# EFFECT OF CAFFEINE ON ACTIVE TRANSPORT OF $Ca^{++}$ IONS IN SKELETAL MUSCLE AND MYOCARDIAL HOMOGENATES

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The fraction of fragments of sarcoplasmic reticulum (SR) from rabbit skeletal muscles can be divided into two subfractions, which differ in their morphological origin: a fraction of terminal cisterns, from which $Ca^{++}$ ions are released during excitation, and a fraction of oblong tubules which, it is considered, take part in the active uptake of $Ca^{++}$ from the sarcoplasm in the relaxation phase only [1, 4, 5, 7, 9].

The relative contribution of the terminal cisterns and oblong tubules to the process of removal of $Ca^{++}$ from the sarcoplasm during relaxation is unknown. The purified fraction of terminal cisterns contains $Ca^{++}$-dependent ATPase and can carry out active $Ca^{++}$ transport. However, the relative content of this fraction in muscle tissue homogenate is quite difficult to estimate, because fragments of terminal cisterns have a higher density than fragments of oblong tubules, and accordingly, unlike the latter, many of them are lost during isolation from the fraction of myofibrils and mitochondria. The purified fraction of SR fragments, which is usually used in studies of active $Ca^{++}$ transport, contains virtually no fragments of terminal cisterns. The relacontribution of terminal cisterns to the process of active $Ca^{++}$ transport in the muscle call can be estimated by measuring $Ca^{++}$ uptake by SR fragments in a homogenate, using a specific inhibitor of $Ca^{++}$ transport into terminal cisterns for this purpose. One such inhibitor is caffeine which, in a concentration of 5-10 mM, specifically inhibits $Ca^{++}$ transport by fragments of terminal cisterns [1].

Accordingly the aim of the present investigation was to study the action of caffeine on $Ca^{++}$ transport in a homogenate of rabbit skeletal muscles and myocardium, measured by means of a Ca-selective electrode.

## EXPERIMENTAL METHOD

Muscle tissue homogenate was prepared by means of a specially made homogenizer of "Polytron" type. A sample of tissue weighing 500-700 mg was homogenized for 40 sec in 10 ml of medium containing 25% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 50 $\mu$M phenylmethylsulfonyl fluoride, and 10 mM histidine (pH 7.4 at 4°C). Tissue samples were taken from the middle part of the m. psoas (fast muscle), m. soleus (slow muscle), and also m. adductor magnus (fast muscle) and the ventricular wall of a rabbit. The time from decapitation of the animal until homogenization of the tissue did not exceed 5 min for the slow muscle and myocardium and 10 min for the fast muscles.

$Ca^{++}$ transport was measured by means of an Orion 92-20 Ca-selective electrode in a constant-temperature cell, with mixing. At the same time the pH was measured in the same cell by means of a G2222C electrode (radiometer, Denmark). Changes in potential of the $Ca^{++}$-electrode were recorded continuously by means of I-120 ionometer, with attached TZ-4221 automatic writer. The incubation mixture, with a volume of 4 ml, contained 100 mM KCl, 15 mM potassium oxalate, 4 mM $MgCl_2$, 2 mM ATP, 5 mM $NaN_2$, and 2.5 mM imidazole (pH 7.0 at 37°C). Three successive doses of $CaCl_2$, each of 50 nanomoles, were added to the reaction mixture, and they were also used to calibrate the electrode, after which the reaction was started by addition of 70-500 $\mu$l of homogenate.

Fractions of terminal cisterns and oblong tubules of SR were isolated from rabbit's white skeletal muscles as described previously [2].

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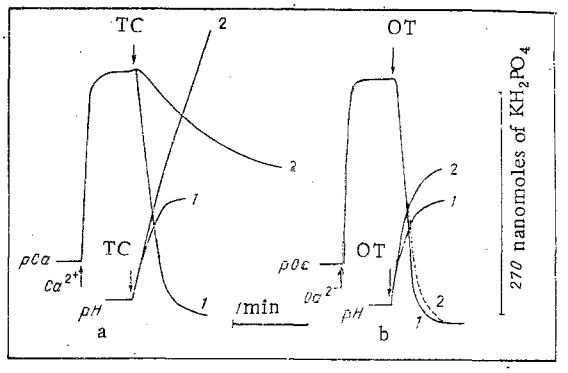


Fig. 1. $Ca^{++}$ transport and ATP hydrolysis by fraction of terminal cisterns (TC) and oblong tubules (OT) of sarcoplasmic reticulum in absence (1) and presence of 5 mM caffeine (2). $Ca^{++}$ added in the form of CaCl (130 nanomoles).

## EXPERIMENTAL RESULTS

A Ca-selective electride was used to record $Ca^{++}$ transport by SR membranes continuously. When different types of electrodes were chosen for this purpose it was found that the Orion 93-20 electrode has sufficiently high sensitivity and a short response time for direct recording of transport [2].

