

Ischemia-related alteration of GABA_A-operated chloride channel properties in gerbil hippocampus and cerebral cortex

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Abstract. The properties of GABA-gated chloride (Cl⁻) channels in ischemia-reperfusion injury were studied by determination of the binding and dissociation kinetics of a specific Cl⁻ channel ligand, tert-butylbicyclophosphoro[³⁵S]thionate (TBPS) and by determination of ³⁶Cl⁻ uptake in the presence of the GABA_A receptor agonist, muscimol. Four days after ischemia a small but insignificant decrease of [³⁵S]TBPS binding to synaptic plasma membranes (SPM) was observed in the hippocampus and cerebral cortex as compared to control. The effect of ischemia was larger and statistically significant after the first and second month of reperfusion, constituting 20% inhibition of [³⁵S]TBPS binding to SPM of sham-operated gerbils. On the other hand, the half-life of fast phase [³⁵S]TBPS dissociation four days after ischemia was markedly diminished by about 40%-50% as compared to its control value and persisted during the first and second month of reperfusion in the hippocampal SPM. A similar but less potent reduction of the half-life of the fast phase of [³⁵S]TBPS dissociation (about 30% *versus* control) appeared one and two months after ischemia in cerebral cortex SPM. One month after ischemia muscimol-stimulated ³⁶Cl⁻ uptake into cerebral cortex synaptoneurosome was lowered as compared with control uptake, but remained statistically insignificant in the whole range of muscimol concentrations tested. Our results indicated that ischemia-reperfusion injury significantly decreases opening time of GABA_A receptor-gated Cl⁻ channels in the hippocampus and cerebral cortex, which may lower the hyperpolarization ability of this receptor complex leading to an imbalance between excitatory and inhibitory neurotransmitter pathways in these brain areas, and in consequence to neuronal dysfunction or degeneration.

Key words: GABA_A receptor, chloride channel, ischemia, gerbil, hippocampus, cerebral cortex

INTRODUCTION

Transient cerebral ischemia results in selective neuronal degeneration in different regions of the brain. Recent evidence suggests that this form of degeneration is, at least in part, induced by excitatory amino acids that stimulate N-methyl-D-aspartate (NMDA) receptors leading to an increase of intracellular calcium level, which is believed to be one of the most important factors in development of cell death (Pellergrini-Giampietro et al. 1990, Ferkany and Enna 1992, Siesjö 1993, Siesjö 1995, Łazarewicz et al. 1995). During the last decade much interest has been concentrated on the subject of excitotoxicity and brain ischemia (Choi 1988, Tecoma and Choi 1989, Weiss and Choi 1991, Moncada et al. 1992). Relatively less attention has been given to inhibitory neurotransmitters.

One of the major inhibitory systems in the brain, which prevents intracellular build up of calcium, consists of GABAergic interneurons. Activation of the GABA_A receptor enhances the influx of chloride ions into the cell, causing membrane hyperpolarization and reduction of cell excitability. The balance between the actions of excitatory and inhibitory neurotransmitters is very important for brain function, including learning and memory processes (Johansen and Diemer 1991).

The GABA_A receptor is a heterooligomeric protein composed of several distinct polypeptide types (α , β , γ , δ , and ρ). These subunits are arranged in pentameric structure and form an ion channel pore (Olsen and Tobin 1990, Schousboe and Redburn 1995, Smith and Olsen 1995, Stephenson 1995). There are multiple GABA_A receptor subtypes, which possess different subunit composition (Angleotti and Macdonald 1993). The receptor subunits contain multiple sites for phosphorylation by different protein kinases (Leidenheimer et al. 1991). Decrease in GABA_A receptor function may also play a role in hyperexcitability states, such as epilepsy and anxiety disorders. The GABA_A receptor, besides having binding sites for many therapeutically potent drugs, has a specific recognition site for convulsants, such as picrotoxin and tert-butylbicyclopheosphorothionate (TBPS). TBPS is a specific ligand of the GABA_A-operated chloride (Cl⁻) channel and is very useful for the study of Cl⁻ channels properties. Miles et al. (1992) have used [³⁵S]TBPS to study its binding to GABA_A receptor quantified by autoradiography and showed that four days and one month after ischemia in gerbils [³⁵S]TBPS binding decreases in the striatum and hippocampus. This study suggests that a lowering of GABAergic neurotransmission may con-

