Is Na,K-ATPase the Target of Oxidative Stress?

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The susceptibility of Na,K-ATPase from bovine brain to various compounds containing active oxygen radicals is assessed. Sodium nitroprusside slightly inhibits Na,K-ATPase, while light-induced NO• radicals (controlled by the rate of ascorbate oxidation) have no effect on the enzyme. When added in concentrations equally effective in the ascorbate oxidation assay, hydrogen peroxide and sodium hypochlorite inhibit Na,K-ATPase by 70 and 25-30%, respectively. The Fe-dinitrosyl-cysteine complex is the most potent ($K_{0.5}$=20 μM) inhibitor of Na,K-ATPase. It is demonstrated that different free oxygen radicals accumulated in the ischemic brain cause different kinds of damage to Na,K-ATPase.

Key Words: *brain Na,K-ATPase; inhibition by free radicals; ischemia; hydrogen peroxide; hypochlorite; S-nitrosoglutathione; Fe-dinitrozyl-cysteine complex*

Interference with the oxygen supply to the brain accompanied by increased formation of highly reactive free radicals (FR) is responsible for a number of undesirable effects, including inhibition of enzymes and modification of ion channels, transporters, and membrane receptors [2,10,17,18]. As a result of FR attack, Na,K-ATPase separates from the active ion transport and loses hydrolytic activity [8,10]. After just 30 min of acute cerebral ischemia, rat Na,K-ATPase is inhibited by 35-40% [16]. From the evidence that tissue damage is attended by increased production of FR [19] and that brain Na,K-ATPase is highly susceptible to FR oxidation [10,11] it can be assumed that the enzyme inhibition results directly from ischemia. The damaging effects of different FR have not yet been evaluated. In this study we compare the effects of hydrogen peroxide, hypochlorite anion, NO•, and its stable complexes with glutathione and cysteine on the activity of bovine brain Na,K-ATPase.

MATERIALS AND METHODS

Na,K-ATPase was isolated from the gray matter of bovine brain [15] or from canine kidneys [11]. The enzyme activity was determined from the increase in inorganic phosphate under optimal conditions [15]. The specific activity of bovine brain and canine kidney Na,K-ATPase was 120-150 and 1500 μmol P_i/mg/h (37°C), respectively. Commercial preparations of hydrogen peroxide, sodium hypochlorite, and sodium nitroprusside (SN, Sigma) were used. Nitrosoglutathione and Fe-dinitrosyl-cysteine complex were synthesized by Dr. A. F. Vanin (Institute of Chemical Physics, Russian Academy of Sciences).

NO• radical was generated by exposing SN [18] to light (wavelength >425 nm, a ZhS-16 filter) from a 300 W bulb. The light was focused with a lens and passed through a water filter to prevent heating of samples. Aqueous solutions of SN (1-10 mM) were exposed to light for 5-30 min in a quartz cuvette with an optical pathway length of 1 mm. The intensity of FR formation was evaluated from the rate of oxidation of 40 μM ascorbate by measuring absorbance at 265 nm.

Na,K-ATPase was preincubated at 4°C with various concentrations of the studied inhibitors, the reaction was terminated by diluting the sample with a buffer-salt solution, and the enzyme activity was measured in medium containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM ATP, and 30 mM Pipes (pH 7.4 at 20°C) as described elsewhere [15]. The inhibitory

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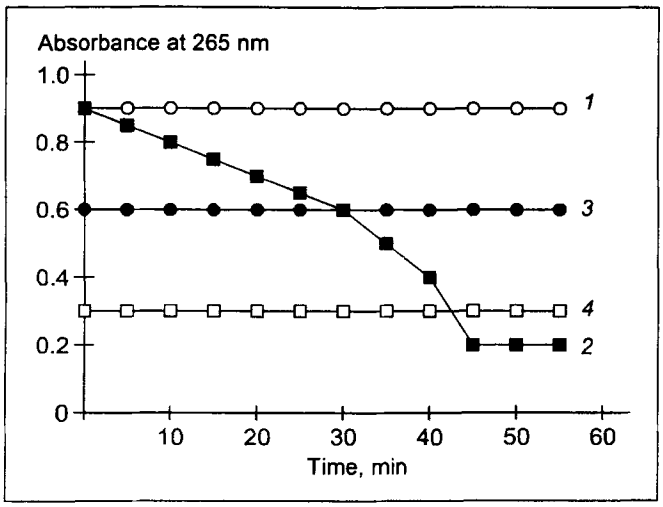

