MEMBRANE FLUIDITY, CHOLESTEROL AND ALLOSTERIC TRANSITIONS OF MEMBRANE-BOUND Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase AND ACETYLCHOLINESTERASE FROM RAT ERYTHROCYTES*

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1. Introduction

Modifications in the fluidity of the lipid phase of mammalian membranes are obtained by changes in its fatty acid composition [1] or cholesterol content [2]. In the case of bacterial membranes, which do not contain sterols, only the first possibility is feasible under physiological conditions [3]. The fluidity of the membrane fatty acids as a physiological regulator for the allosteric behavior of rat erythrocyte [4] and *Escherichia coli* [5] membrane-bound enzymes has been stressed in recent works of this laboratory. These facts prompted investigations about the mechanism of action of cholesterol on the allosteric behavior of animal membrane-bound enzymes.

In a previous paper [4] the determination of the Hill coefficient for the inhibition by F^- of the erythrocyte membrane-bound Mg^{2+}-ATPase, $(Na^+ + K^+)$ ATPase (EC 3.6.1.5) and acetylcholinesterase (EC 3.1.1.7) from rats fed diets differing in their fat supplement, led to the conclusion that there was a correlation between the cooperativity of the two latter enzymes and the fluidity of the membrane fatty acids expressed as the ratio double bond index/saturated fatty acids. A remarkable finding was the opposite-sign character of these correlations: the increasing of the ratio was accompanied by a parallel increase in the acetylcholinesterase cooperativity and a decrease in the cooperativity of the $(Na^+ + K^+)$-ATPase. The Mg^{2+}-ATPase was insensitive to the fluidity parameters.

Several experiences were carried out as described in this paper, to test the cholesterol action on the kinetic parameters of the three membrane-bound enzymes under in vivo physiological conditions.

2. Materials and methods

Male Sprague–Dawley rats (220–320 g) grown after weaning on basic diet supplemented with 5% corn oil were used [4]. While one group continued receiving the same diet (controls), the other was fed basic diet supplemented with 5% corn oil and 1% cholesterol. Details concerning erythrocyte ghost preparation, measurement of enzymatic activities and calculation of the kinetic parameters, as well as protein, cholesterol and lipid phosphorous determinations were given in the preceding paper [4].

Membrane solubilization was carried out by mixing 0.5 ml of membrane suspension in 20 mmol sodium phosphate buffer (pH 7.4) containing 1.5–2.0 mg of ghost protein with 1.5 ml of 1% Triton X-100 in water. After standing for 15 min at room temperature, the sample was centrifuged for 1 hr at 100 000 *g* at 4°C and the pelleted material was discarded.

The in vitro membrane loading with cholesterol was carried out in the following way: 0.1 ml of a chloroformic solution containing 260 nmoles of cholesterol was evaporated under a nitrogen stream. Then

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Table 1
Values of n and K_i for the inhibition by F^- of the acetylcholinesterase, $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase from rats fed corn oil and corn oil plus cholesterol supplemented diets.

Diet	N* Acetylcholinesterase		$(Na^+ + K^+)$-ATPase		Mg^{2+}-ATPase		Activation***
	n	K_i (mM)	n	K_i (mM)	n	K_i (mM)	%
Corn oil	(5) 1.50 ± 0.04**	1.76 ± 0.12	2.07 ± 0.14	1.35 ± 0.20	1.67 ± 0.02	2.00 ± 0.27	40 ± 6
Corn oil + cholesterol	(5) 1.00 ± 0.04	2.50 ± 0.15	3.60 ± 0.18	1.30 ± 0.04	1.32 ± 0.14	2.50 ± 0.50	35 ± 3
	$P < 0.0001$	$P < 0.01$	$P < 0.001$	n.s.	$P < 0.05$	n.s.	n.s.

* Number of experiments.
** The results are expressed as mean ± s.e.m.
*** Calculated as the ratio × 100 of the $(Na^+ + K^+)$-ATPase and the total ATPase.

1.0 ml of membrane suspension containing about 0.4 mg of protein was added. After shaking during 5 min at room temperature, it was transferred to another tube and washed twice with the same buffer. The membranes were pelleted each time during 15 min at 35 000 g. The control preparation was processed in the same way but omitting the cholesterol in the chloroform aliquot. When radioactive cholesterol was employed, the same loading procedure was used. [$^{14}C_4$]cholesterol (spec. act. 58 mCi/mmole) was purchased from New England Nuclear and diluted with cold steroid as indicated in table 3. The final membrane suspension was solubilized in 5 ml of scintillating fluid [6] and counted in a Packard Tri Carb Scintillator Counter.

