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Fe^{2+}-induced inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase: Effect of stobadine, glutathione and combination of both antioxidants

Peter Račay, Ahmed W.A. Qteishat, Hussam M. ElKambergy, Viera Mézešová, Ján Lehotský *

Department of Biochemistry, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, SK-036 01 Martin, Slovak Republic

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Abstract

The incubation of the gerbil forebrain microsomes in the presence of ferrous sulphate and EDTA for either 30 min or for 60 min at a temperature of 37°C led to the inhibition of Ca^{2+}-ATPase in both a concentration- and time-dependent manner. The concentrations of Fe^{2+} which led to the inhibition of 50% of the Ca^{2+}-ATPase activity (IC_{50}-value) at these times were 0.59 mM and 0.07 mM, respectively. The preincubation of microsomes with 0.1 mM of stobadine prevented the inhibition of Ca^{2+}-ATPase, however, the effectivity of prevention was dependent on the Fe^{2+} concentration. The net effect of stobadine was an increase in IC_{50}-value to 0.76 mM. Unlike stobadine, reduced glutathione is a naturally occurring water soluble antioxidant. Glutathione at the concentration of 0.1 mM had no significant protective effect on the inhibition of Ca^{2+}-ATPase. The protective effect of a stobadine–glutathione mixture was also investigated; 0.1 mM of stobadine in combination with 0.1 mM of glutathione was more potent in prevention of Fe^{2+}-induced inhibition of Ca^{2+}-ATPase than stobadine alone (IC_{50} = 1.31 mM). In addition, we have investigated the effect of various stobadine-glutathione molar ratios (the total concentration of both antioxidants being 0.2 mM) on Fe^{2+}-induced inhibition of Ca^{2+}-ATPase. The results indicated that the best stobadine-glutathione ratio was close to 1 : 1. The effect of 0.04 mM stobadine in combination with 0.16 mM glutathione was comparable to the effect of 0.2 mM of stobadine alone, whereas 0.2 mM glutathione was almost ineffective. These results may suggest a possible role of membrane in Fe^{2+}-induced inhibition of Ca^{2+}-ATPase. © 1998 Elsevier Science B.V.

Keywords: Brain; Endoplasmic reticulum; Ca^{2+}-ATPase; Oxidative stress; Lipid peroxidation; Free radical

1. Introduction

Cytoplasmic Ca^{2+} acts as a ubiquitous messenger controlling many different aspects of neuronal cell functioning [1]. Its homeostasis is precisely controlled by various systems including Ca^{2+}-ATPase, which is localized on the endoplasmic reticulum membrane [2,3]. This ATPase is responsible for Ca^{2+} sequestration, leading (together with extrusion of Ca^{2+} to extracellular space) to the decrease of intracellular Ca^{2+} concentration to the resting level. Although iron is a remarkably useful metal in nature, its cytotoxicity is mediated by the potency of iron to induce free radicals and oxidative stress. In the central nervous system, free radicals and oxidative stress are thought to be involved in the pathophysiology of

* Correspondence address. Fax: +421 842 367 70.

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some neurodegenerative diseases such as Parkinson disease [4,5], amyotrophic lateral sclerosis [5], Alzheimer disease [4,6] as well as brain trauma [7], ischemia-reperfusion injury [8,9] and subarachnoidal haemorrhage [10]. Activities of several membrane spanning proteins, including ionic pumps and receptors, are often modulated by the action of free radicals and oxidative stress [11]. Our previous study [12] provided evidence that the Ca^{2+} accumulation mechanism and Ca^{2+} ATPase activity in mammalian brain microsomes are very sensitive to damage induced by free oxygen radicals generated in-vitro. Since the inhibition of the Ca^{2+}-ATPase was delayed in comparison to the decrease of the Ca^{2+} uptake, we supposed that the primary effect of free oxygen radicals is alteration of the lipid component of the membrane; leading probably, first to an increase of passive membrane permeability, and then to the decrease of Ca^{2+}-ATPase activity. In fact, we have also documented the modification of membrane properties [13] by free oxygen radicals and an increase of passive membrane permeability [14]. Since it has been suggested that oxidation of protein cannot be excluded, the aim of this work was to study in more detail the Fe^{2+}-induced inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase. In addition, the protective effects of stobadine, glutathione, and combination of both antioxidants had also been investigated.