tribute to development of neuronal degeneration following cerebral ischemia. Decreasing inhibitory activity during and after ischemia may result in overstimulation of neurons, especially in CA₁ layer of the hippocampus. Li et al. (1993) observed a rapid decline of GABA_A receptor α -1 and β -2 subunits mRNA expression in the hippocampus following transient cerebral ischemia. However, it was not clear if these changes lead to a functional defect in GABA_A receptors. Unlike excitatory neurotransmission, the role of inhibitory neurotransmission in ischemia-evoked neuronal degeneration and death has not been fully characterized during different reperfusion periods after global cerebral ischemia. The study of Li et al. (1993) indicated that a portion of GABA_A receptors in CA₁ layer of the hippocampus, and in other areas that are sensitive to ischemic insult, is associated with degenerating neurons, whereas the other GABA_A receptors are spared.

Up to now the characterization of [³⁵S]TBPS association-dissociation kinetics at different times after transient cerebral ischemia has not been reported. The kinetic parameters of the fast and slow phase of [³⁵S]TBPS dissociation, i.e., open and closed state of chloride channel, define the channel properties. The aim of this study was to investigate the effect of ischemia and the different times of reperfusion after its induction on GABA_A receptor-operated chloride channel properties and function in gerbil brain cortex and hippocampus.

METHODS

Material

Male mongolian gerbils 60-70 g b.w. were supplied from Animal Farm, Lomna, Poland. Tert-butylbicyclopheosphorothionate [³⁵S]thionate ([³⁵S]TBPS; sp.act. 56.4 Ci/mmol) was purchased from New England Nuclear, Germany, and Na[³⁶Cl] (sp.act. 7.8 Ci/mmol) was ordered from Radioisotope Centre, Swierk, Poland. All other reagents, such as Tris, EDTA, HEPES, sucrose, muscimol and picrotoxin were obtained from Sigma Co., St. Louis, MO, USA.

Induction of global cerebral ischemia

Male mongolian gerbils were subjected to bilateral carotid artery occlusion for 5 min under 4% halothane anesthesia, which was subsequently reduced to 2% during the operation. The gerbils were placed on a heating pad and their body temperature was maintained at 37°C.

Both common carotid arteries were exposed through a midline cervical incision and simultaneously occluded with Heifetz clips for 5 min. The animals were allowed to survive for four days, one month or two months after ischemia. They were then decapitated and the brains were quickly removed. Both brain cortex and the hippocampus were isolated on ice. Sham-operated gerbils served as control. The protocol of experimental ischemia in gerbils was approved by the Medical Research Centre Ethical Committee, Warsaw, Poland.

Preparation of synaptic plasma membranes (SPM)

Brain cortex and hippocampal tissues were homogenized in a Dounce-type glass homogenizer in an ice-cold isolation medium (0.32 M sucrose, 20 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA). Homogenates (10%, w/v) were centrifuged at $1,100 \times g$ for 3 min. The resulting supernatant was subsequently centrifuged at $17,000 \times g$ for 10 min to yield a crude synaptosomal fraction (P2). This fraction was lysed in each case in 5 volumes of 1 mM Tris-HCl buffer (pH 7.0) by homogenization and was allowed to stand for 30 min at 4°C . Then it was centrifuged at $10,000 \times g$ for 10 min. The supernatant and light layer pellet were collected into separate centrifuge tubes and the remaining pellet was lysed again and centrifuged at $10,000 \times g$ for 10 min. Both supernatants were combined and centrifuged at $48,000 \times g$ for 20 min. The final supernatant was discarded and the pellet (SPM) was resuspended in 0.05 M Tris-HCl (pH 7.4) and frozen. On the day of the assay it was thawed, centrifuged at $48,000 \times g$ for 20 min and used for TBPS association-dissociation experiments. In each case protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

Preparation of synaptoneurosomes

The synaptoneurosomes were prepared from cerebral cortex using a calcium-free Krebs-Henseleit buffer (pH 7.4) according to the method of Hollingsworth et al. (1985), as described by Strosznajder and Samochocki (1991). Cerebral hemispheres were dissected, slices of gray matter were cut manually on an ice-cold Petri dish with a cooled razor blade and were subsequently homogenized by hand (five strokes) in 7 ml of Krebs-Henseleit buffer. The homogenate was diluted with 28 ml of buffer and centrifuged at $1,100 \times g$ for 15 min. Synaptoneurosomal

pellet was resuspended in 5 ml of Krebs-Henseleit buffer, equilibrated at 37°C for 30 min and then used for Cl^- uptake experiments.

