ACID PHOSPHATASE ACTIVITY AND STATE OF THE LYSOSOMAL MEMBRANES IN A PRIMARY CULTURE OF NEONATAL RAT CARDIOMYOCYTES DURING ANOXIA

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One approach to the study of myocardial function under anoxic and ischemic conditions is to create a model of these states in cardiomyocyte culture [12]. Simulation of anoxia with a cell culture creates a convenient approach to the study of myocardial metabolism under extremal conditions. The investigator is able to use a pure homogeneous cell population, virtually free from contamination with connective tissue cells. In many pathological states there is a change in the permeability of the lysosomal membranes and enzymes escape into the cytoplasm of the cell. It has been shown, for instance, that in myocardial ischemia lysosomal enzymes pass out into the cytoplasm, where they become activated, and this is accompanied by an increase in free enzyme activity in heart muscle homogenates, together with a simultaneous decrease in the lysosome-rich fraction [2]. Investigations have shown that cyclic nucleotides (cAMP and cGMP) help to regulate stability of lysosomal membranes [2, 4, 8]. It has been suggested that cAMP and cGMP have opposite actions on permeability of lysosomal membranes. cAMP and its analogs have a marked stabilizing effect on lysosomal membranes, whereas elevation of the cGMP level leads to their labilization and enhances the outflow of acid hydrolases into the cell cytoplasm. This action of the cyclic nucleotides has been demonstrated in various pathological states (traumatic shock, hypokinesia, myocardial infarction) [27]. However, these investigations have been conducted on the whole organism, during exposure of the myocardium to various neurohumoral and hormonal factors, and also during long-term exposure to traumatic influences [1, 2].

The aim of this investigation was to study disturbances of the stability of lysosomal membranes and changes in cyclic nucleotide and ATP levels in a model of anoxia with a primary culture of cardiomyocytes after oxygen deprivation for 1 h.

EXPERIMENTAL METHODS

Experiments were carried out on a primary culture of cardiomyocytes obtained from the hearts of 3-day-old rats after maceration in 0.1% collagenase solution [9] for 2.5 h at 37°C. The cells were grown in Carrel's flasks in Eagle's medium with the addition of 10% serum (a mixture of equal quantities of embryonic calf serum and bovine serum), and used in a state of confluent monolayer on the 4th-6th day of culture, when the cells could actively contract. Anoxia was simulated in a constant-temperature gas-flow chamber, through which a gas mixture consisting of 5% O_2, 5% CO_2, and 90% N_2 was passed for 1 h. To aggravate the conditions of anoxia, in some experiments the cell culture was incubated in Hanks's salt solution. The control culture was incubated in an atmosphere with 5% CO_2 and 20% O_2. To determine acid phosphatase (AP) activity the cell culture was homogenized in 0.25 M sucrose, containing 1 mM EDTA (pH 7.4). The homogenate was subjected to differential centrifugation. The residue obtained after centrifugation at 25,000g was resuspended and treated with Triton X-100 in a final concentration of 0.1%, after which the bound enzyme activity was determined. Free activity was measured in the supernatant, and total AP activity was investigated in the primary homogenate, treated with Triton X-100 in a final concentration of 0.1%. AP activity was determined spectrophotometrically (4-nitrophenyl phosphate was used as the substrate [7]) and expressed in micromoles of product formed during hydrolysis of the substrate in 1 h per

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TABLE 1. AP Activity in Culture of Cardiomyocytes under Anoxic Conditions (in μmoles/mg total protein/h)

Conditions of incubation	AP activity		
	total	free	bound
Eagle's medium with add. of 10% serum			
control	2,37±0,30 (5)	0,30±0,005 (5)	0,435±0,075 (5)
anoxia	2,22±0,27 (7)	0,60±0,008* (7)	0,240±0,060* (7)
Hanks's soln.			
control	2,16±0,20 (5)	0,30±0,045 (5)	0,195±0,015 (5)
anoxia	1,89±0,15 (7)	0,63±0,008* (7)	0,135±0,008 (7)

Legend. Here and in Table 2: number of observations shown in parentheses; *$p < 0.001$.