Data on the effect of caffeine on $Ca^{++}$ transport by different fractions of SR membranes are given in Fig. 1. As Fig. 1 shows, caffeine in a concentration of 5 mM reduced the rate of uptake of $Ca^{++}$ ions by the fraction of terminal cisterns by more than 70% but had virtually no effect on $Ca^{++}$ transport by the fraction of oblong tubules. Since, according to electron-microscopic data, the fraction of terminal cisterns contains 30% of vesicles formed from oblong tubules [1], it can be calculated that caffeine, in a concentration of 5 mM, causes 100% inhibition of $Ca^{++}$ transport by terminal cisterns of SR. Thus, by measuring the effect of caffeine on $Ca^{++}$ transport, the relative contribution of each of the fractions to the total process can be calculated.

Investigations [3, 6, 10] have shown that $Ca^{++}$ transport by fragments of SR in the presence of oxalate can be measured directly in muscle tissue homogenate as binding of $^{45}Ca$. Such measurement is possible because oxalate does not penetrate inside the vesicles of the sarcolemma, and this makes the contribution of sarcolemmal $Ca^{++}$ transport to the total process negligible. To suppress $Ca^{++}$ transport by mitochondria, sodium azide was added to the incubation medium. Thus in the presence of oxalate and azide in the homogenate $Ca^{++}$ transport is measured only in vesicles of SR. By using caffeine as a specific inhibitor of $Ca^{++}$ transport into terminal cisterns, we measured the relative contribution of fragments of terminal cisterns to the total process of $Ca^{++}$ transport in rabbit myocardial and skeletal muscle homogenate. The medium and conditions of homogenization were chosen in preliminary experiments. It was found that if homogenization is carried out in medium containing glycerol and the proteolysis inhibitor phenylmethylsulfonyl fluoride, the transport function of SR in the homogenate was unchanged during keeping of the homogenate at 4°C for 24 h. Maximal $Ca^{++}$-transporting activity was observed after homogenization of the tissue for 30-40 sec. Increasing the time of homogenization to 1 min had no effect. The critical factor for obtaining reproducible results is the time from circulatory arrest until homogenization of the tissue. Tissue from the myocardium and m. soleus is particularly sensitive to this factor. A delay of 10 min before homogenization of the tissue leads to substantial injury to the Ca-transporting function of SR in the myocardium and m. soleus.

Typical traces of $Ca^{++}$ transport in homogenate of m. psoas, m. soleus, and the myocardium are illustrated in Fig. 2. They show that in the presence of oxalate in the maximally activating concentration (15 mM) in the incubation medium the rate of uptake of $Ca^{++}$ in the homogenate of the fast skeletal muscle with a predominantly glycolytic type of energy metabolism was much higher than in homogenate of the myocardium and of the slow skeletal muscle with oxidative energy metabolism. This is in good agreement with the degree of development of the sarcoplasmic reticulum in muscles of this type and the rate of their relaxation [11, 12]. Addition of 5 mM caffeine to the incubation mixture reduced $Ca^{++}$ transport in homogenate of the fast muscle by more than 50%. The action of caffeine on $Ca^{++}$ transport in the homogenate of the slow muscle and myocardium was rather weaker (Fig. 2). Elevation of the caffeine concentration to 10 mM caused no additional inhibition of $Ca^{++}$ transport in homogenate of all three types of muscle.

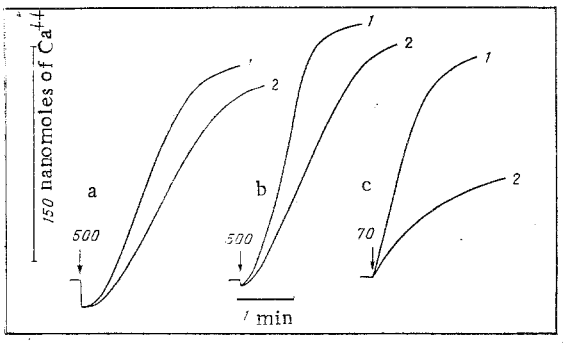


Fig. 2. $Ca^{++}$ transport in homogenate of myocardium (a), slow muscle (b), and fast muscle (c) in absence (1) and in presence of 5 mM caffeine (2). Number above arrows indicates volume of homogenate added (in $\mu$l).

$Ca^{++}$ transport in homogenate of the slow muscle and myocardium was quite complex in its kinetics. Transport did not begin at once, but after some delay. This delay could also be observed with homogenate of the fast muscle if the oxalate concentration in the incubation medium was reduced to 2-5 mM. This suggests that delay with the beginning of $Ca^{++}$ uptake is connected with delay of precipitation of calcium oxalate within the vesicles of SR. Delayed precipitation of calcium oxalate, with low oxalate concentrations, also was observed on isolated vesicles of SR [13]. The writers were unable to abolish the delay and to accelerate $Ca^{++}$ transport in homogenate of the slow muscle and myocardium by increasing the oxalate concentration from 15 to 25 mM. Accordingly, the important advantage of continuous recording of $Ca^{++}$ transport with the aid of a Ca-electrode will be noted. Continuous recording of $Ca^{++}$ transport enabled the error in determination of the velocity of $Ca^{++}$ transport due to the lag phase to be eliminated, for the velocity of transport can be determined in the stationary phase of the transport process.

