CHANGES IN MOLECULAR PROPERTIES OF Ca-ATPase OF THE SARCOPLASMIC RETICULUM FOLLOWING MEMBRANE MODIFICATION BY CHOLESTEROL

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One method of modifying membranes by cholesterol (Ch) in vivo is by the development of hypercholesterolemia (HCh), as a result of which a marked rise of the plasma Ch level is accompanied by an increase in its concentration in membranes of rabbit erythrocytes [9], rat brain microsomes [1], mitochondria [11] and microsomes of the heart [13], and sarcoplasmic reticulum (SR) of rabbit skeletal muscles [7, 13]. Depending on the period of cholesterol feeding, significant disturbances are found in the functioning of membrane-bound enzymes, especially of Na,K-ATPase of brain microsomes [1] and erythrocyte membranes [9], of Ca^{++}-dependent ATPase (Ca-ATPase) of cardiac microsomes [13] and SR of skeletal muscles [7, 13]. In the course of its function Ca-ATPase undergoes conformational transformations connected with ion transport through the membrane [3, 4, 6, 14]. One indicator of conformational transformations in the Ca-ATPase molecule is a change in reactivity of the SH-groups of the enzyme during their interaction with thiol modifiers, for the ability of protein SH-groups to interact with thiol reagents is determined by their immediate environment: by the presence of charged groups and hydrophobic regions in the vicinity, and by participation of SH-groups in hydrogen or hydrophobic interaction [8]. It can be postulated that even minor conformational changes in the region surrounding SH-groups can result in sharp changes of their reactivity.

The aim of the present investigation was to determine the number and reactivity of SH-groups of SR Ca-ATPase during HCh and to study their properties on binding of ATP and Ca^{++} by the enzyme. NBD chloride (4-chloro-7-nitrobenzo-2-oxo-1,3-diazole) was used as the thiol reagent.

EXPERIMENTAL METHOD

Experiments were carried out on 24 male chinchilla rabbits weighing 2.5-3 kg. Control rabbits (n = 9) were kept on the standard animal house diet. Experimental animals (n = 15) received a diet with the addition of Ch (1 g/kg body weight) for 1 month (n = 5), 3 months (n = 5), and 6 months (n = 5). The Ch concentration in the serum was determined by the method described previously [11, 12] and in SR membrane preparations by thin-layer chromatography [2]. The SR fraction was obtained from white muscles of the hind limbs of the decapitated rabbits by differential centrifugation [5] and kept at −20°C in 1 M sucrose with 25 mM imidazole, pH 7.0, with the addition of serum albumin to the keeping medium in a dose of 5 mg/10 mg SR protein. Ca^{++} transport into SR vesicles and Ca-dependent ATPase activity were measured in the presence of oxalate by pH-metry [10]. The protein composition of SR was analyzed by polyacrylamide gel electrophoresis with sodium dodecylsulfate [15]. The gels were then treated as described previously [4] and scanned at a wavelength of 580-590 nm on a Chromoscan-200 instrument (England). Protein was determined by Lowry's method with 1% sodium deoxycholate [10]. The reaction between NBD chloride (concentration 750 μM) and free SH-groups of SR Ca-ATPase was carried out in medium containing 100 μM) KCl, 0.5 mM EGTA, 30 mM imidazole, pH 7.0, at 20-22°C, with SR protein concentration of 0.1-0.2 mg/ml medium. The measurements were made on an SP-1700 spectrophotometer (Pye Unicam, England). The change in optical density through formation of the S derivative of NBD chloride was calculated as the difference between optical densities at wavelengths of 420 and 550 nm. Subsequent calculations were as

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TABLE 1. Kinetic Parameters of SH-Groups of SR Ca-ATPase in Normal Blood and in Hypercholesterolemia, and Effect of Ca^{2+} Ions and ATP on the Reaction of SH-Group and NBD Chloride (M ± m)

