PHOSPHORYLATION OF PHOSPHOLAMBAN IN EXPERIMENTAL MYOCARDIAL INFARCTION AND RESISTANCE TO PROTEOLYSIS DURING PHOSPHORLYATION

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One of the most characteristic features of pathological processes in the myocardium is disturbance of contractility of the muscles. It has recently been shown that Ca^{++} ions and cyclic nucleotides control the contractility of heart muscle. This control is effected in particular, by cAMP-dependent and Ca-dependent (in the presence of calmodulin) phosphorylation of target proteins in the myofibrils, sarcolemma, and sarcoplasmic reticulum (SR).

For instance, close interaction has been demonstrated between Ca-ATPase of SR and phospholamban – a low-molecular-weight component of the SR membrane. The essence of this interaction is evidently that cAMP-dependent phosphorylation of phospholamban changes the velocity of active Ca^{++} transport through the membrane of SR cisterns [5, 6]. Phosphorylation of heart muscle phospholamban is thus evidently of great physiological importance, but this process in myocardial ischemia remains virtually unstudied.

An attempt was accordingly made to investigate cAMP-dependent phosphorylation of phospholamban in experimental myocardial infarction and to study the resistance of phospholamban to proteolysis.

EXPERIMENTAL METHOD

Experiments were carried out on mongrel dogs weighing 15-18 kg. Operations on the animals were carried out under intravenous thiopental anesthesia with controlled respiration. Myocardial infarction was induced by ligation of the descending branch of the left coronary artery. The dogs were killed 24 h after ligation of the coronary artery by respiratory arrest induced by injection of muscle relaxants. Necrotic tissue of the left ventricle was studied and intact areas of myocardium from animals undergoing the operation served as the control.

Microsomal fraction was isolated by our modification of Bidlack and Shamoo's method [4]. cAMP-dependent protein kinase was obtained as described previously [2]. Microsomal proteins were phosphorylated in medium of the following composition: 0.1 M KCl, 0.5 mM $MgCl_2$, 0.15 mM [γ-^{33}P]-ATP (3.3 Ci/mmole), 2.5 mM EGTA, 15 mM NaN_3, 20 mM NaF, 40 mM Tris-HCl, pH 7.0, in the presence or absence of 10^{-6} M cAMP or cGMP, and 0.2 mg/ml of protein kinase. Incorporation of ^{33}P into microsomal proteins was determined in the residue after triple washing with 10% TCA. To estimate incorporation of ^{33}P into phospholamban, microsomes were separated by disk electrophoresis [7], using 12% concentrating and 6% separating polyacrylamide gel in the presence of sodium dodecylsulfate. Radioactivity of phospholamban was measured as described previously [3]. Protein kinase activity in the microsomal fraction was measured as accumulation of arginine in the medium [1].

EXPERIMENTAL RESULTS

The experiments showed (Fig. 1a) that the mean basal phosphorylation level in preparations of intact microsomes after incubation for 5 min at 30°C was 0.22 nmole ^{33}P/mg protein, and it was increased about 3.1 times by the presence of 1 μM cycle AMP (endogenous phosphorylation) and by 4.7 times in the presence of 1 μM cAMP and 0.2 mg/ml of protein kinase (exogenous phosphorylation). A different picture was observed in experimental infarction (Fig. 1b). In this case activation of phosphorylation by cAMP led to an increase of only 1.6 times in the

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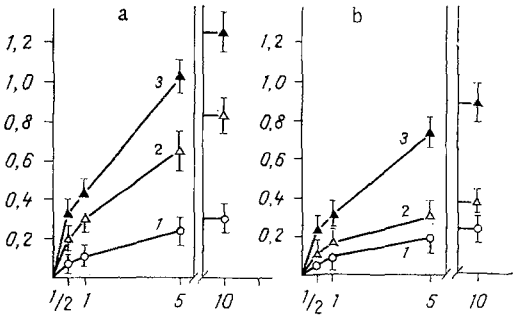

Fig. 1. Incorporation of ^{33}P into microsomal proteins in myocardial infarction: a) normal, b) infarction. 1) Basal level of phosphorylation; 2) phosphorylation in presence of cAMP; 3) phosphorylation in presence of cAMP and protein kinase. Abscissa, time (in min); ordinate, incorpation of ^{33}P (in nmoles/mg protein).

