ANTIOXIDANTS AS STABILIZERS OF THE Ca^{++} TRANSPORT ENZYME SYSTEM IN SARCOPLASMIC RETICULUM MEMBRANES *IN VIVO*

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Extreme activation of two physiologically important methods of modification of biological membranes, namely lipid peroxidation (LPO) and hydrolysis of phospholipids by endogenous phospholipase A_2, causes a disturbance of structural and functional organization of cell membranes and may ultimately be the cause of irreversible cell damage with the development of various pathological processes [15]. Damage to membrane enzyme systems involved in Ca^{++} transport due to these effects is a key pathogenetic stage in ischemia and stress, a combination of which plays a principal role in the genesis of the most important heart diseases [6]. It has recently been shown that antioxidants, which are inhibitors of free-radical oxidation, can not only prevent the accumulation of LPO products in biomembranes, but can also stabilize them against the action of phospholipase A_2, forming complexes with free fatty acids [10, 13]. Experiments *in vitro* have shown that the enzyme system of Ca^{++} transport can be stabilized in membranes of the sarcoplasmic reticulum (SR) from skeletal muscles by antioxidants, both during induction of LPO and during the action of phospholipase A_2 or of free fatty acids [1, 4, 11].

The aim of this investigation was to study the ability of antioxidants with varied chemical structure (natural and synthetic) to stabilize the enzyme system of Ca^{++} transport in membranes *in vivo*.

EXPERIMENTAL METHOD

Fragments of SR membranes from rat skeletal muscles were isolated by differential centrifugation from a homogenate of the hind limb muscles [14]. The ATPase activity of the SR membrane fraction was determined by pH-metry [8]. Thermal denaturation of the SR membranes was carried out in medium containing 5% sucrose and 50 mM phosphate buffer (pH 6.8, 20°C) with a protein concentration of 5 mg/ml. Incorporation of arachidonic acid (20 μg/mg protein, from Sigma, USA) into the SR membranes was carried out directly in medium for thermal inactivation in solution in ethanol, the mixture being incubated for 10 min at 37°C (the final ethanol concentration did not exceed 2%). Ca^{++} transport in SR of sleketal and heart muscles was recorded by means of a Ca^{++}-selective electrode (Orion 93-20, Finland) [9], with the addition of NaN_3 (5 mM) to the muscle homogenate to prevent Ca^{++} transport by the mitochondria. To obtain the muscle homogenate 0.5 g of tissue was placed in 10 ml of medium containing 25% glycerin, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 mM $CaCl_2$, and 10 mM histidine (pH 7.4, 4°C), and homogenized in a Polytron-type homogenizer for 40 sec, with a voltage of 100 V. The medium for recording Ca^{++} transport (4 ml) contained: 100 mM KCl, 15 mM potassium oxalate, 4 mM $MgCl_2$, 2 mM ATP, 10 mM HEPES (pH 7.0, 37°C), and 1.2 mg protein of homogenate, D,L-α-tocopherol (TP, from Sigma and 4-methyl-2,6-di-tert-butylphenol (MDBP, from "Serva," West Germany) were added to the diet of the Wistar rats at the rate of 50 and 80 mg/kg body weight, respectively, once every two days for 3 weeks. 2-Ethyl-6-methyl-3-hydroxypyridine (synthesized at the Institute of Pharmacology, Academy of Medical Sciences of the

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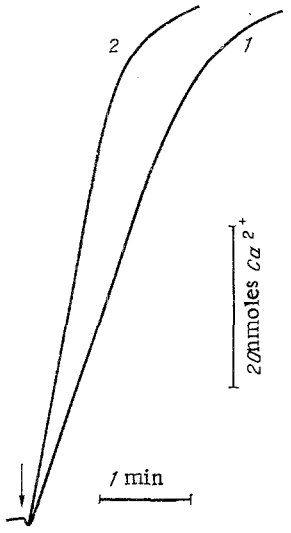

Fig. 1

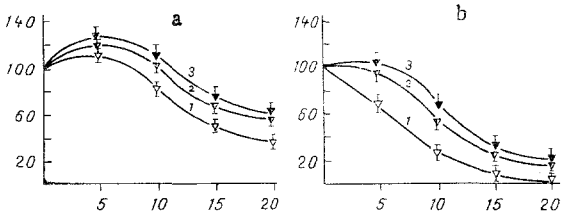

Fig. 2

Fig. 1. Kinetic curves of Ca^{++} transport in skeletal muscle homogenates of control rats (1) and rats fed for 12 weeks with 2-ethyl-6-methyl-3-hydroxypyridine (2). Arrow indicates time of introduction of muscle homogenate (1.2 mg protein) into recording medium. Calibration: 20 nmoles $CaCl_2$.

