PROTECTION OF Ca^{++} TRANSPORT BY CARNOSINE AGAINST DISTURBANCES INDUCED BY LIPID PEROXIDATION

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Carnosine, an endogenous component of the muscle cell whose concentration in the myoplasm amounts to some tens of millimoles, is evidently important for muscular function. It has recently been shown that it has a stabilizing action on the system of the sarcoplasmic reticulum (SR), and also increases the working capacity of fatigued muscle [2, 6, 12]. However, the molecular mechanism of the increase in the efficiency of work of the Ca pump of the SR produced by carnosine have not been explained. We know that during exhausting physical exertion lipid peroxidation (LPO) processes in the membranous structures of the muscle cell are activated [8, 9]. It has also been recently shown that accumulation of LPO products in the membranes of SR results in inhibition of their Ca-transporting ability [4]. It has accordingly been suggested that the stabilizing action of carnosine may be due to its ability to inhibit LPO. The ability of carnosine to inhibit accumulation of LPO products interacting with 2-thiobarbituric acid was demonstrated previously on suspensions of mitochondria [5].

The aim of this investigation was to study the ability of carnosine to prevent accumulation of LPO products in membranes of SR and thus to stabilize the enzyme system for Ca^{++} transport.

EXPERIMENTAL METHOD

Fragments of SR were isolated from skeletal muscles of the hind limbs of *Rana temporaria* by differential centrifugation [2, 11]. The medium for preserving membrane vesicles contained 0.25 M sucrose, 10 mM histidine, pH 6.8, at 2°C and bovine serum albumin (5 mg/ml). The preparations of SR were kept at −25°C. ATPase activity was measured by a pH-metric method [2]. Fe^{++} + ascorbate-dependent LPO in SR was induced as described previously [1]. Carnosine was determined quantitatively by thin-layer chromatography of its NBD* derivative [7], followed by scanning under dual beam difference conditions (470 nm against 600 nm) on a Hitachi 557 (Japan) spectrophotometer. Protein was determined by Lowry's method [10]. The carnosine (β-alanyl-L-histidine) was obtained from Fluka (Switzerland), the piperazine-N,N-bis-2-ethanesulfonic acid (PIPES) was from Serva (West Germany), the NBD chloride from Sigma (USA), and the 2-thiobarbituric acid and $FeSO_4$ (analytical grade) and the remaining reagents were from Reakhim (USSR).

EXPERIMENTAL RESULTS

On incubation of fragments of frog SR in the presence of the LPO induction system (Fe^{++} + ascorbate) both Ca^{++} transport and Ca-ATPase activity were inhibited. The first process was disturbed by a greater degree, so that the Ca/ATP ratio fell (Fig. 1). Incidentally, in SR membranes isolated from mammalian skeletal muscles, LPO induced much more rapid inhibition of the Ca^{++}-transporting function than of Ca-ATPase activity [1]. Addition of carnosine to the incubation medium in the process of LPO prevented damage to the Ca^{++} transport enzyme system; the protective effect of carnosine, moreover, was exhibited equally both on the efficiency of transport and on ATPase activity. For instance, whereas in the absence of carnosine total inhibition of Ca^{++} transport and of Ca-ATPase activity was recorded after incubation for only 10 min, in the presence of carnosine (25 mM) a complete inhibitory effect was achieved only after 40 min.

*4-chloro-7-nitrobenzo-2-oxa-1,3-diazole.

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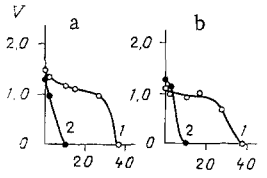

Fig. 1

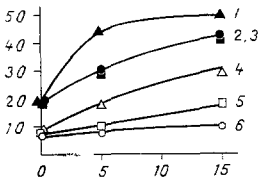

Fig. 2

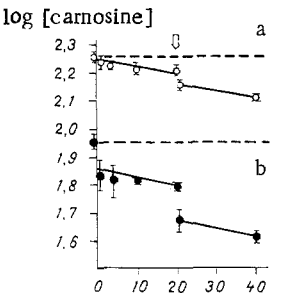

Fig. 3

Fig. 1. Ca-ATPase activity of SR (a) and efficiency of Ca^{++} accumulation (b) as functions of preceding LPO. Abscissa, incubation time (in min); ordinate, a) Ca-ATPase activity (in μmoles P_i/mg protein of SR/min); b) Ca/ATP. Incubation medium KCl 100 mM, $MgCl_2$ 10 mM, ATP 1 mM, potassium oxalate 10 mM, $CaCl_2$ 200 nanomoles per sample, SR protein 0.3 mg, PIPES 20 mM (pH 6.8); volume of samples 2.2 ml. LPO was induced in medium with 20 mM carnosine (1) and 20 mM PIPES (2), $FeSO_4$ 10 nmoles/mg SR protein, ascorbate 100 μM.