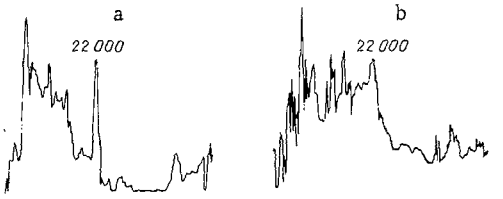

Fig. 2. Densitograms of gels with microsomal fractions: a) normal, b) infarct.

basal phosphorylation level. Addition of protein kinase to the incubation medium caused a marked increase in the degree of phosphorylation of microsomal proteins — by about fourfold compared with the basal level. Comparison of levels of phosphorylation of microsomal proteins from intact and damaged regions revealed an about equal basal degree of phosphorylation, a substantial difference (by 2.3 times) in the case of cAMP-stimulated endogenous phosphorylation, and a tendency toward equalization of the levels of exogenous phosphorylation, although in this case also the ability of the microsomes of the affected myocardium to undergo phosphorylation remained lower than in the control. This last fact is evidence that disturbance of the ability of microsomal proteins to undergo phosphorylation in myocardial infarction can be explained not only by the different cAMP-stimulated activity of endogenous protein kinases in the intact and damaged zones, but also by the different ability of molecules of the target proteins in these regions of the myocardium to undergo phosphate modification.

To determine phosphorylation of phospholamban, microsomes were subjected to electrophoretic separation and phospholamban was identified by its relative molecular weight of 22,000 daltons. Analysis of the densitograms of the stained gels showed partial degradation of several protein components of the microsomes in the infarct including phospholamban (Fig. 2). This fact calls for an investigation of the proteinase activity in microsomes of the affected muscle. The results of these experiments are given in Fig. 3. The values of the activities measured and the character of their dependence on pH of the medium were virtually unchanged in the presence of 1% histone as substrate. Proteinase activity in microsomes from undamaged areas of the myocardium was relatively low, namely 6-8 nmoles arginine/mg protein/h, and the sensitivity of the method used did not permit its dependence on pH of the medium to be established. The partial degradation of microsomal protein components which was observed was thus evidently associated with the marked increase in endogenous proteinase activity in infarction.

The results of investigation of cAMP-dependent phosphorylation of phospholamban are given in Table 1.

The addition of cGMP stimulated phosphorylation, although not by the same degree as cAMP. The fall in the levels of endogenous and exogenous phosphorylation is evidence, just as in the case of unfractionated microsomes, of the phosphate modification of phospholamban in infarction.

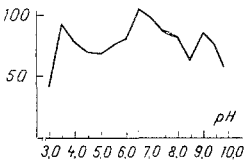

Fig. 3. Protein kinase activity (in nmoles arginine/mg protein/h) in microsomes from myocardial infarct at different pH values.

TABLE 1. Phosphorylation of Phospholamban in Myocardial Infarction (M ± m)

Composition of samples	% of protein-bound ^{33}P	
	normal	infarct
Control	22±4 (n=8)	20±5 (n=9)
cGMP (1 μM)	49±8 (n=9)	24±6 (n=9)
cAMP (1 μM)	68±7 (n=12)	26±8 (n=12)
cAMP(1 μM) + protein kinase	100±6 (n=9)	71±8 (n=9)

Legend. Here and Table 2, n denotes number of experiments.

TABLE 2. Phosphorylation of Phospholamban in Myocardial Infarction Combined with Trypsin Treatment (M ± m)

Composition of samples	Protein-bound ^{33}P (phosphorylation in control for normal and affected muscle taken as 100)	
	normal	infarct
cAMP followed by trypsin	94±8 (n=11)	67±8 (n=12)
cAMP + protein kinase followed by trypsin	97±9 (n=12)	81±7 (n=12)
Trypsin followed by inhibitor, followed by cAMP + protein kinase	7±0,9 (n=9)	9±1,3 (n=9)

This fact, together with the observed increase in proteinase activity in preparations of microsomes from the necrotic myocardium justifies the undertaking of a series of experiments in which phosphorylation of phospholamban was combined with its treatment with trypsin (Table 2).