3. Results

3.1. *Cholesterol feeding and cooperative parameters of membrane-bound enzymes.*

The values of n and K_i for the inhibition by F^- of the Mg^{2+}- ATPase, $(Na^+ + K^+)$-ATPase and acetylcholinesterase from animals fed a corn oil plus cholesterol supplemented diet are shown in table 1. As can be seen, the Hill coefficient for the two latter enzymes shift in an opposite way when compared with the control animals: from 2.0–3.6 and from 1.5–1.0 respectively. The variations in the Mg^{2+}-ATPase are of doubtful significance. The presence of cholesterol in the diet did not influence the % of activation, K_i and specific activity (not shown) for the ATPase system. On the other hand, while acetylcholinesterase specific activity remains unchanged, a distinct increase in the K_i is observed. No changes were found in the cholesterol and lipid phosphorous content (not shown). The data presented in table 1 were obtained from five pairs of animals fed control and cholesterol-supplemented diet for a period of 1–4 weeks. When a rat grown on corn oil supplemented diet was analyzed (1 ml of blood was obtained by cardiac puncture under ether anesthesia) and was then fed the cholesterol-supplemented diet for 24 hr and analyzed again, the following results were found for acetylcholinesterase: the value of n decreased from 1.5–0.9 and the K_i increased from 1.6–2.3 mM. This fact indicates that the effect of cholesterol feeding is soon reflected in the kinetic parameters.

3.2. *Effect of membrane solubilization on the cooperative kinetic of acetylcholinesterase*

As shown in table 2, when the erythrocyte membrane is solubilized by Triton X-100, significant changes in the values of n and K_i for acetylcholinesterase from cholesterol-fed animals were observed. The values changed from 0.9–1.6 and from 3.0–1.6 mM respectively, while the same kinetic parameters of the enzyme from control animals were not modified by similar treatment. These results show that cholesterol feeding influences the allosteric behavior of the enzyme only in the membrane-bound state.

3.3. *In vitro cholesterol loading and cooperative kinetic of acetylcholinesterase*

The effect of in vitro cholesterol loading of the membrane was tested. As further can be seen in table

Table 2
Changes in the kinetic parameters of acetylcholinesterase by different in vitro treatment.

Diet	Enzyme preparation	n	K_i (mM)
Corn oil	Membrane	1.4	1.9
	100 000 g supernatant of solubilized membrane	1.6	1.8
Corn oil + cholesterol	Membrane	0.9	3.0
	100 000 g supernatant of solubilized membrane	1.6	1.6
Corn oil	Membrane	1.4	2.0
	in vitro cholesterol-loaded membrane	0.9	2.9
	100 000 g supernatant of solubilized membrane after cholesterol loading	1.3	2.2
Corn oil	Membrane	1.6	2.3
	in vitro cholesterol-loaded membrane	0.8	3.5

Conditions for supernatant obtention and cholesterol loading of the erythrocyte membranes are given in the text.

Table 3
In vitro cholesterol loading capacity of erythrocyte membrane from rats fed corn oil and corn oil plus cholesterol supplemented diets.

Diet	Enzyme preparation	Total cpm incorporated	Net cholesterol incorporation (μmoles/mg protein)
Corn oil	Membrane	21 900	
	Membrane after treating with cold steroid	4 200	0.026
Corn oil + cholesterol	Membrane	9 600	
	Membrane after treating with cold steroid	7 500	0.003

0.2 ml fo membrane suspension (0.082 mg of protein) were treated with 5.2 nmol of radioactive cholesterol (spec. act. 8190 cpm/nmol) as described in the text. The same procedure was carried out on samples previously loaded with the same amount of cold steroid. The radioactivity were measured on aliquots of membrane suspension which were washed before dissolving in the scintillator fluid.

2, when membranes from control animals were loaded, a decrease in the value of n and an increase in the K_i for the inhibition by F^- of the acetylcholinesterase were obtained. Also it can be observed that the reversion of values of the kinetic parameters by solubilization is also obtained in this case. It is interesting to note that in spite of marked changes in the kinetic parameters obtained after shaking the membranes with cholesterol, we failed to detect a significative increase in the membrane cholesterol content by ordinary colorimetric methods. All these facts closely resemble the in vivo situation.