[^{35}S]TBPS binding to brain cortex and hippocampal SPM

The incubation mixture consisted of 0.4 mg of SPM protein, 3 nM [^{35}S]TBPS, 5 mM Tris-HCl buffer pH 7.4, containing 200 mM KBr, in a final volume of 1 ml. This mixture was incubated for 100 min at 22°C in the absence or presence of 100 nM muscimol. The reaction was stopped by addition of 50 μM picrotoxin and then 0.5 ml aliquots were filtered in duplicate on Whatman GF/C filters and washed with 10 ml of buffer. Radioactivity of [^{35}S]TBPS on the filters was measured in dioxan-based scintillation mixture (Bray's fluid) with an efficiency of about 85%.

[^{35}S]TBPS dissociation kinetics in cerebral cortex and hippocampal SPM

The effect of agonist on TBPS dissociation was studied according to the method described earlier (Samochocki and Strosznajder 1994). In brief, the SPM suspension (0.6 mg protein/ml) was incubated with 3 nM [^{35}S]TBPS in 5 mM Tris-HCl buffer pH 7.4, containing 200 mM KBr, for 100 min at 22°C in the absence or presence of 100 nM muscimol in a final volume of 10 ml. Dissociation of bound [^{35}S]TBPS was initiated by addition of 50 μM picrotoxin, then 0.5 ml aliquots were filtered in duplicate at 10-min intervals on Whatman GF/C filters and washed with 10 ml of buffer. Radioactivity of [^{35}S]TBPS on the filters was measured in Bray's fluid. The Bt/Bo ratio (specific [^{35}S]TBPS binding at time t related to the beginning of dissociation at $t = 0$) was plotted against time. The half-lives of fast and slow phase of [^{35}S]TBPS dissociation and their percentage contribution were evaluated by the method of nonlinear regression, using IBM PC Graph-Pad InPlot software (version 3.01, copyright 1985-1989 by H. Motulsky), according to the following biphasic exponential decay equation: $Y = A e^{-(B \cdot X)} + C e^{-(D \cdot X)}$, where A and C are the percentage contributions of the fast and slow phase of [^{35}S]TBPS dissociation respectively, and B and D are the corresponding half-lives of these phases. The significance of the differences of two dissociation plots was determined by a pairwise comparison of the data using Student's t -test.

Muscimol-evoked $^{36}\text{Cl}^-$ uptake into brain cortex synaptoneurosomes

Muscimol-evoked $^{36}\text{Cl}^-$ uptake was measured according to the procedure described by Miles et al. (1992). The synaptoneurosomal fraction (6–8 mg protein/ml) was resuspended in a Krebs-Henseleit buffer, containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 10 mM HEPES and equilibrated to pH 7.4 with Tris base. Approximately 1 mg of synaptoneurosomal protein was incubated for 20 min at 30°C. Reaction was initiated by simultaneous addition of 0.2 μCi of Na^{36}Cl and muscimol (in a concentration varied from 1 to 100 μM) to the incubation tube. Uptake was stopped 5 s later by dilution of the samples with 10 ml of ice-cold Krebs-Henseleit buffer, containing 50 μM picrotoxin, and then samples were filtered through Whatman GF/C filters and washed with 10 ml of buffer. Radioactivity of ^{36}Cl on the filters was measured in 8 ml of Bray's fluid. The significance of the differences of muscimol-stimulated Cl^- uptake into control and ischemic synaptoneurosomes was evaluated by Student's *t*-test.