Fig. 2. Activity of Ca^{++}-dependent ATPase in SR membranes from skeletal muscles of control rats (1) and also of rats kept for 3 weeks on a diet with added MDBP (2) or vitamin E (3). Abscissa, time (in min); ordinate, Ca^{++}-ATPase activity (in $_{50}$. a) In absence of arachidonic acid; b) after addition of arachidonic acid (20 µg/mg protein).

USSR) was added to the diet at the rate of 100 mg/kg body weight, also once every two days for 12 weeks. The control rats were kept on a standard laboratory diet.

EXPERIMENTAL RESULTS

In the experments of series I the effectiveness of the Ca^{++}-transporting function was tested in SR of cardiac and skeletal muscles in young (aged 6 months) and old (aged 20 months) rats, receiving the antioxidant 2-ethyl-6-methyl-3-hydroxypyridine, and also in rats of the corresponding age in the two control groups. The rate of Ca^{++} transport in SR in the skeletal muscle homogenate from rats of the experimental group was shown to be significantly greater than in the control: The rate of removal of added Ca^{++} from the external medium into vesicles of SR was almost 1.7 times greater than in the control preparation (Fig. 1). In the control the rate of Ca^{++} transport into the myocardial SR of old and young rats was virtually identical, whereas in skeletal muscles the rate of Ca^{++} transport into SR of the young animals was twice as high as the value for old rats (Table 1). In rats receiving the antioxidant the rate of Ca^{++} transport into SR of both sleketal muscle and myocardium was significantly higher in both age groups than in the corresponding control samples. Whereas the increase in the rate of Ca^{++} transport for the myocardium did not exceed 25-35% in both young and old animals, for the sleketal muscles the action of the antioxidant was stronger in the old animals (the rate of transport was increased by 70%) than in young animals (by 20%).

In the experiments of series II resistance of Ca^{++}-ATPase to heat inactivation was compared in SR membranes isolated from control rats and from rats kept on a diet enriched with natural TP or with the synthetic antioxidant MDBP. Specific Ca^{++}-ATPase activity in SR membranes of animals of the control and experimental groups was identical, namely 4.0 ± 0.5 µmole P_i/mg protein/min at 37°C. On heat inactivation of the SR membranes significant differences were found between the control and experimental groups. Preincubation of SR membranes at 48°C led to inactivation of Ca^{++}-ATPase (Fig. 2). The low level of activation of the enzyme in the initial period was due to increased membrane permeability for Ca^{++} and was not observed when activity of the enyme was modified in the presence of the Ca^{++}-ionophore A 23187 (3 µg/ml). Inhibition of ATPase by 50% in the control preparations of SR membranes was achieved after preincubation for 15 min at 48°C. Ca^{++}-ATPase in SR membranes of animals receiving the antioxidant was more thermostable. The stabilizing effect of the antioxidants

TABLE 1. Rate of Ca^{++} Transport (in μmoles/min/g tissue) in SR Vesicles from Heart and Skeletal Muscles of Old and Young Rats, Kept on a Standard Laboratory Diet or on a Diet with Addition of 2-Ethyl-6-methyl-3-hydroxypyridine (M ± m)

Experimental conditions	Control	2-Ethyl-6-methyl-3-hydroxypyridine
Skeletal muscles		
old rats	25,8±0,9	43,8±0,7
young rats	53,9±2,6	64,3±4,1
Heart muscle		
old rats	1,29±0,05	1,61±0,07
young rats	1,18±0,04	1,58±0,09

was more marked in the presence of added exogenous fatty acid. Addition of arachidonic acid to the membrane suspension in the control accelerated thermal denaturation of Ca^{++}-ATPase more effectively than in both experimental groups (Fig. 2). It must be emphasized that both antioxidants used in the experiments were similar in the effectiveness of their stabilizing ability. It must be pointed out in this connection that both TP and MDBP can form complexes with free fatty acids [3, 10].

It can be concluded from these results as a whole that antioxidants are stabilizers of the enzyme system responsible for Ca^{++} transport in SR membranes of cardiac and skeletal muscles *in vivo*. It can be tentatively suggested that antioxidants exert their stabilizing effect both through their antiradical properties and through their ability to form complexes with hydrolysis products of phospholipids by type A_2 phospholipases. The practical importance of these effects of antioxidants on the Ca^{++}-transporting system is determined, on the one hand, by the marked activation of LPO and of A_2 phospholipases in cardiac and skeletal muscles recently discovered during intensive physical work [5, 12, 15], stress, ischemia, and infarction [6, 15], and on the other hand, by experience of the successful use of antioxidants to increase endurance in man and animals [5, 7], and also as therapeutic agents in clinical cardiology [2].

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