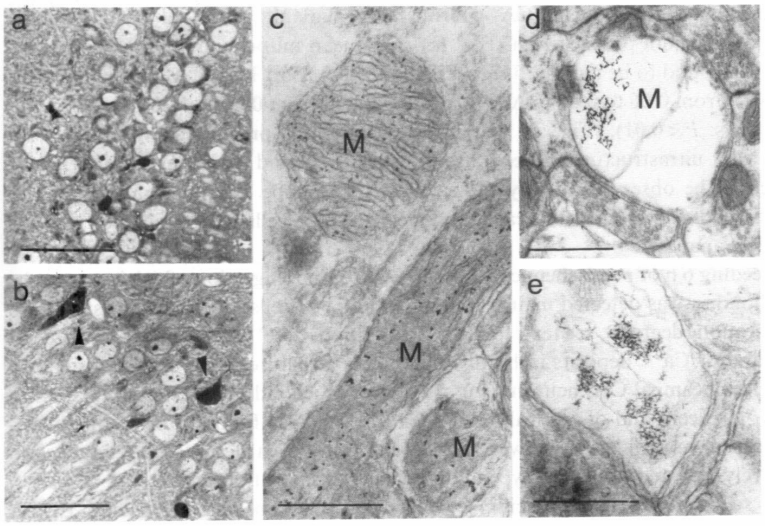

Fig. 1. a and b: semi-thin sections of hippocampal CA_1 subfield in gerbil (Bar = 50 μm). a: control. b: 5 min bilateral carotid occlusion followed by 6 h recirculation. The arrowheads point at the 'dark' cells. c–e: electron cytochemical localization of calcium after 5 min carotid occlusion (M: mitochondrion). Bar = 0.5 μm. c: after 15 min recirculation in the parietal cortex, mitochondrial calcium uptake index: 1. d: calcium accumulation in a degenerated mitochondrion in the CA_1 subfield of hippocampus after 6 h recirculation, calcium uptake index: 3. e: calcium-containing precipitates in hardly identifiable structures after 24 h recirculation in the hippocampal CA_1 region.

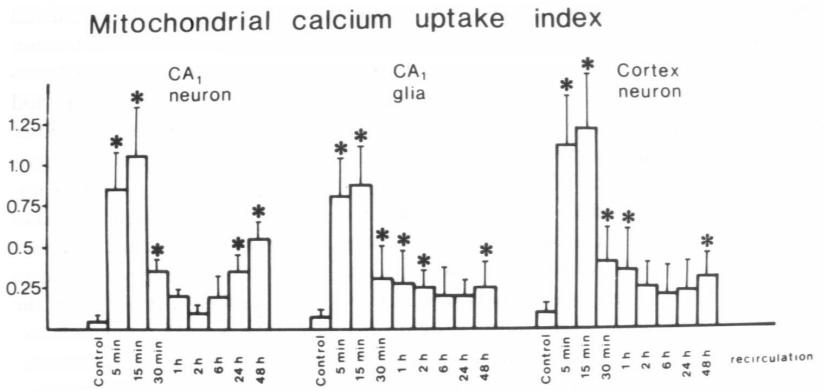

Fig. 2. Cytochemical evaluation of calcium uptake in the CA_1 subfield of hippocampus (pyramidal and glial cells) and in the parietal cortex (pyramidal cells). Mean $\pm$ S.D. Significant differences from the control values ($*P < 0.01$, confidence level 95%) were found in all investigated cells in the early phase with a maximum of 15 min and in the later phase of recirculation (48 h). The second increase in mitochondrial uptake of calcium was mostly expressed in the CA_1 pyramidal neurons.

appeared in mitochondria of CA_1 neurons, CA_1 glia and in cortical pyramidal cells 5 min and 15 min postischemia (Fig. 1c). The mean mitochondrial calcium uptake index was found to be increased significantly from 0.05 to 1.05 (CA_1 pyramidal cells: $P<0.01$), from 0.06 to 0.85 (CA_1 glia: $P<0.01$) and from 0.09 to 1.20 (cortical pyramidal cells: $P<0.01$), (Fig. 2). These dust-like calcium precipitates were not associated with ultrastructural degenerations. After 6, 24 and 48 h, degenerated 'dark' cells could be observed also by the electron microscope examination with hardly identifiable fine structure and calcium deposits. These cells were excluded from the statistical analysis.