TABLE I

Effect of ischemia-reperfusion injury on muscimol-dependent [^{35}S]TBPS binding to hippocampal and cerebral cortex synaptic plasma membranes

Time of reperfusion	Muscimol-dependent [^{35}S]TBPS binding (% of control)	
	Hippocampus	Cerebral cortex
Four days	76.7 \pm 23.85	93.2 \pm 18.82
One month	83.7 \pm 8.32*	89.2 \pm 7.50*
Two months	79.5 \pm 3.33*	80.5 \pm 7.78*

The [^{35}S]TBPS binding was carried out for 100 min at 22°C in the presence of 100 nM muscimol. Synaptic plasma membranes (SPM) were isolated from the brains of sham-operated gerbils (control) and gerbils subjected to 5 min of global cerebral ischemia following four days, one month or two months reperfusion period. Results are expressed as percent of [^{35}S]TBPS binding in control SPM. The control values from typical experiment were as follows: 1,255 \pm 216 fmol/mg protein and 1,088 \pm 277 fmol/mg protein in cerebral cortex and hippocampal SPM, respectively. Data are means \pm SD derived from 16 animals. * $P < 0.05$ as compared to controls.

RESULTS

Effect of ischemia-reperfusion on [^{35}S]TBPS binding to cerebral cortex and hippocampal SPM

The specific binding of [^{35}S]TBPS was investigated in the presence of 100 nM muscimol in SPM isolated from brain cortex and hippocampus four days, one month or two months after 5 min of global cerebral ischemia. On the fourth day of reperfusion no statistically significant changes of [^{35}S]TBPS binding to SPM from either cerebral cortex or hippocampus was observed as compared with [^{35}S]TBPS binding to SPM isolated from these structures of sham-operated gerbils (control). One month after ischemic insult, however, a significant decrease of [^{35}S]TBPS binding to SPM from these brain areas was seen. This alteration of [^{35}S]TBPS binding persisted over a period of two months of reperfusion. Two months after ischemia a similar, about 20% decrease of [^{35}S]TBPS binding to cerebral cortex and hippocampal SPM was found as compared to respective controls (Table I).

Effect of ischemia-reperfusion on [^{35}S]TBPS dissociation kinetics in cerebral cortex and hippocampal SPM

Ischemia-reperfusion injury induced reduction of the half-life of fast phase of muscimol-dependent [^{35}S]TBPS dissociation in cerebral cortex and hippocampal SPM. A visible change was initially detected in the hippocampus four days after ischemia. At this time the half-life of fast phase of [^{35}S]TBPS dissociation was shortened by about 40% as compared with that observed in SPM from the hippocampus of sham-operated gerbils (control, 7.2 \pm 1.88 min). Reduced half-life of fast phase of [^{35}S]TBPS dissociation in the hippocampal SPM was maintained during further two months of reperfusion (Table II). Interestingly, the same alteration evoked by ischemia-reperfusion injury of the half-life of fast phase of muscimol-dependent [^{35}S]TBPS dissociation was found in cerebral cortex SPM. However, a significant effect of ischemia was detected one month later than in the hippocampal SPM and persisted over the next month. There was a 20% reduction of the half-life of fast phase of [^{35}S]TBPS dissociation in cerebral cortex SPM one and two months after ischemia as compared to control, 5.2 \pm 0.55 min (Table II). Moreover, a significant reduction of the half-life of fast phase of [^{35}S]TBPS dissociation one and two months after ischemia was observed in cerebral

cortex SPM in the absence of muscimol as compared to appropriate control, by about 25% and 50%, respectively (data not shown).

Effect of ischemia-reperfusion on muscimol-evoked [³⁶Cl⁻] uptake into cerebral cortex synaptoneurosomes

At 1-100 μ M muscimol concentration, there was apparent decrease of muscimol-stimulated Cl⁻ uptake into cerebral cortex synaptoneurosomes subjected to ischemia following one month reperfusion as compared to its value found in synaptoneurosomes from cerebral cortex of sham-operated gerbils (control). However, the evaluated effect of ischemia was not statistically significant in respect to control as determined by Students *t*-test. The values of basal (muscimol-independent) Cl⁻ uptake were also not significantly different (0.3 ± 0.08 nmol/mg protein/min and 0.3 ± 0.06 nmol/mg protein/min in control and ischemic synaptoneurosomes, respectively). It was further observed that muscimol, when applied at 100 μ M

concentration, exerted less stimulatory effect on basal Cl⁻ uptake than its lower concentrations in both control and ischemic synaptoneurosomes (Fig. 1).