3.4. *Determination of the cholesterol loading capacity of the membranes*

It has been stated that the amount of cholesterol in the rat red cells is not dependent on the unsaturation of the dietary fat [4, 7]. Monsen et al. [8] did not find any cholesterol increase in erythrocytes from cholesterol-fed rats. These facts and our own failure in detecting any increment in the cholesterol content of the membrane in vivo as well as in vitro, induced us to think that the amount of the steroid eventually incorporated would be under the level of detection of the colorimetric methods. We then decided to test the in vitro cholesterol loading capacity of the cell membranes using radioactive steroid. The experimental conditions were the same as those used for testing the in vitro change of the kinetic parameters.

As can be seen in table 3, the total cpm incorporated by the membrane of rats fed with a cholesterol-supplemented diet is less than half of the quantity incorporated by the membrane from control animals. The total radioactivity incorporated includes marcation due to the turnover of the cholesterol already present in the membrane, as well as incorporation of additional amounts of the steroid. The net incorporation can be calculated considering that the marcation due to turnover is the radioactivity incorporated after loading the membranes with a saturating quantity of cold steroid. The difference between the cpm incorporated before and after the treatment with cold cholesterol represents a net increment of 1–3% in the total cholesterol content of the membrane from animals fed the control diet. It is important to observe that the net incorporation into the membranes of cholesterol-fed animals is about nine-fold less.

4. Discussion

Modifications in the fatty acid composition of red cell phospholipids change the allosteric behavior of the $(Na^+ + K^+)$-ATPase and acetylcholinesterase [4]. This mechanism for the dietary cholesterol action was ruled out in our experiments since we did not detect any difference in the fatty acid composition of the erythrocyte lipids from the animals used in the experiments of table 1 (not shown). This was a plausible possibility taking into account the plasmatic acetylcholine–cholesterol acyl transferase activity [9] and the active exchange of fatty acyl residues between erythrocytic and plasmatic phospholipids [10]. Furthermore, the in vitro experiments clearly show that additional cholesterol modifies the kinetic parameters of the enzymes in presence of the same membrane fatty acid composition. The behavior of the solubilized acetylcholinesterase from cholesterol-loaded membranes indicate that the effect of the steroid is an indirect one, requiring a particulate structure to which the lipid and the enzyme are attached. The lower in vitro incorporation of cholesterol to membranes from cholesterol-fed animals shows that the dietary cholesterol is incorporated to the erythrocyte membrane in vivo. All these facts make unlikely the possibility that the cholesterol feeding affects the enzymes in another way than by cholesterol incorporation into the membrane.

Although we use only F^- as allosteric effector in these experiments, it is worthwhile to take into account that the cooperative behavior of the ATPase system with the ligands Na^+ and K^+ is also responsive to changes in the phospholipid fatty acid composition of the membrane to which the enzyme is bound [11, 12].

Current models of biological membranes point out that although most of the phospholipids are forming a bilayer, 20–30% of them would be sharing specific associations with functionally active proteins [13]. Several recent findings agree with this hypothesis [14–16]. A high cholesterol–phospholipids molar ratio of about 0.8 is found in rat erythrocytes [17] and unpublished results). It is known that cholesterol interacts with erythrocyte phospholipids reducing its molecular area [18, 19]. Electron spin resonance studies indicate that these interactions cause a diminution in the local fluidity of the lipid matrix of the erythrocyte membrane [2]. In addition, the fact that the interactions between phospholipids and cholesterol are function of the unsaturation and distribution of the double bonds in the acyl chains [20] as well as the chain length [21] and the evidence that cholesterol is localized in certain areas of the erythrocyte membrane [22] make likely that a slight increment in the cholesterol content would decrease the fluidity of strategic zones of the membrane. This hypothesis, coupled with the finding that the allosteric behavior of membranous enzymes is influenced by membranal fatty acid composition [4, 5, 11, 12, 23–27] and by slight variations in its cholesterol content as reported here, suggest that the in vivo local changes in the lipid zone where the membrane-bound enzymes are located might be a physiological control for the biological processes in which cooperative changes take place. According to this suggestion, it would be interesting to evaluate the allosteric parameters of the enzymes from membranes under some pathological conditions where their fatty acid composition and/or cholesterol content are altered, as hereditary spherocitosis [28], obstructive jaundice [29], phosphatidylcholine–cholesterol acyltransferase deficiency [30], atherosclerosis [31] and others.

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