Experimental conditions	Kinetic parameters of SH-group				
	n	n_1	k_1	n_2	k_2
Normal					
without addition	12,20±0,78	6,50±0,63	0,110±0,004	5,70±0,68	0,016±0,001
with 30 μM ATP	—	5,50±0,06	0,090±0,017	—	—
with 3 mM ATP	11,00±0,87	2,90±0,60*	0,070±0,004*	7,30±0,95	0,020±0,003
with 0.5 mM $CaCl_2$	13,1±0,4	7,40±0,50	0,170±0,028*	5,60±0,41	0,022±0,005
Cholesterol diet					
1 month					
without addition	8,85±0,51*	4,40±0,39*	0,090±0,010*	4,40±0,78	0,022±0,002
with 30 μM ATP	—	4,80±0,26	0,090±0,012	—	—
with 3 mM ATP	9,40±0,59*	2,80±1,07*	0,060±0,006*	6,50±1,19	0,020±0,001
with 0,5 mM $CaCl_2$	8,40±0,32*	4,30±0,42	0,12±0,03*	4,10±0,22	0,030±0,003
3 months					
without addition	13,10±0,99	8,00±0,54*	0,09±0,006*	5,1±0,63	0,020±0,002
with 30 μM ATP	—	8,10±0,38	0,11±0,009	—	—
with 3 mM ATP	14,70±0,94*	3,30±0,43*	0,05±0,009*	11,30±0,93*	0,026±0,005
with 0,5 mM $CaCl_2$	13,6±0,72	10,10±1,04	0,17±0,40*	3,50±0,40	0,030±0,002
6 months					
without addition	8,30±0,52*	5,00±0,58	0,130±0,018	3,70±0,17	0,013±0,003
with 30 μM ATP	—	5,40±0,35	0,140±0,004	—	—
with 3 mM ATP	8,70±0,61*	2,30±0,10*	0,080±0,002*	5,4±0,6	0,015±0,005
with 0,5 mM $CaCl_2$	9,60±0,75*	5,30±0,84	0,130±0,018	4,3±0,7	0,020±0,002

Note. n — Total amount of SH-group; n_1 and n_2 — amount of fast and slow SH-groups (in moles/10^5 g protein); k_1 and k_2 are the modification constants corresponding to fast and slow SH-groups (in min^{-1}); asterisk — $P < 0.001$.

described in [4]. The concentration of SH-groups was determined by using the coefficient of molar extinction of the S-NBD derivative, namely $13 \cdot 10^3$ M^{-1}. To determine the number of types of SH-groups and velocity constants of their interaction with NBD chloride, a graph of concentration of free SH-groups as a function of time was plotted in semilogarithmic coordinates. The experimental data were subjected to statistical analysis (by Student's t test). The ATP, histidine, serum albumin, and reagents for electrophoresis were obtained from Reanal, Hungary; the sucrose, Tris-buffer, and NBD chloride from Serva (West Germany); the $MgCl_2$, $CaCl_2$, and oxalate from Merck (West Germany); the EDTA, EGTA, and imidazole from Sigma (USA); and the remaining reagents were from the "Soyuzreaktiv" Combine (USSR) and were of the chemically pure or optically pure grades.