DISCUSSION

Our study indicates for the first time that 5 min of global cerebral ischemia induces significant and progressive alteration of GABA_A receptor-operated Cl⁻ channel properties. The half-life of the fast phase of [³⁵S]TBPS dissociation, which corresponds to the opening time of the Cl⁻ channel, is significantly decreased in hippocampal SPM four days after ischemic insult. This change is consolidated up to two months of reperfusion. The same alteration takes place in brain cortex, however, it appears more significant after one month of reperfusion. There is an apparent link between ischemia-reperfusion-induced changes of the half-life of fast phase of muscimol-stimulated [³⁵S]TBPS dissociation and this agonist-dependent [³⁵S]TBPS binding to both hippocampal and cerebral cortex SPM. The reduction of muscimol-activated ³⁶Cl⁻ uptake into cortical synaptoneurosomes, although not statistically significant, suggests ischemia-induced alteration of GABA_A receptor-stimulated Cl⁻ uptake. It may result from the changes of Cl⁻ channel properties and therefore may be responsible for the dysfunction of GABA_A receptor-operated Cl⁻ channels.

It is now accepted that TBPS binding correlates well with the number of Cl⁻ channels in the "open" state and this fact was considered for determination of this receptor function (Maksay and Simonyi 1986, Erdö and Wolff 1989). Thus, the quantity of TBPS binding sites is proportional to the number of Cl⁻ channels in the "open" state. Moreover, fast and slow kinetic phases of TBPS dissociation are related to the state of the Cl⁻ channel, that is "open" and "closed", respectively (Maksay and Simonyi 1986). A previous study of Miles et al. (1992) showed that there is no change in muscimol-stimulated Cl⁻ uptake and in [³⁵S]TBPS binding to brain cortex membranes during a one month reperfusion period. Using autoradiography they were not able to evaluate the properties of Cl⁻ channels. In contrast, our data suggest that ischemia alters GABA_A receptor-operated Cl⁻ channel properties and probably also their function. On the other hand, Li et al. (1993) found that GABA_A receptor α -1 and β -2 subunits mRNA expression was suppressed before degeneration of CA₁ pyramidal cells. Nevertheless, there is no information regarding whether the loss of GABA_A receptor subunit mRNA expression

TABLE II

Effect of ischemia-reperfusion injury on the half-life of the fast phase of muscimol-stimulated [³⁵S]TBPS dissociation in hippocampal and cerebral cortex synaptic plasma membranes

Time of reperfusion	Half-life of fast phase of muscimol-stimulated [³⁵ S]TBPS dissociation (% of control)	
	Hippocampus	Cerebral cortex
Four days	$66.7 \pm 18.62^*$	88.5 ± 18.12
One month	$69.4 \pm 4.65^*$	$80.8 \pm 8.55^*$
Two months	$44.4 \pm 10.48^*$	$86.5 \pm 7.30^*$

Dissociation of [³⁵S]TBPS was induced by 50 μ M picrotoxin and carried out in 10 min intervals at 22°C in the presence of 100 nM muscimol. Synaptic plasma membranes (SPM) were isolated from the brains of sham-operated gerbils (control) and gerbils subjected to 5 min of global cerebral ischemia following four days, one month or two months reperfusion period. Results are expressed as percent of the half-life of the fast phase of [³⁵S]TBPS dissociation in control SPM. The control values from typical experiment were as follows: 7.2 ± 1.88 min and 5.2 ± 0.55 min in hippocampal and cerebral cortex SPM, respectively. Data are means \pm SD derived from 16 animals. **P*<0.05 as compared to controls.

