BLOCKING EFFECT OF CYCLOHEXIMIDE ON LOWERING OF MITOCHONDRIAL RESISTANCE BY THE ACTION OF THYROID HORMONES

A. I. Marzoev, S. L. Turchina, and V. A. Pechatnikov

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The writers showed previously that the transmembrane potential (TMP) of mitochondria is increased in the organelles of hyperthyroid rats [4] and reduced in those of hypothyroid rats [1]. At the same time, those investigations showed that the resistance of the system of mitochondria maintaining TMP to Ca^{++} ions correlates negatively with the level of thyroid hormones in the body. For instance, when the organelles were equally loaded with Ca^{++} ions the decline of TMP took place more rapidly in organelles of hyperthyroid animals and later in mitochondria of thyroidectomized rats [1, 4]. On the basis of our own data [5] and results obtained by other workers [2, 6] we suggested that the lowering of resistance of the mitochondria to the harmful action of Ca^{++} ions associated with hyperthyroidism is based on activation of the endogenous phospholipase of these organelles. The increase in lipase activity of liver homogenates from hyperthyroid rats was abolished if the animals were given actinomycin D simultaneously with thyroxine [5].

The aim of the present investigation was to study the effect of cycloheximide, an inhibitor of cytoplasmic protein synthesis, on the lowered resistance of mitochondria to the action of Ca^{++} ions, induced by thyroid hormones.

EXPERIMENTAL METHOD

Wister rats weighing 150-180 g were used. In the animals of group 1 hyperthyroidism was induced by a single intraperitoneal injection of L-thyroxine into the animals in a dose of 300 μg/100 g body weight 48 h before isolation of the mitochondria. The rats of group 2 received this same dose of the hormone simultaneously with cycloheximide (40 μg of the antibiotic to each rat). The injection of cycloheximide was repeated 24 h after the first injection. Mitochondria were isolated from the liver by the method in [8] in medium containing 0.3 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. The mitochondria were washed in the same medium, but without the EDTA. The level of TMP was judged from the intensity of fluorescence of the dis-C_3-(5) probe (3,3'-dipropylthiodicarbocyanin) in a mitochondrial suspension [10]. Fluorescence of NADPH in the mitochondria was excited with a wavelength of 360 nm and recorded in the 430 nm region, using a slit 10 nm wide. The fluorescence studies were undertaken on a special kind of spectrofluorometer, reducing the effect of the light-scattering properties of the object [9]. The protein concentration was determined by the biuret reaction.

EXPERIMENTAL RESULTS

The system of mitochondrial TMP of rats which, besides thyroxine, received cycloheximide, proved to be more resistant to Ca^{++} ions than in preparations from animals receiving the hormone only (Figs. 1 and 2). The concentration of Ca^{++} ions required to induce the decline of TMP was greater in preparations from the animals of group 2 than in the mitochondria of the rats of group 1 (Fig. 1). Moreover, during limiting loading of the organelles with Ca^{++} ions the decline of the mitochondrial TMP of animals treated with the antibiotic began later than in organelles of animals with pure hyperthyroidism (Fig. 2). At the same time, recording the parameter $\Delta F/F_0$ of fluorescence of the dis-C_3-(5) probe (Fig. 1, inset), the value of which is proportional to

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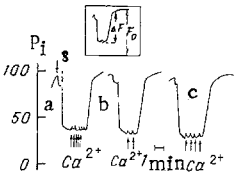

Fig. 1

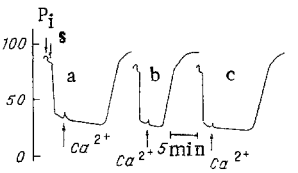

Fig. 2

Fig. 1. Effect of Ca^{++} on mitochondrial TMP. Fluorescence of dis-C_3-(5) probe in mitochondrial suspension from normal rats (a), animals of group 1 (b), and animals of group 2 (c). Here and in Fig. 2, incubation medium contained 0.1 M KCl, 0.1 M sucrose, 5 mM Tris-HCl, pH 7.4 (20°C); protein 1 mg/ml. Times of addition of inorganic phosphate (P_i 1 mM) and succinate (S, 5 mM) to suspension indicated by arrow. Ca^{++} added in portions of 20 nmoles. Ordinate, intensity of fluorescence (F, in relative units). Inset shows parameters of fluorescence used to calculate relative value of TMP.