Exceeding 6 h of postischemic recirculation a second migration of calcium into the mitochondria was detected mainly in mitochondria of cells revealing ultrastructural degeneration (degree 3, see text; Fig. 1d). In addition, 'free' cytoplasmic Ca^{2+} precipitates and/or Ca^{2+} deposits in hardly identifiable structures were also seen in some of the hippocampal CA_1 neurons (Fig. 1e). The mitochondrial calcium uptake index of CA_1 glial cells and of cortical pyramidal cells was also found to be distinctly elevated above control levels. However, 'free' and not ultrastructurally bound calcium was rarely seen and 'dark' cell type of degeneration was not observed.

The specificity of tissue calcium cytochemical reaction was tested with EGTA treatment after which dense precipitates could not be detected, in contrast to those tissue blocks which were incubated with distilled water only. X-ray microanalysis in respective preparations confirmed that optically dense precipitates were associated with calcium and antimony.

Since the Mongolian gerbils lack a connection between the carotid and vertebrobasilar arterial circulation at the age of 5 weeks or more, cerebral ischemia could be easily produced experimentally by occluding the common carotid arteries [14]. The selective vulnerability of hippocampal CA_1 pyramidal neurons has been described and characterized morphologically after 5 min bilateral occlusion of the carotid arteries followed by recirculation [9–11]. In our present investigation the localization of calcium in the hippocampal and cortical regions of control gerbils was similar to that found in rats by others using the same method [5]. In the recirculation period after 5 min bilateral carotid occlusion calcium entry into mitochondria occurred in two different phases. Shortly after the release of occlusion calcium was found to massively enter mitochondria of neurons and glial cells in the CA_1 region of hippocampus but also mitochondria of the cortical neurons. At that time the ultrastructure of mitochondria was preserved. The fine mitochondrial precipitates disappeared after 2 h recirculation with exception of those which were localized in ischemic damaged 'dark' cells. This finding corresponds well with the results of Simon et al. [21] in the rat, who also found an early and persistent calcium uptake with the same method in vulnerable hippocampal CA_1 and CA_3 neurons and in degenerated 'dark' cells. After recovery of cellular energy metabolism, Ca^{2+} is sequestered by mitochondria which represents an active process. As obvious, the initial cytosolic Ca^{2+} overload occurring during ischemic depolarization of cells is reversible in both ischemic vulnerable and resistant neurons.

The transient disturbance of cellular Ca^{2+} homeostasis, however, may impair or

damage the capacity of metabolic compartments. Interestingly, the early migration of calcium into mitochondria correlates with the accumulation of cyclooxygenase products in brain measured in the same animal model [4]. After 2 h of postischemic recirculation, cyclooxygenase activity returns to normal in parallel with recovery from intracellular Ca^{2+} overload. Following ischemic brain injury, disturbed Ca^{2+} homeostasis was suggested to affect cerebral phospholipid and polyunsaturated fatty acid metabolism of the brain [23]. It remains uncertain whether such Ca^{2+}-related metabolic disturbances can be made responsible for the onset of neuronal damage.

Calcium accumulation in mitochondria of hippocampal CA_1 neurons two days after postischemic recirculation was found to be associated with damage of mitochondrial ultrastructure and signs of cellular degeneration. This suggest that the second rise of cellular calcium occurs in parallel with the loss of cellular integrity.

It has been postulated that calcium with phosphate and electron transport are together necessary for ischemic injury in the brain [12]. In our experiment, the mitochondrial calcium uptake of selectively vulnerable neurons did not differ from that of glial cells or cortical neurons. This finding agrees well with the result of Hossmann et al. [8] observed in cat, suggesting that calcium alone may not be sufficient in determining the final outcome of the ischemic pathological alterations.

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