2. Materials and methods

2.1. Chemicals

Stobadine dihydrochloride, (-)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido [4,3-b] indole dihydrochloride, was synthetized by the Štolc et al. method [15] and was provided through the courtesy of Dr. S. Štolc (Institute of Experimental Pharmacology, Slovak Academy of Sciences Bratislava). All other chemicals were of research grade.

2.2. Microsome preparation

The brains of adult gerbils were removed after decapitation and the cerebrum separated from the cerebellum and mesencephalon. The microsomal fraction was isolated from the cerebrum according to Edelman et al. [16] with slight modifications. Briefly, brain tissue was minced into small pieces and homogenized in 10 vol. of cold buffer A (0.32 M sucrose, 5 mM Hepes, 0.1 mM phenylmethanesulphonyl fluoride, pH = 7.4) with a wide-clearance Teflon Potter–Elvehjem homogenizer. The homogenate was centrifuged at $900 \times g$ for 10 min. The pellet was re-homogenized in five volumes of ice cold buffer A and centrifuged at $900 \times g$ for 10 min. The supernatants were collected and the crude mitochondrial fraction was removed by centrifugation at $17\,000 \times g$ for 45 min. The microsomal pellet, which was obtained by centrifugation of post-mitochondrial supernatant at $100\,000 \times g$ for 60 min, was suspended in 10 ml of ice cold buffer A and centrifuged at $100\,000 \times g$ for 60 min. All steps were carried out at 4°C. The final pellet was suspended in 0.5 ml of ice cold buffer A to give a membrane protein concentration of ≈ 3 mg/ml and was then stored in liquid nitrogen. Microsome isolated from six independent membrane preparations were used in this study. The protein concentration was determined by the Lowry et al. method [17]. Edelman et al. [16] demonstrated that microsomal fraction isolated by this technique was enriched in endoplasmic reticulum membranes by utilizing a membrane marker enzyme characterization profile.

2.3. Membrane treatment

Isolated microsomes (2 mg/ml) were preincubated with various amounts of freshly prepared equimolar $FeSO_4$-EDTA solution at 37°C for 30 and 60 min. The final concentration of the membrane protein was 1 mg/ml, and the final concentrations of $FeSO_4$-EDTA are indicated in the figures. To study the effect of antioxidants, microsomes were preincubated with antioxidants for 15 min at 0°C prior to the incubation with $FeSO_4$-EDTA. Final concentrations of used antioxidants are indicated in the figure and table legends.

2.4. Ca^{2+}-ATPase measurement

Ca^{2+}-stimulated ATPase activity was determined by the coupled enzyme assay. Microsomes (10 μg per assay) were incubated in a medium containing 20 mM HEPES with pH 7.0, 5 mM $MgCl_2$, 1 mM

EGTA, 5 mM NaN_3, 0.44 mM phosphoenolpyruvate, 0.4 mM NADH, 2 mM ATP, 32.5 μg of $C_{12}E_8$, pyruvate kinase (10 units) and lactate dehydrogenase (22 units) in a total volume of 0.5 ml. ADP production was monitored by measurement of NADH oxidation at 340 nm (Pharmacia LKB Ultrospec III). The Ca^{2+}-stimulated activity was calculated by subtracting the activity, in the absence of Ca^{2+} (basal rate), from that obtained after addition of the appropriate concentration of Ca^{2+} to yield the free Ca^{2+} concentration of 10 μM. Free Ca^{2+} concentration was calculated by Cabuf software, generously provided by Dr. G. Droogmans (K.U. Leuven, Belgium).

2.5. Evaluation of results

The results from experiments measuring the dependence of Ca^{2+}-ATPase activity (v) upon Fe^{2+} concentration ([Fe^{2+}]) were best fitted by the following equation:

$$v = 100/\left\{1 + \left([Fe^{2+}]/IC_{50}\right)^{n}\right\}$$

where IC_{50} is the concentration of Fe^{2+} which led to the inhibition of the Ca^{2+}-ATPase activity to 50% of control and n the exponent, which resembles the Hill coefficient; however, its exact meaning is unknown. The parameters of such a curve were calculated by computer.