Fig. 2. "Retention" time of Ca^{++} by mitochondria of normal rats (a), and rats of group 1 (b) and group 2 (c).

TMP [10], revealed no differences between preparations from animals of the two groups. For instance, in the group of animals receiving cycloheximide the value of $\Delta F/F_0$ was 101 ± 3% of the corresponding parameter for rats of group 1 (data obtained from separate preparations of four hyperthyroid rats and four rats receiving the antibiotic, with four or five repetitions for each preparation).

Injection of cycloheximide into hyperthyroid rats blocked the development of another manifestation of thyroid poisoning: the more rapid oxidation of mitochondrial pyridine nucleotides. Figure 3 shows that the rate of decline of fluorescence of mitochondrial NADPH was proportional to the number of Ca^{++} ions accumulated by organelles. The same degrees of loading of the mitochondria with Ca^{++} were accompanied by a decline of fluorescence of pyridine nucleotides with higher velocities in the mitochondria of rats of group 1 than in the organelles of the rats of group 2.

Incidentally, the NADPH concentration, judging from the intensity of fluorescence, was higher in the mitochondria of the animals of group 2, namely 137 ± 14% of its value in the rate of group 1 (results from three separate mitochondrial preparations from each group with three repetitions for each preparation).

Intensity of fluorescence of mitochondrial pyridine nucleotides is determined by their reduced forms. Furthermore, the main contribution to fluorescence is due to those NADPH molecules which are located in the hydrophobic region of the mitochondrial membrane [11]. Consequently, the rate of decline of the intensity of fluorescence of the membrane components under discussion reflects not only the rate of oxidation of NADPH, but also at the same time, the decrease in hydrophobicity of the mitochondrial membrane. One cause of this decline may be endogenous phospholipase activity. In fact, hydrolysis of phospholipids, accompanied by the formation of lysophospholipids and free fatty acids, ought to induce the appearance of polar sites in the hydrophobic region of the membranes. Correlation between the rate of oxidation of mitochondrial pyridine nucleotides and mitochondrial phospholipase A_2 activity also is shown by data obtained in Pfeiffer's laboratory. It was shown [6], for instance, that inhibition of endogenous mitochondrial phospholipase by the local anesthetic nupercaine prevents both oxidation of mitochondrial NADPH and the decline of TMP with the accompanying outflow of Ca^{++} ions. Consequently, inter alia, recording fluorescence of mitochondrial pyridine nucleotides can be used as a test reflecting mitochondrial phosphilipase activity. In that case the effects of cycloheximide observed, namely an increase in resistance of the mitochondria to the action of Ca^{++} and a decrease in the rate of oxidation of mitochondrial pyridine nucleotides – may be the result of the inhibitory action of the antibiotic on mitochondrial phospholipase activity. A weighty argument in support of this hypothesis may also be the results of parallel experiments which showed that the increase in mitochondrial phospholipase activity is abolished if the animal is given thyroxine together with cycloheximide.

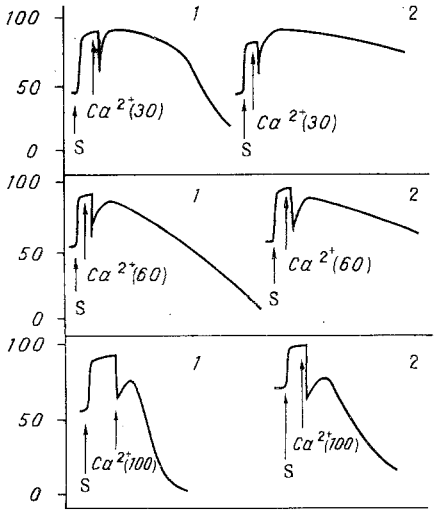

Fig. 3. Effect of hyperthyroidism and cycloheximide on fluorescence of mitochondria "loaded" with Ca^{++} ions. Composition of medium the same as in Figs. 1 and 2, except that the P_i concentration was 0.3 mM. Quantity of Ca^{++} added (in nmoles/mg protein) shown in parentheses. 1) Mitochondria from rats of group 1; 2) mitochondria of group 2. Ordinate, intensity of fluorescence of NADPH (in relative units).

Cycloheximide thus blocks the development of the decline in the resistance of mitochondria to Ca^{++} ions that is specific for hyperthyroidism. However, the details of this blocking remain unexplained and require further study.

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