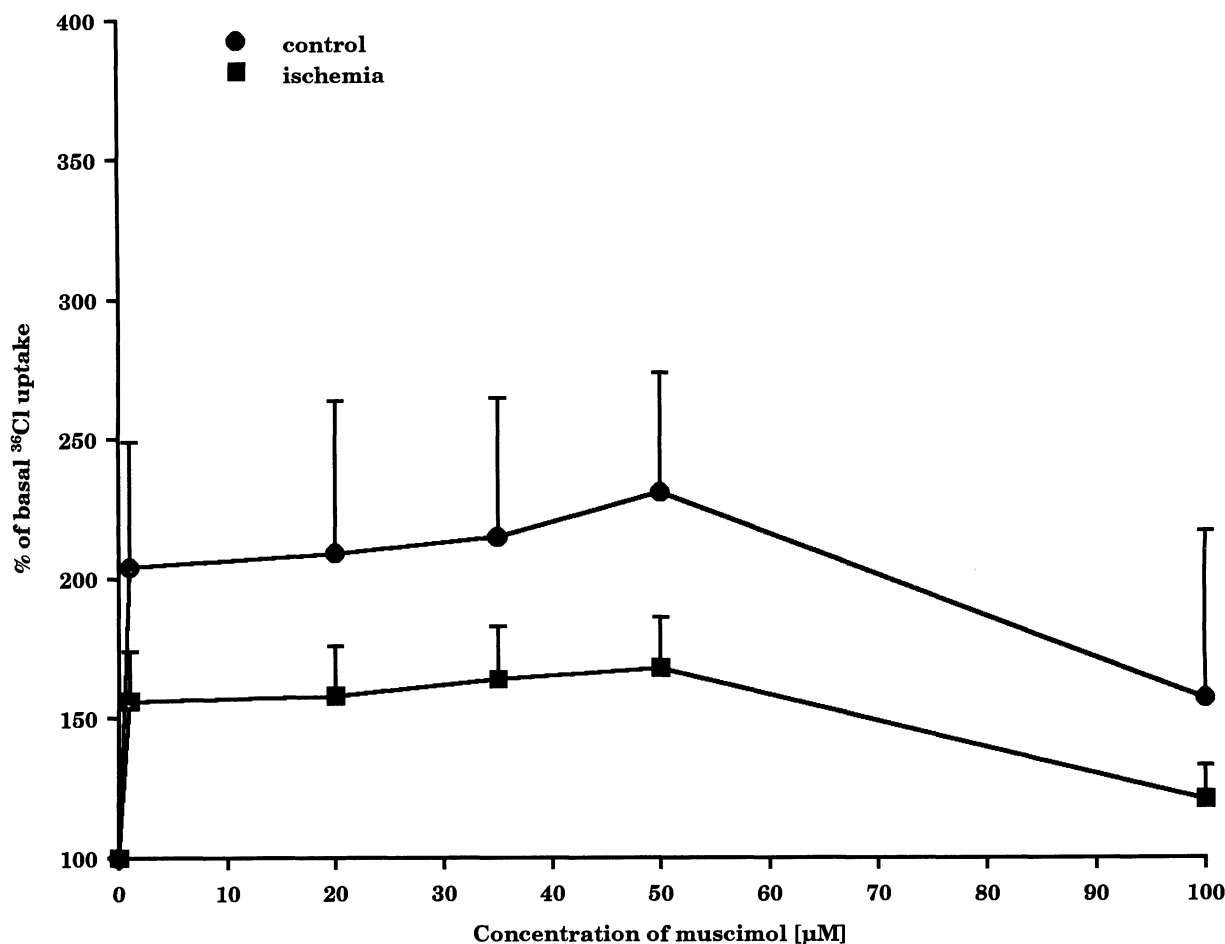


Fig. 1. Effect of one month ischemia-reperfusion injury on muscimol-stimulated chloride $^{36}\text{Cl}^-$ uptake into cerebral cortex synaptoneurosomes. The uptake was conducted for five seconds at 30°C using $0.2 \mu\text{Ci } ^{36}\text{Cl}^-$ and 1.5 mg protein in the presence of muscimol in a concentration range of $1 \mu\text{M}$ - $100 \mu\text{M}$. Synaptoneurosomes were isolated from the brains of sham-operated gerbils (control) or gerbils subjected to 5 min of global cerebral ischemia following one month reperfusion (ischemia). Results are expressed as a percent of corresponding basal, muscimol-independent, $^{36}\text{Cl}^-$ uptake (control- $0.3 \pm 0.08 \text{ nmol/mg protein/min}$, ischemia- $0.3 \pm 0.06 \text{ nmol/mg protein/min}$). Data are means \pm SD derived from 16 animals.