The results were presented as mean ± S.E.M. and the Student's *t*-test was used to determine the differences between the means.

3. Results

The incubation of the gerbil forebrain microsomes in the presence of $FeSO_4$-EDTA for either 30 min or for 1 h at a temperature of 37°C led to the inhibition of Ca^{2+}-ATPase in both a concentration- and time-dependent manner (Fig. 1). The calculated IC_{50}-values were 0.59 and 0.07 mM, respectively, of the incubation time. The exponents were 3.4 ± 0.1 and 0.84 ± 0.03, respectively.

Stobadine, with known chain-breaking antioxidant properties [18], and being both membrane and water soluble [19] was included into the membranes to prevent the Fe^{2+}-induced inhibition of Ca^{2+}-ATPase. The preincubation of microsomes with 0.1 mM of stobadine influenced the inhibition of Ca^{2+}-ATPase in the Fe^{2+} concentration-dependent manner (Fig. 2). While 0.1 mM of stobadine conferred protection of

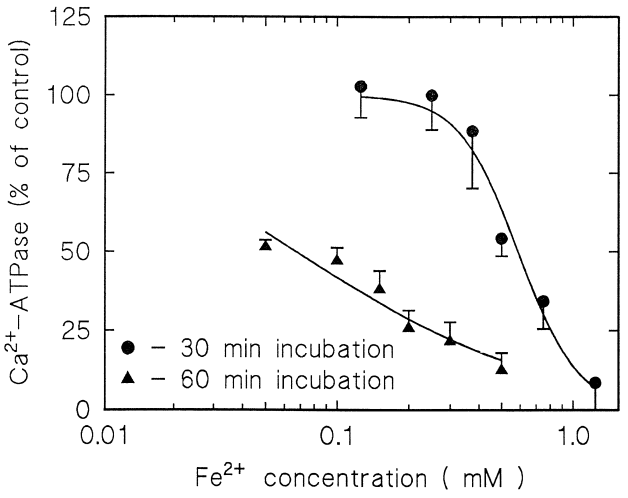

Fig. 1. Concentration-dependent inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase by Fe^{2+}. Microsomal membranes were incubated at 37°C for 30 or 60 min in the presence of varying amounts of Fe^{2+}. Ca^{2+}-ATPase activity is expressed as a percentage of control activity. The results are shown as mean ± S.E.M. of 4 independent measurements.

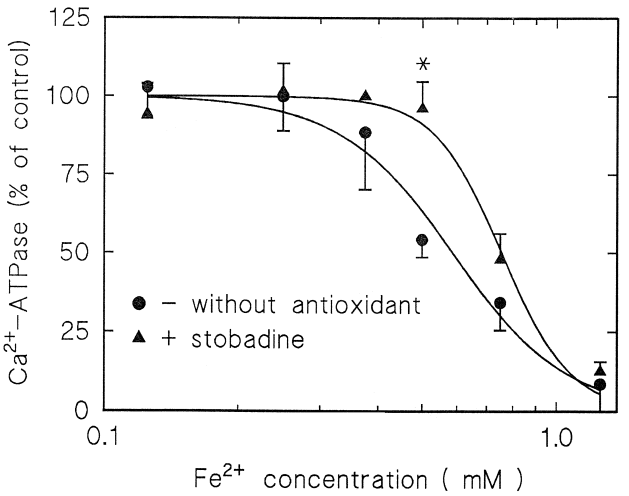

Fig. 2. Effect of stobadine on the inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase by Fe^{2+}. Microsomal membranes were incubated at 37°C for 30 min in the presence of varying concentrations of Fe^{2+} and 0.1 mM stobadine. Ca^{2+}-ATPase activity is expressed as a percentage of control activity. The results are shown as mean ± S.E.M. of four independent measurements. * ($p < 0.01$) significantly different from corresponding parallel incubated without antioxidant.