Fig. 1. Oxidation of ascorbate (40 μM) in the presence of sodium nitroprusside (SN, 170 μM) under the influence of visible light. *1*) control (mixture of aqueous solutions of ascorbate and SN without exposure to light); *2*) oxidation of ascorbate in the presence of SN during exposure to light (a mixture of aqueous solutions was exposed to light as described under Materials and Methods, and light absorbance was measured every 5 min); *3*) control (ascorbate solution exposed to light); *4*) control (SN solution exposed to light).

effects were evaluated by the decrease in enzyme activity expressed as percentage of the original activity.

RESULTS

All the inhibitors produced an irreversible effect on Na,K-ATPase: the lost enzyme activity was not recovered after incubation with inhibitor and dilution. The inhibitory effects of hydrogen peroxide and hypochlorite anion reached the maximum 10-30 min after incubation, depending on the inhibitor concentration. Bovine brain Na,K-ATPase was more sensitive to hypochlorite than to hydrogen peroxide, as was shown for Na,K-ATPase isolated from the kidneys. $K_{0.5}$ measured under comparable conditions was 50 μM for hydrogen peroxide and 1.5 mM for sodium hypochlorite.

Although SN by itself slightly inhibited Na,K-ATPase, this effect did not increase after exposure of the samples to light and cannot be attributed to liberated NO• (Table 1). In order to evaluate the intensity of radical formation during exposure of SN to light, we measured the rate of ascorbate oxidation under the conditions used to generate FR (the ascorbate test, Fig. 1). Under conditions analogous to those used for protein preparations the release of FR corresponded to ascorbate oxidation at a rate of 5 nmol/min. A 10-min preincubation of sample under conditions providing for the formation of at least 50 nmol NO• induced no modification of Na,K-ATPase. This is probably due either to the fact that chemical groups of Na,K-ATPase are not accessible to modification or to a low sensitivity of the enzyme to this compound. Although NO• persists in an aqueous solution for several seconds [12,13], a low probability of its interaction with Na,K-ATPase due to the low concentration of the enzyme in the preparation cannot be ruled out. Therefore, we repeated the experiments with Na,K-ATPase purified from the external medulla of dog kidney. This preparation had a specific activity of 1500 μmol P_i/mg/h, and was 80% pure by protein, i.e., it contained 10 times more Na,K-ATPase [11]. However, we failed to detect any inhibitory activity of nitrogen oxide using this preparation.

TABLE 1. Inhibition of Brain Na,K-ATPase by Sodium Nitroprusside under Various Conditions

Conditions	Exposure to light	Activity, % of original
1 mM SN, 30 min at 4°C	[-, +]	89±3
1 mM SN, 25 min at 20°C	[-, +]	77±5
10 mM SN, 5 min at 20°C	[-, +]	70±4

The effects of various FR were compared in the ascorbate oxidation test by determining the equally effective concentrations. It was found that the same rate of ascorbate oxidation (5 nmol/ min) is achieved by the addition of 2 mM H_2O_2 and 40 μM sodium hypochlorite. In this test system, a microsomal preparation of Na,K-ATPase added instead of ascorbate was inhibited 25-30% by hypochlorite and 70% by hydrogen peroxide. Consequently, NO• has no effect on Na,K-ATPase because the enzyme is resistant to this radical. The effect of H_2O_2 was stronger than that of hypochlorite if we compare their equally effective concentrations.