EXPERIMENTAL RESULTS

The development of HCh in the rabbits was accompnaied by elevation of the serum Ch level from 20-25 to 1200-1500 mg%. The normal Ch/phospholipid ratio in the SR membranes was 0.025 mg/mg and it rose after cholesterol feeding for 1 month to 0.034 mg/mg (i.e., by 36%), for 3 months to 0.055 mg/mg (by 120%), and for 6 months to 0.06 mg/mg (by 136%). It was shown electrophoretically that 78-80% of the SR membrane preparations in control animals was accounted for by a polypeptide chain with molecular weight of 100 kilodaltons, and by a very small quantity of minor proteins, consisting mainly of Ca^{++}-binding proteins. The Ca^{++}-accumulating capacity, Ca^{++}-dependent ATPase activity, and efficiency of work of the Ca-pump of SR during HCh were reduced almost to half the normal level [7, 13]. On modification of the SR membranes by NBD chloride, the total number of free SH-groups accessible for NBD chloride normally (in the absence of substrate and activator ion in the incubation medium) averaged 12 moles/10^5 g protein, in agreement with data in the literature for this modifier [4, 6, 14, 16]. According to their modification rate, the reacted SH-groups could be subdivided into two kinetic types: fast and slow; the velocity constant of interaction (modification constant) of the fast SH-groups, moreover, was much higher than that of the slow SH-groups (Table 1). Addition of ATP to the incubation medium led to changes in reactivity of the SH groups. In the presence of low ATP concentrations (30 μM), sufficient to saturate the hydrolytic center of Ca-ATPase in the absence of its hydrolysis (Ca^{++} not present in the incubation medium), the changes affected 1 mole/10^5 g protein of SH groups of the fast type. In HCh this was not found. It can be postulated that this SH-group is "masked" by binding of ATP in the hydrolytic center of Ca-ATPase [3, 4, 14]. The presence of excess Ch in the SR membranes in the animals with HCh may perhaps prevent binding of ATP in the hydrolytic center of Ca-ATPase. In the presence of high ATP concentrations (3 mM), sufficient for binding of ATP with the allo-

steric site of Ca-ATPase, changes take place in the conformation of the enzyme, namely a significant decrease in reactivty under normal conditions by a further 3 moles/10^5 g protein of SH-groups of the fast type and their transition into the slow type of SH-groups, and a decrease in the modification constant (Table 1). In HCh, after 1 and 6 months on a high cholesterol diet, changes were found in reactivity of SR SH-groups on their interaction with NBD chloride, accompanied by a decrease in the total number of free SH-groups accessible for the thiol reagent, on average by 3-4 moles/10^5 g protein, mainly on account of a decrease in the number of SH-groups of fast type. SR preparations obtained from skeletal muscles after 3 months of cholesterol feeding were the exception: The total number of SH-groups in them which reacted with NBD chloride was practically the same as normal, on account of an increase in the number of SH-groups of fast type. The presence of 3 mM ATP in the incubation medium in the experiments with HCh led to a decrease in reactivity of SH-groups of the fast type. Compared with the control (incubation medium without substrate) after 1 month of cholesterol feeding the number of SH-groups of fast type which reacted with NBD chloride was 2 moles/10^5 g protein less, after 3 months it was 5 moles/10^5 g protein less, and after 6 months of cholesterol feeding 3 moles/10^5 g protein less. The modification constant of these SH-groups was significantly lowered. Addition of Ca^{++} ions ($5 \cdot 10^{-4}$ M) to the incubation medium in the absence of ATP led under normal conditions to an increase in reactivity of the fast type of SH-groups by 1 mole/10^5 g protein and to an increase in the modification constant from 0.11 to 0.17 min^{-1}, in all probability reflecting changes in conformation of Ca-ATPase taking place during binding of Ca^{++} in the activation site of Ca-ATPase, but not during its transport through the membrane [4, 17]. Ca^{++} ions had no significant effect on modification of SH-groups of the slow type. In HCh, after 1 and 6 months of cholesterol feeding, Ca^{++} ions had virtually no effect on the number of fast SH-groups but accelerated reactivity of 2 moles/10^5 g protein of SH-groups of the fast type after 3 months of cholesterol feeding as a result of their transfer from the number of slow SH-groups. The modification constant of these SH-groups increased from 0.09 to 0.17 min^{-1}.

The results are thus evidence of a change in the kinetic properties of the SH-groups under the influence of substrate and activator ion, accompanied by changes in conformation of the SR Ca-ATPase. During modification of SR membranes by Ch a decrease in reactivity of the SH-groups of Ca-ATPase was found during their interaction with NBD chloride and the "masking" effect of high ATP concentrations was intensified. It can be tentatively suggested that excess Ch, modifying the SR membranes, changes the conformation of Ca-ATPase, and thereby renders the works of the Ca pump less efficient.

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