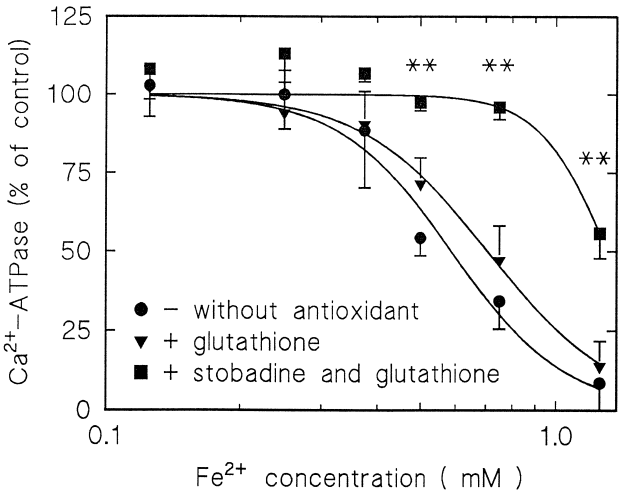

Fig. 3. Effect of glutathione and stobadine–glutathione mixture on the inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase by Fe^{2+}. Microsomal membranes were incubated at 37°C for 30 min in the presence of varying concentrations of Fe^{2+} and 0.1 mM glutathione or 0.1 mM stobadine plus 0.1 mM glutathione. Ca^{2+}-ATPase activity is expressed as a percentage of control activity. The results are shown as mean ± S.E.M. of four independent measurements. $^{**}(p < 0.001)$ significantly different from corresponding parallel incubated without antioxidant.

the Ca^{2+}-ATPase at an Fe^{2+} concentration of 0.5 mM ($p < 0.01$), it was almost ineffective at Fe^{2+} concentrations higher than 1 mM. The parameters of the curve describing the inhibition of Ca^{2+}-ATPase in the presence of 0.1 mM stobadine have also been calculated. The net effect of stobadine was the increase of both, IC_{50}-value to 0.76 mM of Fe^{2+} and the exponent to 5.7 ± 0.2.

Unlike stobadine, reduced glutathione is a naturally occurring water soluble thiol group containing antioxidant [20]. To test the hypothesis that protein thiol group oxidation may be involved in the process of ATPase inhibition, we have investigated the possible protective effect of reduced glutathione alone. Glutathione at the concentration of 0.1 mM had no significant protective effect on the Fe^{2+}-induced inhibition of Ca^{2+}-ATPase (Fig. 3). Although the IC_{50} value had been shifted to 0.71 ± 0.01 mM of Fe^{2+}, all experimental points are not significantly different in comparison to their corresponding parallels measured in the absence of antioxidant. Based on these results, we suppose that the increase of IC_{50} is within experimental error. The exponent value, 3.1 ± 0.01, is comparable to that obtained in the case of incubation without glutathione.

It is well known that combination of membrane soluble tocopherol with water-soluble ascorbic acid leads to the profound protection of membranes against lipid peroxidation [20]. The protective effect of stobadine–glutathione mixture was investigated in order to test the potency of such a mixture to prevent the Ca^{2+}-ATPase against Fe^{2+}-induced inhibition (Fig. 3). We have omitted the usage of ascorbic acid since its combination with Fe^{2+} may also lead to the potentiation of lipid peroxidation [20]. A 0.1 mM aliquot of stobadine in combination with 0.1 mM of glutathione was more potent in prevention the Fe^{2+}-induced inhibition of Ca^{2+}-ATPase than stobadine alone ($IC_{50} = 1.31 \pm 0.01$ mM). Under these conditions, the exponent value also increased to 5.7 ± 0.5. In addition, we have investigated the effect of stobadine–glutathione mixture with different molar ratios