The biological significance of the different susceptibility of Na,K-ATPase to different FR will remain unknown until the actual concentrations of FR in normal and ischemic tissues are determined. The concentrations of the major free radicals in an ischemic cell are reported to vary considerably: from 10 to 100 pM for oxygen superoxide anion, 70-100 nM for NO•, and 100-240 nM for ONOO•, the total steady-state concentration of FR not exceeding 10 μM [14,20]. The last value is comparable to the minimal effective dose of hypochlorite anion and is too low to suppose that compounds such as hydroxyl radical or superoxide anion, which display a low inhibitory activity toward Na,K-ATPase [8,10], are potentially hazardous. This study has shown that hydrogen peroxide causes no serious damage to Na,K-ATPase. The data on the effect of NO• are contradictory. Both inhibitory and activatory effects of this radical on Na,K-ATPase have been de-

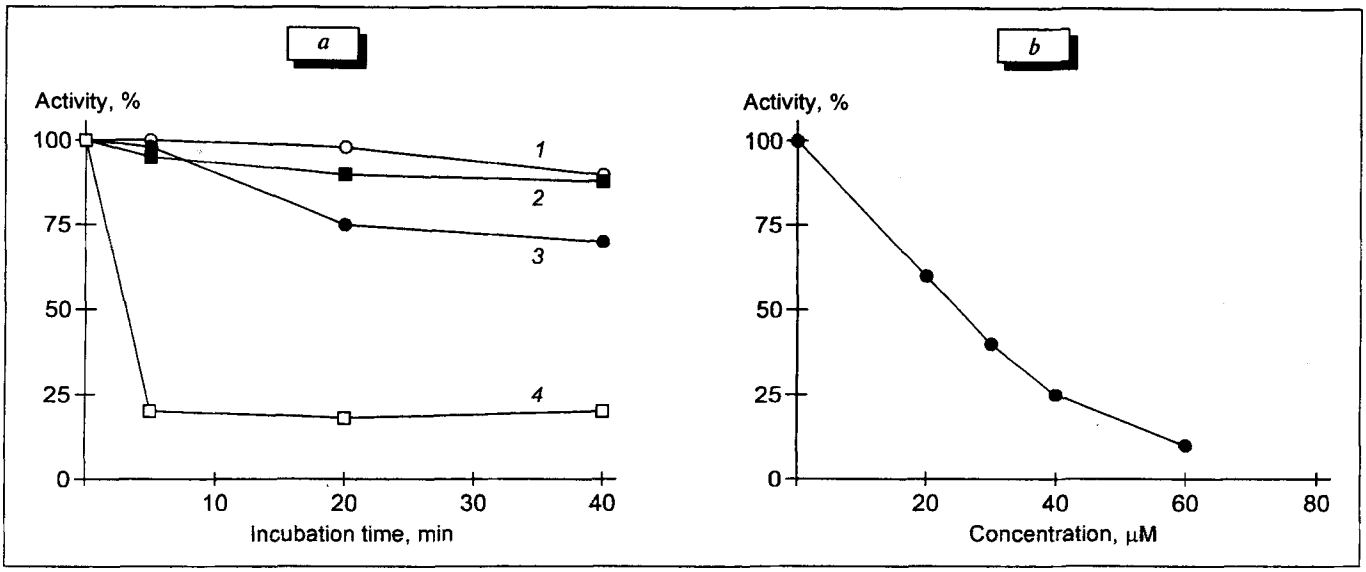

Fig. 2. Effects of stable complexes of NO• with cysteine and glutathione on the activity of bovine brain Na,K-ATPase. The protein concentration in the preincubation medium was 200 μg/ml at 4°C. *a*) enzyme activity as a function of the duration of preincubation with 100 μM L-cysteine (*1*), reduced glutathione (*2*), S-nitrosoglutathione (*3*), and the Fe-dinitrozyl-cysteine complex (*4*). *b*) effects of various concentrations of the Fe-dinitrosyl-cysteine complex on the activity of bovine brain Na,K-ATPase. Preincubation was carried out for 10 min.

scribed [7]. Recently, it was reported that NO• stimulates Na,K-ATPase via the activation of Na/Ca exchange [9]. Our results indicate that NO• has no direct effect on Na,K-ATPase.

