7. P. S. Brady, L. J. Brady, and D. E. Ullrey, J. Nutr., 109, 1103 (1979).
8. D. L. Gree and A. L. Tappel, Life Sci., 28, 2425 (1981).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, et al., J. Biol. Chem., 193, 257 (1951).
10. A. M. Rubtzov, O. D. Lopina, and A. A. Boldyrev, Gen. Physiol. Biophys., 1, 161 (1982).

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

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KEY WORDS: calcium ions, protein kinase, heart, NAD-isocitrate dehydrogenase.

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.

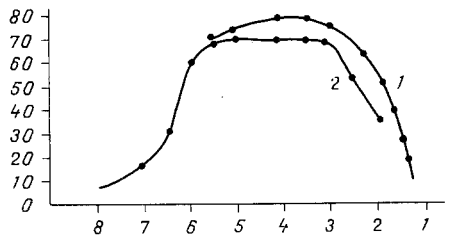

Fig. 1. Effect of various Ca^{++} concentrations on ICDH activity. Abscissa, Ca^{++} concentration; ordinate, ICDH activity (in nanomoles/min/mg protein). 1) Medium without EGTA; 2) medium with EGTA.

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TABLE 1. Activation of NAD-Isocitrate Dehygrogenase by Ca^{++} Ions at Different States of Purification of the Enzyme and Influence of Trifluoroperazine on This Effect (M ± m)

Reaction conditions	Enzyme preparation		
	after fractionation with ammonium sulfate		after blue sepharose
	– trifluoperazine	+ trifluoperazine	
Nothing added	5,74±1,32	5,61±1,41	7,28±1,15
ADP – 2 mM EGTA – 5 mM Ca^{2+} < 1 pM	4,85±1,11	5,85±1,63	5,56±1,72
ADP – 5 mM EGTA – 5 mM $CaCl_2$ — 5 mM Ca^{2+} — 15,5 μM	20,81±2,58	20,84±1,92	27,81±3,92

Legend. Reaction system contained 0.1 mM isocitrate, 2 mM NAD^+, 8 mM $MgCl_2$ in 50 mM Tris-acetate buffer, pH 7.0. Activity of the enzyme after fractionation with ammonium sulfate expressed in nanomoles NADH/min/mg protein, after blue sepharose in micromoles NADH/min/mg protein.

EXPERIMENTAL RESULTS

Although PH is known not to possess strict specificity for protein substrates, three different kinds of PK were tested. PK from rabbit muscles did not phosphorylate ICDH. No effect likewise was obtained by the use of PK from pig brain, so that any possible objection on the grounds of species specificity could be rejected. Mitochondrial PK, which differs from cytoplasmic, likewise did not incorporate ^{32}P from P-γ-ATP into ICDH.

PK from different sources, both in the presence and in the absence of 10^{-6} M cAMP, likewise did not effect enzyme activity of the highly purified ICDH. cAMP itself in this concentration likewise had no effect.

Activation of ICDH, incidentally, is observed only after preincubation of cAMP with intact mitochondria [2]. The activating effect of cAMP disappears if incubated with mitochondria disintegrated beforehand with Triton X-100, although such treatment solubilizes and activates endogenous mitochondrial PK [7]. Some other, not protein-kinase, mechanism of activation of ICDH by catecholamines and cAMP may perhaps exist.

The second stage of the work was to investigate the effect of Ca^{++} on ICDH. In a concentration of 15 μM Ca^{++} increased activity of the enzyme at all stages of purification, including after blue sepharose (Table 1), when the enzyme was already electrophoretically homogeneous [1]. Activation of ICDH was manifested as a sharp decrease in the Michaelis constant (K_m) for isocitrate (by 3-5 times at different stages of purification, $P < 0.001$); V_{max} was unchanged. These data are in good agreement with results obtained with crude mitochondrial preparations from mammalian tissue [4].

The range of activating concentrations of Ca^{++} was very wide: from $2.7 \cdot 10^{-7}$ to 10^{-3} M (Fig. 1). At higher Ca^{++} concentrations (10^{-3} M) ICDH was inhibited in both the presence and absence of EGTA. However, such high Ca^{++} concentrations have no physiological significance. The efficiency of very low Ca^{++} concentrations suggested involvement of an intermediate protein, most probably calmodulin, for it is the most universal Ca^{++} receptor and has recently been found in mitochondria [6]. However, trifluoperazine (100 μM), an inhibitor of calmodulin, caused absolutely no change in the activating effect of Ca^{++} after fractionation with ammonium sulfate, i.e., before separation of the accompanying proteins from ICDH.