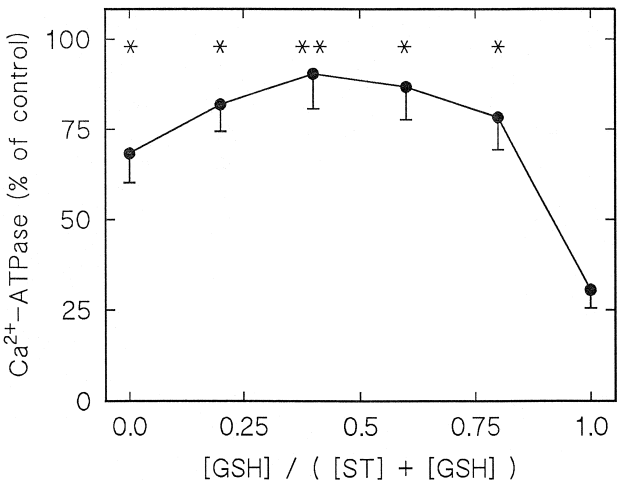

Fig. 4. Molar ratio-dependent protective effect of stobadine–glutathione mixture on the inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase by Fe^{2+}. Microsomal membranes were incubated at 37°C for 30 min in the presence of 0.75 mM Fe^{2+} and varying molar ratios of glutathione and stobadine. The different molar ratios of both the antioxidants are expressed as molar fractions of glutathione: [GSH]/([ST]+[GSH]), where [GSH] is concentration of glutathione and [ST] the concentration of stobadine. The total concentration of stobadine and glutathione ([ST]+[GSH]) was 0.2 mM. Ca^{2+}-ATPase activity is expressed as a percentage of control activity. The results are shown as mean ± S.E.M. of four independent measurements. $*(p < 0.01)$, $**(p < 0.001)$ significantly different from sample incubated with 0.2 mM glutathione {[GSH]/([ST]+[GSH]) = 1}.

Table 1
Effect of antioxidants on the gerbil forebrain microsomal Ca^{2+}-ATPase [a]

	Ca^{2+}-ATPase activity (nmol P_i /min per mg protein)
Control	173.7 ± 8.6
+ 0.1 mM stobadine	198.4 ± 34.4
+ 0.1 mM glutathione	163.4 ± 4.7
+ 0.1 mM stobadine + 0.1 mM glutathione	176.8 ± 16.1

[a] Microsomal membranes were preincubated at 0°C for 30 min with antioxidant and then the activities of Ca^{2+}-ATPase were measured at 37°C. The results are shown as mean ± S.E.M. of four triplicate measurements.

of both antioxidants (the total concentration of both being 0.2 mM) on the Fe^{2+}-induced inhibition of Ca^{2+}-ATPase (Fig. 4). An obtained curve indicates that the best ratio of stobadine/glutathione is close to 1 : 1.

To assess the possible effects of stobadine, glutathione, and combination of both antioxidants together on Ca^{2+}-ATPase, the activity of ATPase was measured after preincubation of microsomes with stobadine without further treatment with Fe^{2+}. As shown in Table 1, the activity of Ca^{2+}-ATPase measured under different conditions was slightly different; however, these differences were not statistically significant.

Table 2 summarizes the presented results. The effect of 0.1 mM stobadine in combination with 0.1 mM glutathione was significantly higher than the effect of 0.2 mM stobadine ($p < 0.05$). The effect of 0.04 mM stobadine in combination with 0.16 mM glutathione ($p < 0.01$, in comparison to the sample without antioxidant) was comparable (if not higher) to the effect of 0.2 mM stobadine ($p < 0.05$). Both 0.1 mM of stobadine and 0.1 mM of glutathione led to the increase of Ca^{2+}-ATPase activity, however, these changes are not statistically significant. Glutathione at a concentrations of 0.2 mM appears to be almost ineffective.

Table 2
Effect of antioxidants on the Fe^{2+}-induced inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase[a]

	Ca^{2+}-ATPase activity (% of control)
Without antioxidant	34.3 ± 8.7
0.1 mM stobadine	47.9 ± 8.3
0.2 mM stobadine	68.3 ± 8.1[b]
0.04 mM stobadine + 0.16 mM glutathione	78.3 ± 9.0[c]
0.1 mM stobadine + 0.1 mM glutathione	95.9 ± 3.9[d,e]
0.1 mM glutathione	47.1 ± 11.0
0.2 mM glutathione	30.5 ± 5.0

[a] Microsomal membranes were first preincubated at 0°C for 30 min with antioxidants and then incubated at 37°C for 30 min in the presence of 0.75 mM $FeSO_4$–EDTA. The results are shown as mean ± S.E.M. of four independent measurements. The values significantly different from the value obtained after incubation without antioxidant ([b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$) and from 0.2 mM stobadine value ([e] $p < 0.05$) are indicated.