However, the possibility that intermediate FR products are accumulated in the cell as more stable metabolites with their own biological activities cannot be ruled out. For example, NO• can form stable complexes with amino acids which can be regarded as a transport form of this short-lived FR and as independently existing inhibitors of the thiol groups of macromolecules [1,6]. Since Na,K-ATPase is a classical SH enzyme, we examined the effect of NO• complexes with cysteine and glutathione on its activity. The effects of these complexes were compared with those of cysteine and glutathione (Fig. 2). Preincubation of Na,K-ATPase with these amino acids (100 μM) caused a slight decrease in enzyme activity. Nitrosoglutathione had a more pronounced, though still rather weak, effect. The Fe-dinitrosyl-cysteine complex had a marked inhibitory effect ($K_{0.5}$=20 μM, Fig. 2, *b*), which developed within a short time period (Fig. 2, *a*). Thus, the Fe-containing NO•-cysteine complex has the most pronounced inhibitory effect on Na,K-ATPase. Presumably, this agent attacks SH groups of the enzyme. In fact, Na,K-ATPase is an SH enzyme susceptible to thiol poisons, and it is known that SH-containing membrane proteins are highly susceptible to oxidation stress [5].

Thus, we have demonstrated that FR inhibit brain Na,K-ATPase by directly acting upon the enzyme and that the inhibitory potential of diferent FR is different. Therefore, is seems important to assess the threat of various damaging factors to the neuronal membranes in ischemic tissue so as to make a correct choice of selective antioxidants. In the present case, hypochlorite anion was one of the most dangerous ones, and therefore carnosine and taurine, hydrophilic antioxidants inhibiting the myeloperoxidase system [3,4], should be the most effective protectors against tissue ischemia.

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Hemispheric Peculiarities of Serotonin Involvement in the Processing of Relevant and Irrelevant Information in Mice

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Stimulus-dependent hemispheric-regional changes are found in the activity of the serotoninergic system. Determination of stimulus novelty is provided by a decrease of serotonin and its metabolite content in the frontal cortex of the left hemisphere as well as in the striatum without the effect of lateralization. Presentation of an extinct stimulus is attended by a serotonin increase in the hippocampus of the left hemisphere and in the amygdaloid complex of both hemispheres.

Key Words: *serotonin; 5-hydroxyindoleacetic acid; brain asymmetry; orienting reaction*

At the present time there is strong evidence of the existence of biochemical brain asymmetry in animals. The data mainly concern catecholamines [5,11,14], while much less is known about serotonin [4], even though its involvement in cognitive functions and behavior has been demonstrated repeatedly. Specifically, stimulation of or damage to the nuclei of the midbrain suture enhances or disturbs, respectively, the process of habituation to information presented repeatedly [1,3,7,12,13].

In addition, the interaction found in various behavior tests [10] between biochemical asymmetry and spatial preference behavior implies that neurotransmitter asymmetry is more than an accidental phenomenon, but rather is a brain characteristic which may play an important role in the central organization of behavior. Studies taking account of such interaction would make a valuable contribution to the concept of neurochemical mechanisms of cognitive function. The promise of such investigations, on the one hand, and the scant evidence of serotoninergic lateralization, on the other, prompted us to perform this study, the aim of which was to reveal the laterality of serotoninergic activity and the peculiarities of its manifestation in brain structures in response to the presentation of relevant and irrelevant information.

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MATERIALS AND METHODS

Experiments were carried out on 32 female Wistar rats weighing 180-200 g. The behavioral procedure of habituation of the orienting reaction performed in a Jarvik and Kopp chamber [8] was essentially similar to the earlier described [1] stage of preliminary exposure to a situational stimulus preceding the elaboration of the conditioned passive avoidance re-

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