TABLE 2. ATP and Cyclic Nucleotide Concentrations in Culture of Cardiomyocytes in a Model of Anoxia

Conditions of incubation	ATP, μmoles/mg total protein	cAMP	cGMP
		pmoles/mg total protein	
Eagle's medium with add. of 10% serum			
control	17,90±0,87 (8)	19,60±0,84 (25)	1,66±0,13 (12)
anoxia	11,95±1,70 (6)	22,80±2,80 (7)	1,47±0,32 (8)
Hanks's soln.			
control	15,87±0,83 (10)	20,25±1,72 (8)	1,83±0,16 (17)
anoxia	4,70±1,20* (6)	24,90±1,65 (13)	1,57±0,23 (12)

mg total protein. The concentrations of cAMP, cGMP, and ATP were measured in $HClO_4$ extracts. Cyclic nucleotide levels were determined by the standard method, using kits from Amersham International (England); the ATP concentration was measured by the luciferin–luciferase method [13] and expressed in picomoles per milligram protein. Protein was determined by Lowry's method. The functional capacity of the cells was estimated by the presence of contraction.

EXPERIMENTAL RESULTS

During incubation of the monolayer culture of cardiomyocytes in a gas flow with 5% O_2 in normal growth medium for 1 h the cells ceased to contract but remained viable. In fixed preparations stained with hematoxylin, morphological changes in the form of vacuolation, contractures, and evaginations on the cell surface were found in about 10% of the cells under the microscope. However, these changes were reversible in character. After rehabiliation of the cell cultures for 24 h under normal conditions, the cells resumed their contractile activity.

Under anoxic conditions, lysosome-bound activity in cardiomyocytes kept in normal growth medium was reduced by about half, whereas free activity was increased ($p < 0.001$); total enzyme activity was unchanged (Table 1). When the normal growth medium was replaced by Hanks's salt solution, a further significant decrease took place in lysosome-bound AP activity. Thus after incubation of the cardiomyocytes under anoxic conditions for 1 h a disturbance of permeability of the lysosomal membranes was observed, and this was aggravated as the conditions of anoxia were made more stringent.

Table 2 gives the results of investigation of ATP and cyclic nucleotide concentrations in cardiomyocytes incubated under the same conditions. When cells kept in growth medium were subjected to anoxia, a very small fall of the ATP level was observed. Replacement of the growth medium by Hands's salt solution under anoxic conditions led to a reduction of the ATP concentration by two-thirds compared with the control level ($p < 0.01$). There is evidence that during incubation of a suspension of adult rat cardiomyocytes under anoxic conditions, with exclusion of glucose from the medium, the ATP concentration falls sharply; when the ATP level fell below 40% of its initial value, moreover, the structural integrity of the cardiomyocytes was disturbed and release of some cytosol enzymes, including LDG and MDH, into the incubation medium was observed [11]. It will be clear from Tables 1 and 2 that the sharp fall of the ATP level was accompanied by more marked labilization of the lysosomal membranes.

Investigation of the cyclic nucleotide concentrations in a primary culture of neonatal rat cardiomyocytes under anoxic conditions showed (Table 2) that when the cells were incubated in normal growth medium or in Hanks's salt solution, no sharp changes in the cAMP or cGMP levels were observed after 1 h of oxygen deprivation. In other words, no clear relationship could be found between the concentrations of cyclic nucleotides and changes in permeability of the lysosomal membranes of the cardiomyocytes under these experimental conditions. It will be recalled that primary cultures of cardiomyocytes on newborn animals have a high level of cyclic nucleotides [6], about 10 times higher than in adult rat heart muscle. We know from the literature that in early postnatal development the cAMP level in the cardiomyocytes rises sharply [3] and sensitivity of the adenylate cyclase system to the action of β-adrenomimetics is reduced [10].

It has been shown that cAMP-dependent protein kinases are found not only in the cytosol, but also in various subcellular fractions: nuclear, mitochondrial, and microsomal. They are firmly bound with subcellular membranes and are not adsorbed on them [14]. However, investigations have been carried out which have led to doubts about the presence of cAMP-dependent protein kinases firmly bound to lysosomal membranes. Nevertheless, the authors cited consider that phosphorylation of the proteins of lysosomal membranes with the participation of cAMP-dependent protein kinases is possible, and that this mechanism plays an important role in regulation of the functions of lysosomes [7].

It can also be postulated that the change in permeability of the lysosomal membranes in pathological states is due to damage to cAMP-dependent protein kinases and to weakening of their ability to phosphorylate proteins, including membrane proteins [7].

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