4. Discussion

Fe^{2+}-induced oxidative stress of neuronal cells is important from several points of view [21]. The brain is an abundant source of iron and several areas of the brain are enriched in iron content. The capacity of cerebrospinal fluid to bind iron is poor, therefore the release of iron by haemorrhage, or its mobilization from ferritin by acidosis, may trigger lipid peroxidation of neuronal membranes and lead to the damage to the cells and/or death of neurones. In addition, brain tissue possesses limited natural antioxidant capacity. Although the central nervous system has a rather high ascorbic acid content, this property may not always be beneficial, especially in the case of Fe^{2+}-induced lipid peroxidation; ascorbate may potentiate the cytotoxity of free iron [20]. The artificial antioxidant intervention is thought to be important in the treatment of head injury [7], ischemia-reperfusion injury [22], Parkinson disease [23] and subarachnoid haemorrhage [10]. Using gerbil forebrain microsomal membranes, we examined a model system for Fe^{2+}-induced inhibition of Ca^{2+}-ATPase and tested the antioxidant properties of stobadine and glutathione. Freshly prepared equimolar solution of $FeSO_4$-EDTA in deionized water has been used in this study. Such solution, as had been discussed by Rohn et al. [24], contains 92–95% of iron in the form of Fe^{2+} and is widely used as lipid peroxidation initiator. Since we have documented earlier that Fe^{2+}-induced lipid per-

oxidation depresses the maximal velocity of microsomal Ca^{2+}-stimulated ATP hydrolysis reaction without effect on the Ca^{2+} affinity [12], we measured the effect of various Fe^{2+} concentrations on inhibition of Ca^{2+}-ATPase only at saturated concentration of Ca^{2+}.

The incubation of the microsomes with Fe^{2+}-EDTA led to the inhibition of Ca^{2+}-ATPase activity in both a concentration- and time-dependent manner. The prolonged incubation time was with the intention of investigating the effect of time in correlation with Fe^{2+} concentration. Although the estimated IC_{50}-values are far from the physiological concentrations of non-haem iron, which was found to be 0.074 μg/mg protein in the brain [25], our experiments clearly showed that prolonged incubation time increased the extend of Ca^{2+}-ATPase inhibition. For comparison, following preincubation of red blood cells with ferrous sulphate and EDTA for 4 h, the Na^+/K^+-ATPase, basal Ca^{2+}-ATPase, and calmodulin activated Ca^{2+}-ATPase were inhibited in a concentration-dependent manner, with an apparent IC_{50} for all three ATPases of $\approx$ 0.025 mM iron [24].