No effect of any kind of PK on purified ICDH, neither activation nor phosphorylation of the enzyme, could thus be established. Activation of Ca^{++} in a homogeneous preparation was demonstrated for the first time and is of considerable importance for the elucidation of the

mechanisms of this phenomenon. The effect of Ca^{++} is evidently not mediated by calmodulin, for it is unchanged by trifluoperazine. The impression is gained that Ca^{++} acts directly on ICDH. However, more complex mechanisms cannot yet be ruled out: phosphorylation of some unknown protein intermediary or the participation of another Ca^{++}-receptive protein. For instance, it has recently been shown that phosphorylation of a mitochondrial protein with molecular weight of 3500 daltons is increased by glucagon [12]. Ca^{++} metabolism in the mitochondria is modified by stimulators not only of α-adrenoreceptors, but also of β-adrenoreceptors [5], and also by cAMP [8]. Perhaps Ca^{++} ions activate ICDH through the intermediary of catecholamines and cAMP.

LITERATURE CITED

1. T. V. Kolpakova and V. V. Mezhevikin, in: Regulatory Effects and Metabolism of Monoamines and Cyclic Nucleotides [in Russian], No. 3, Krasnoyarsk (1981), pp. 31-35.
2. V. I. Kulinskii and L. V. Trufanova, Dokl. Akad. Nauk SSSR, 239, No. 6, 1479 (1978).
3. B. A. Bulos and B. Sactor, Anal. Biochem., 95, 62 (1979).
4. R. M. Denton, D. A. Richards, and J. G. Chin, Biochem. J., 176, 899 (1978).
5. T. P. Goldston and M. Crompton, Biochem. J., 204, 369 (1982).
6. O. Hatasse, M. Tokuda, T. Itaho, et al., Biochem. Biophys. Res. Commun., 104, 673 (1982).
7. T. Henriksson and B. Jergil, Biochim. Biophys. Acta, 588, 380 (1979).
8. H. A. Juzu and E. S. Holdsworth, J. Membr. Biol., 52, 185 (1980).
9. J. G. McCormack and R. M. Denton, Biochem. J., 196, 619 (1981).
10. M. V. Nesterova, L. P. Saschenko, V. Yu. Vasiliev, et al., Biochim. Biophys. Acta, 377, 271 (1975).
11. W. C. Shen, L. Mauck, and R. F. Colman, J. Biol. Chem., 249, 7942 (1974).
12. A. H. Vargas, A. P. Halestrap, and R. M. Denton, Biochem. J., 208, 221 (1982).
13. H. Vaughan and E. A. Newsholme, FEBS Lett., 5, 124 (1969).
14. V. A. Zammit and E. A. Newsholme, Biochem. J., 154, 677 (1976).

EFFECT OF CHOLERA ENTEROTOXIN ON THE ANTITOXIC SYSTEM OF THE RAT SMALL INTESTINE AND LIVER

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An important aspect of the study of the effect of cholera toxin (CT) *in vivo* is the state of the detoxication enzyme system (DES). This system has evolved in order to activate potentially harmful cytotoxic substances formed during metabolism and to render harmless toxins entering from outside. The first function has been studied reasonably well. Activity of enzymes of the mono-oxygenase system (stage I of detoxication) and activity of epoxide hydratase (EH) and glutathione-SH-transferase (GT) (stage II) has been investigated in the liver, kidneys, lungs, and intestine [4, 5, 10, 13], and in the blood cells [12]. The second function of DES is only beginning to be investigated [6, 8]. In our view, to understand the role of the antitoxic system in mobilization of the defensive forces of the body in infectious pathology it is essential to examine the state of those of its enzymes, such as EH, cytochrome-450, uridine disphophoglucuronyl-transferase, GT, superoxide dismutase (SOD), and glutathione peroxidase (GP), in the small intestine, which is the target for bacterial toxins, and also in the liver, the principal detoxicating organ.

The aim of this investigation was to study the principal enzyme of nonspecific defense of the body against the toxic action of oxygen (SOD) and of GP in cytosols of the mucous membrane of the small intestine and in the liver of rats exposed to the action of CT.

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