Fig. 1. Accumulation of MDA during LPO in frog SR membranes. Abscissa, incubation time (in min); ordinate, MDA concentration (nanomoles/mg protein). Concentration of SR protein 1 mg/ml. 1-3) PIPES, 5, 10, and 25 mM respectively; 4-6) carnosine, 5, 10, and 25 mM respectively. $FeSO_4$ 10 nanomoles/mg protein; ascorbate, 200 μM.

Fig. 3. Changes in carnosine concentration during ascorbate-dependent LPO. Abscissa, incubation time (in min); ordinate, carnosine content in sample (in relative units). a) 1 mg SR protein (in ml), 10 nanomoles $FeSO_4$/mg SR, 100 μM ascorbate, intial carnosine concentration 5 mM; b) 2 mg SR protein/ml, 40 nanomoles $FeSO_4$/mg SR, 200 μM ascorbate, initial carnosine concentration 2.5 mM. Arrow indicates addition of more ascorbate.

Carnosine had a similar protective action on the enzymic Ca^{++} transport system during LPO developing in the course of "aging" of SR preparations during keeping *in vitro*.

The question arises, what is the mechanism of action of carnosine? It follows from the data given in Fig. 2 that the protective action of carnosine is based on its ability to inhibit the accumulation of LPO products. In fact, accumulation of carbonyl products of LPO (especially malonic dialdehyde), found in the absence of carnosine, is inhibited by increasing concentrations of carnosine; its addition in a concentration of 25 mM, for instance, completely prevented induction of LPO in SR membranes. It should be pointed out that carnosine can not only prevent activation of LPO, but can also depress the original concentration of malonic dialdehyde (MDA) in SR membranes (Fig. 2).

The concentration of carnosine in the incubation medium decreased as a result of the action of LPO inducers. For instance, after induction of LPO for 40 min in SR membranes (2 mg/ml) the carnosine concentration fell by 30% (Fig. 3).

It can be concluded from these data that the protective effect of carnosine on the Ca^{++} enzyme transport system against LPO products is based on a mechanism consisting of inhibition of free-radical LPO in the membrane, in the course of which carnosine is utilized. It can be tentatively suggested that it is this property which lies at the basis of the physiological function of dipeptides in skeletal muscles [2, 3, 12].

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REGULATION OF PURIFIED PIG HEART NAD-ISOCITRATE DEHYDROGENASE BY CALCIUM IONS AND cAMP-DEPENDENT PROTEIN KINASE

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Activation of NAD-dependent isocitrate dehydrogenase (ICDH), the key enzyme of the Krebs cycle, by catecholamines and cAMP has been discovered in recent years and the suggestion has been made that the effect of catecholamines is realized through cAMP-dependent protein kinase (PK) [2]. Ca^{++} ions do not affect ICDH from potato [9] and inhibit the enzyme from muscles of insects [3, 9, 14] and mollusks [14]. Inhibition of ICDH from rat heart also was reported initially [13], but activation of ICDH from the mammalian heart, liver, and other tissues by Ca^{++} ions was later described [4, 9]. All these investigations were conducted on whole mitochondria or crude mitochondrial preparations.

The aim of this investigation was to verify, on a purified preparation of the enzyme, the view that PK participates in the activation of ICDH by catecholamines and cAMP and to study the effect of Ca^{++}, and also the possible involvement of calmodulin.

EXPERIMENTAL METHOD

ICDH from pig heart was purified in three stages, with the method described previously as the basis [11]. The technique adopted included fractionation with ammonium sulfate, chromatography on DEAE-Sephadex, and affinity chromatography on blue sepharose [1]. The enzyme was purified by 2500 times to a specific activity of 28 μmoles NADH/min/mg protein. The homogeneity of the resulting preparation was confirmed by polyacrylamide gel electrophoresis. The holoenzyme from pig brain was generously provided by E. S. Severin, from rabbit brain by M. D. Kurskii, and from rat liver mitochondria by B. Jergil. The phosphorylation reaction was conducted by the method in [7, 10]. Preparations of ICDH after each stage of purification were used as substrates. PK activity was monitored by determining incorporation of the label into histones and protamine. ICDH activity was measured at 340 nm by the method in [11]. When the effect of Ca^{++} was studied its concentration was controlled by means of EGTA buffers. Mg^{++} and not Mn^{++} was used as the coenzyme for ICDH, for the latter can displace Ca^{++} from the EGTA–Ca complex. The concentration of ionized Ca^{++} was calculated by an equation taking account of the binding of Ca^{++} by isocitrate and ADP [3]. In each series of experiments between four and seven samples of ICDH were used.

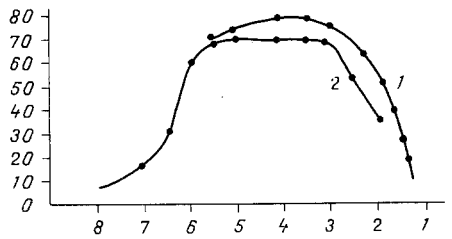

Fig. 1. Effect of various Ca^{++} concentrations on ICDH activity. Abscissa, Ca^{++} concentration; ordinate, ICDH activity (in nanomoles/min/mg protein). 1) Medium without EGTA; 2) medium with EGTA.

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