In this series of experiments microsomes were treated with trypsin (0.25 mg/ml) in incubation medium for phosphorylation not containing cAMP, protein kinase, and ATP. After 5 min of incubation the trypsin inhibitor phenylmethylsulfonyl fluoride (0.5 mM) was added, followed after 5 min by cAMP and ATP and, in some of the experiments, protein kinase. In those cases when phosphorylated microsomes were treated with trypsin, phosphorylation was carried out as described above. Trypsin was added after 10 min, and treatment with the inhibitor followed 5 min later. In control series the trypsin and inhibitor were added simultaneously after phosphorylation in the presence of protein kinase.

It will be clear from Table 2 that preliminary treatment with trypsin led to the almost complete inability of phospholamban to undergo phosphorylation, in both necrotic and intact areas of the myocardium. Phosphorylation of phospholamban of healthy muscle had an absolute protective effect against trypsin; this was not observed in infarction in the case of both endogenous and exogenous phosphorylation. These data are evidence of correlation between the degree of phosphorylation of phospholamban from affected and intact myocardium and of its vulnerability to trypsin: whatever the result of structural reorganization of phospholamban due to its phosphorylation, it will be evidence that under these circumstances sites of the protein molecule that are sensitive to proteinase attack are modified. The disturbance of the ability of phospholamban to undergo phosphorylation in myocardial infarction discovered in

these experiments is evidently due to its greater vulnerability to trypsin compared with protein from the intact muscle, and it is a particularly important discovery because of the increased proteinase activity in SR of the affected muscle.

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BIOCHEMICAL PARAMETERS OF ERYTHROCYTES PROTECTED FOR CRYOPRESERVATION BY 1,2-PROPANEDIOL AND GLYCEROL

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During long-term low-temperature conservation of blood cells [1, 2, 11, 13, 14] protective media containing glycerol are used most often. However, the need to remove the glycerol from the cells before transfusion limits the use of this method on a wide scale, and this has led to the search for less laborious and more efficient ways of preservation. The method of low-temperature preservation of erythrocytes, protected by 1,2-propanediol (1,2-PD), developed at the Institute for Problems in Cryobiology and Cryomedicine, Academy of Sciences of the Ukrainian SSR [3], is interesting in this respect. It allows considerable simplification of the procedure of removal of the cryoprotector from the cells before use after preservation.

This paper gives comparative results of a study of the time course of the concentrations of ATP, 2,3-diphosphoglycerate (2,3-DPG), and also Na^+ and K^+ ions in erythrocytes after keeping for 5 days at 4°C in suspending media 8b and 8c of the Central Research Institute of Hematology and Blood Transfusion [6] after freezing under protection of 1,2-PD and glycerol. These biochemical parameters determine the structural integrity of the erythrocytes and the degree to which they perform their oxygen-transport function in the recipient's blood stream [7, 15].

EXPERIMENTAL METHOD

Blood from donors preserved at 4°C for not more than 24 h after collection was used. Low-temperature preservation of blood under protection by 1,2-PD and glycerol, including freezing to −196°C, warming on a water bath, and removal of the cryoprotectors, was carried out in accordance with the procedures described [3, 6]. The blood, after removal of the preservatives, was resuspended in media 8b and 8c. After storing for 0, 12, 36, and 120 h at 2-4°C the ATP [8] and 2,3-DPG levels in the cells were determined. The intracellular concentration of Na^+ and K^+ cations was determined at the same periods of storage by flame photometry. To remove extracellular Na^+ and K^+ the blood was washed three times at 4°C with a solution containing 30 mM Tris-HCl, pH 7.4, and 90 mM $MgCl_2$ [9].

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