leads to a decrease of GABA_A receptor function and to further progression of neuronal degeneration. It is well known that pyramidal neurons in the CA₁ region of the hippocampus are vulnerable to cerebral ischemia that leads to biochemical and morphological alterations. Disaggregation of polyribosomes associated with suppression of polypeptide or impairment of protein synthesis were described (Morimoto and Yamagihara 1981, Kirino 1982). Moreover, Maruno and Yamagihara (1990) found a progressive decline of mRNA in the CA₁ region of the hippocampus prior to evolution of morphologic damage. This could be a result of suppression of mRNA synthesis in neuronal nuclei, or its accelerated degradation in the cytoplasm by ribonucleases, or both.

Up to now, little has been known about biochemical or functional changes in brain cortex appearing at different times of reperfusion. Despite the lack of morphological signs of neuronal degeneration in brain cortex and in contrast to the neuronal death occurring in CA₁ layer of the hippocampus after ischemic insult, our results demonstrate alteration of GABA_A receptor-operated Cl^- channel properties in brain cortex up to two months of reperfusion. There are some findings that support the hypothesis that the lowering of GABAergic neurotransmission may contribute in delayed neuronal death occurring after ischemic insult. A decrease of inhibitory action of GABAergic system can cause impairment of existing pathways diminishing excitatory activity in the brain. Johansen and Diemer (1991) found that increasing

postischemic GABAergic neurotransmission during the first two/three days after ischemic insult may reduce ischemic damage of CA₁ layer of the hippocampus, through a continuous increase of extracellular levels of GABA using GABA uptake inhibitor (No-328) or through an enhancement of GABAergic neurotransmission by diazepam. A growing body of evidence suggests that in gerbils GABAergic agents protect against delayed death of CA₁ hippocampal neurons elicited by ischemia (Sternau et al. 1989, Lyden and Hedges 1992, Shuaib et al. 1996). The hyperpolarization of neuronal membranes induced by muscimol may depress the voltage- and NMDA-evoked influx of calcium ions as a consequence of excitatory amino acid action. Muscimol, through GABA_A receptor activation, displays a protective effect against NMDA-induced neuronal injury (Ohkuma et al. 1994). Our results indicate, however, that ischemia disturbs the properties and function of GABAergic system too. Thus, it seems that an enhancement of activity of this neurotransmitter system during ischemia may not protect the brain against this injury.

It is known that, apart from accumulation and overactivity of excitatory amino acids, the other unfavourable processes occur during ischemia and reperfusion, for example liberation of arachidonic acid, decrease of cellular pH, generation of oxygen radicals, and lipid peroxidation (White et al. 1993). Our previous studies indicated that arachidonic acid modifies GABA_A receptor-Cl⁻ channel complex. Arachidonic acid reduces TBPS binding and exerts inhibitory action on GABA-stimulated Cl⁻ uptake (Samochocki and Strosznajder 1993) and modifies Cl⁻ channel properties (Samochocki and Strosznajder 1994). These results suggest that arachidonic acid is important in modulation of the GABAergic system during ischemia.

It has been proposed that oxidation of proteins could lead to inactivation of enzymes or modification of ligand-gated channel receptors, such as the NMDA receptor (Hahn 1988). Hall et al. (1995) demonstrated that free radicals, generated in ischemia-reperfusion injury, react with and damage synaptosomal membrane proteins and lipids. Recent evidence suggests that alterations in membrane phospholipids and proteins might induce changes of GABA_A receptor-gated Cl⁻ channels (Schwartz et al. 1988). Our unpublished data also show significant changes of TBPS dissociation due to lipid-protein peroxidation and to lowering of cellular pH.

Summarizing, our results indicate that ischemia-reperfusion injury induces alteration of Cl⁻ channel properties

by decreasing its opening time, which subsequently may affect the capability of the GABA_A receptor to promote membrane hyperpolarization. The dysfunction of the GABAergic inhibitory system may produce the imbalance between inhibitory and excitatory systems in ischemic brain.

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