In our previous studies, we published the ability of stobadine to prevent both, the loss of the rabbit forebrain endoplasmic reticulum membrane's effectivity to sequester Ca^{2+} [26] and the changes in membrane integrity [13] due to free-radical attack. The concentration of stobadine leading to the prevention of Ca^{2+}-accumulation to 50% of control (EC_{50}-value) was as low as 0.012 mM, and stobadine at a concentration of 0.2 mM conferred full protection against Fe^{2+}-induced depression of Ca^{2+}-accumulation [26]. In this study, we used a fixed concentration of stobadine while the concentrations of Fe^{2+} were varied. The effectivity of prevention was dependent on the Fe^{2+} concentration. Since stobadine is considered as a potent antioxidant and inhibitor of lipid peroxidation [18], the results presented in this paper suggest that, at higher concentrations of Fe^{2+}-EDTA, the membrane damage is either too extensive so that addition of 0.1 mM stobadine is not able to prevent membrane disruption, or that a secondary mechanism, possibly different from lipid peroxidation, is involved. Production of secondary free radicals has been documented during the antioxidant action of stobadine (for a review, see Ref. [18]). It is possible that such radicals themselves could trigger lipid peroxidation presumably with significantly slower kinetics. In comparison, at higher concentrations of iron, butylated hydroxytoluene also provided only partial protection of erythrocyte Ca^{2+}-ATPase from Fe^{3+}-induced inhibition [27]. The results obtained by Rohn et al. [28] showed that neither butylated hydroxytoluene nor stobadine were able to confer full defence to the erythrocyte Ca^{2+}-ATPase against *t*-butyl peroxide induced inhibition. The ability of thiol compounds to protect heart and skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPases from free-radical-mediated inhibition has been documented earlier [29,30]. Based on these results, we assume that the oxidation of Ca^{2+}-ATPase protein thiol groups might play a role in Fe^{2+}-inhibition of gerbil brain Ca^{2+}-ATPase. Although reduced glutathione at a concentration of 0.15 mM was able to protect heart microsomal Ca^{2+}-ATPase from thiol oxidation-mediated inhibition [29], inclusion of 0.1 mM reduced glutathione had no significant protective effect on brain microsomal Ca^{2+}-ATPase, and 0.2 mM of glutathione was almost ineffective. It seems that with respect to Fe^{2+}-induced inhibition of brain reticular ATPases, the oxidation of thiol groups does not play the role, as we suggested earlier, based on the ineffectivity of hydrogen peroxide to inhibit both Ca^{2+} uptake and Ca^{2+}-ATPase [12]. On the other hand, the action of thiol compounds appears to be controversial. Rohn et al. [28] documented the ability of dithiothreitol to protect Ca^{2+}-ATPase from *t*-butyl peroxide induced inhibition. The initial assumption that the protective effect of dithiothreitol is caused by reduction of oxidized Ca^{2+}-ATPase protein thiol groups has not been proven and the effect has been shown to be mediated by the action of glutathione peroxidase. The recent study documented even the involvement of iron-catalyzed oxidation of thiol compounds in the initiation of free radicals [31].

Modification of Ca^{2+}-ATPase polypeptide amino acids could also be considered in the process of Fe^{2+}-induced inhibition of Ca^{2+}-ATPase. In the process of Fe^{2+}-induced enzyme inhibition, Stadtman and Oliver [32] proposed the formation of carbonyl groups by the mechanism requiring the presence of hydrogen peroxide. Although the reaction of Fe^{2+} with oxygen could lead to the formation of hydrogen peroxide [24] earlier presented data documented the inability of catalase to protect the Ca^{2+} pump of red blood cells [24] and smooth muscle sarcoplasmic

reticulum [33]. Similarly, the recently published protein fragmentation of the sarcoplasmic reticulum Ca^{2+} pump has been mediated by Fe^{2+}/H_2O_2/ascorbate [34]. Although other processes leading to the amino acid modifications [35] may also play a role in Fe^{2+}-induced inhibition of Ca^{2+}-ATPase, our results concerning combination of stobadine and glutathione support the view that probably the membrane is a primary site of Fe^{2+} attack. Inclusion of a glutathione–stobadine mixture significantly protects the membrane even at such Fe^{2+} concentrations at which the stobadine alone was ineffective. In support of this, another possibility exists, that of interaction between Ca^{2+}-ATPase protein amino acids and membrane-derived compounds produced during the progression of lipid peroxidation. Although the inhibition of Na^+/K^+-ATPase by 4-hydroxynonenal has been recently documented [36], this question in respect of Ca^{2+}-ATPase should be clarified by additional experiments.

In conclusion, the evidence has been presented that the incubation of the microsomes with Fe^{2+}-EDTA led to the inhibition of Ca^{2+}-ATPase activity in both a concentration- and a time-dependent manner. This inhibition may be prevented by the inclusion of membrane soluble stobadine, however, the effectivity of such prevention depends on the Fe^{2+} concentration. The stobadine effectivity is enhanced by a combination of stobadine with reduced glutathione. Since reduced glutathione alone in the concentrations used is almost ineffective, we suppose that the interaction between stobadine and glutathione is involved in the mechanism of enhanced stobadine antioxidant effectivity. The exact mechanism of such an interaction is not yet clear and several explanations are possible in this aspect, but they require additional experiments for clarification. The beneficial action of drug combinations might be very important from the therapeutic point of view, allowing the decrease of antioxidant doses and, thus, minimizing their possible side effects.

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