For comparison with known data on $Ca^{++}$ transport in homogenate of rat skeletal muscle and myocardium, we calculated the velocity of $Ca^{++}$ transport in homogenate of rabbit muscle tissue. Specific Ca-transporting activity, expressed in micromoles $Ca^{++}$/min/g tissue, was 40-60 units for fast muscles, 4-5 units for slow muscle, and 3-5 units for the myocardium. The appropriate values for the rats were 18, 6, and 2 units [10]. The velocity of $Ca^{++}$ transport on the myocardial homogenate could be increased by 1.5 times by the addition of 5 mM creatine phosphate to the incubation mixture. Creatine phosphate had virtually no effect on $Ca^{++}$ transport in skeletal muscle homogenate. The effect of creatine phosphate of $Ca^{++}$ transport in myocardial homogenate agree with the hypothesis that this energy substrate plays an important role in metabolism of heart muscle [8]. The degree of inhibition of $Ca^{++}$ transport in myocardial homogenate by caffeine is indepent of the presence of creatine phosphate.

These results are evidence that caffeine, a specific inhibitor of $Ca^{++}$ transport in the terminal cisterns, has a powerful inhibitory action on $Ca^{++}$ uptake in homogenate of fast and slow skeletal muscles and myocardium of rabbits. This suggests that the terminal cisterns of SR not only serve as a store for $Ca^{++}$ required for triggering contraction, but may also play an active role in muscle relaxation.

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# INDUCTION OF LIPID PEROXIDATION IN ERYTHROCYTES DURING CHOLESTEROL OXIDATION CATALYZED BY CHOLESTEROL OXIDASE

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Lipid peroxidation (LPO) is a universal method of modification of biomembranes under normal conditions and when injured by the development of pathological processes [6, 10]. That is why it is particularly important to study mechanisms of initiation of the LPO reaction in vivo. LPO reactions can be initiated in vivo both enzymatically and nonenzymatically [2, 6]. The enzyme systems that participate in the formation of LPO products in biomembranes include various NADPH- and NADH-dependent generators of active forms of oxygen [2, 8, 12] and, in particular, the system of mixed-function microsomal oxygenases. Much less is known about the participation of oxidase systems generating $H_2O_2$ in the induction of LPO. It has recently been shown that deamination of biogenic amines, catalyzed by monoamine oxidase, may under certain conditions be accompanied by accumulation of LPO products [9]. Hence the importance of studying to what extent the participation of oxidases could be a source of lipid peroxides in vivo.

The aim of this investigation was to study the ability of cholesterol oxidase (ChO), which catalyzes oxidation of cholesterol (Ch) to cholest-4-en-3-one and, at the same time, reduction of $O_2$ to $H_2O_2$, to induce the LPO in erythrocyte membranes, for we know that Ch is a natural inhibitor of LPO in plasma membranes [3].

## EXPERIMENTAL METHOD

Erythrocyte ghosts were obtained from guinea pig blood by the method in [13]. The reaction of oxidation of Ch in the erythrocyte ghosts or in micelles with Triton X-100 was carried out in the following medium: Tris-HCl 0.2 M, pH 7.0 (at 37°C), Triton X-100 0.25%, and ChO 0.05 U/ml. The activity of ChO was determined by the chemiluminescence method which detected $H_2O_2$ with the presence of luminol ($10^{-4}$ M) and horseradish peroxidase (1.0 U/ml) [4, 11]. To separate Ch from cholest-4-en-3-one the method of thin-layer chromatography was used in a solvent system of chloroform – methanol (98 : 2) as described in [7]. For densitometry of the plates in ER165M densitometer was used. The level of LPO products interacting with 2-thiobarbituric acid (TBA) was determined as described previously [5].

## EXPERIMENTAL RESULTS

ChO catalyzes the reaction of Ch oxidation with simultaneous formation of $H_2O_2$:

$$\text{Ch} + O_2 \xrightarrow{\text{ChO}} \text{cholest-4-en-3-one} + H_2O_2.$$

Activity of the enzyme can be estimated by determing accumulation of either reaction product. It was shown (Fig. 1) that during incubation of Ch dispersed in Triton X-100 with ChO the concentration of Ch (the spot with $R_f = 0.36$) falls and that of cholest-4-en-3-one (the spot with $R_f = 0.50$) rises. $H_2O_2$ formation can be recorded highly sensitively by a chemiluminescence method, using a luminol – peroxidase system. Curves showing the kinetics of chemiluminescence arising during incubation of Ch, dispersed if Triton X-100, in the presence of ChO, luminol, and peroxidase, are given in Fig. 2. In the absence of ChO or of Ch, and also of

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