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Increased Na^+,K^+-pump activity in erythrocytes of rabbits fed cholesterol

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Summary. Na^+,L^+-pump activity, intracellular sodium, potassium and magnesium concentrations and membrane cholesterol content were studied in erythrocytes of rabbits fed cholesterol. The average activity of the Na^+,K^+-pump in erythrocytes of rabbits with high plasma cholesterol was twice that in erythrocytes of control animals. Analysis showed a positive correlation between the pump activity and plasma cholesterol. The sodium content in erythrocytes correlated negatively with plasma cholesterol, as well as with the Na^+,K^+-pump activity. No significant differences in potassium and magnesium concentrations or in the membrane cholesterol content were observed between the two groups. The results indicate that modulation of the pump activity by cholesterol is not necessarily mediated by changes in the membrane viscosity.

Keywords: erythrocytes, Na^+,K^+-pump, intracellular sodium, plasma cholesterol, membrane cholesterol, cholesterol-fed rabbits

Membrane cholesterol has been shown to modulate the activity of numerous membrane-associated processes such as glucose transport, anion transport, ATP–ADP-exchange, etc. its effect being either inhibition or activation (review by Yeagle 1985). The Na^+,K^+-ATPase of plasma membranes (the enzyme responsible for maintaining low intracellular sodium and high intracellular potassium concentrations) has been reported to be inhibited by high levels of membrane cholesterol *in vitro* in human erythrocytes (Giraud *et al.* 1981; Yeagle 1983) and in basolateral membranes from bovine kidney (Yeagle *et al.* 1988). The inhibition is most probably caused by the increase in the anisotropic motional ordering of the lipid bilayer of the membrane which may lead to an increase in the ordering of the Na^+,K^+-ATPase conformation (Yeagle 1989). Indirect evidence for possible sodium pump activation by plasma cholesterol reduction in lovastatin-treated patients which could be associated with changes in membrane lipid composition has been presented by Weder *et al.* (1990).

Yeagle *et al.* (1988) found, however, that when low levels of cholesterol are present in the membrane, cholesterol is capable of activating the enzyme (Yeagle *et al.* 1988), which is best explained by a direct sterol–protein interaction (Yeagle 1989). Some other data also indicate that cholesterol is essential for Na^+,K^+-ATPase function. Cholesterol reactivated the Na^+,K^+-ATPase separated from the electrical organ of *Electrophorus electricus* (Järnefelt 1972); oxidation of cholesterol in human erythrocyte ghosts reduced the enzyme activity (Seiler & Fiehn 1976); the ouabain-inhibited K^+ influx in human red cells was significantly lowered by the partial removal of cholesterol from the membranes (Poznansky *et al.* 1973).

The inhibition of Na^+,K^+-ATPase activity by high levels of membrane cholesterol has been found in erythrocytes of rabbits kept on a cholesterol-rich diet

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(Torkhovskaya *et al.* 1980; Uysal 1986). However, an enhanced Na^+,K^+-ATPase activity in erythrocyte ghosts from rabbits fed cholesterol has also been reported previously (Wells & Tsubone 1969). Therefore, although it is an established fact that high membrane cholesterol inhibits Na^+,K^+-ATPase activity in erythrocytes and in some other tissues (Yeagle 1989), probably because of an increase in the membrane viscosity, the possibility of cholesterol stimulation of the enzyme not mediated by changes in the motional ordering of the membrane, appears to require further investigation.

In the present work we studied the activity of the Na^+,K^+-ATPase-mediated Na^+,K^+-pump represented by the ouabain-inhibited component of $^{86}Rb^+$ influx in erythrocytes of rabbits fed cholesterol. Apart from significant differences in the mean pump activity between the animals with diet-induced hypercholesterolaemia and the controls, correlations of the pump activity with plasma cholesterol and with intracellular sodium were observed.

Materials and methods

Animals and reagents

Male chinchilla rabbits weighing 2.3–2.5 kg were obtained from the Institute's vivarium. Eight rabbits received daily, apart from the standard rabbit chow, 0.5 g of cholesterol in vegetable oil; 3 control animals were given the same amounts of vegetable oil. The animals of the two groups were kept on their respective diets for 2 months.

Cholesterol (Reakhim, Russia) was purified by double recrystallization from hot ethanol. $^{86}RbCl$ was purchased from NPO Izotop (St Petersburg, Russia). Ouabain was from Sigma Chemical Company (St Louis, MO, USA); heparin from Biochemi (Vienna, Austria); n-hexane, isopropanol, HCl, trichloroacetic acid, liquid scintillation cocktail ZS-8M – from Reakhim (Russia). All other chemicals were from Serva Feinbiochemica (Heidelberg, Germany).

Preparation of erythrocytes

Blood was collected from an ear vein of the animals into heparinized (50 U/ml of blood) medium containing either 150 mM NaCl and 5 mM Na^+-phosphate buffer, pH 7.4 (for ion transport and membrane cholesterol measurements) or 160 mM choline chloride (for intracellular ion concentration measurements). Erythrocytes were sedimented (1000 ***g***, 10 min) and washed several times in the respective mediums. All operations with erythrocytes were carried out at low temperatures (0–2°C).

Na^+,K^+-pump activity

Fifty μl of packed erythrocytes were put into 0.5 ml of medium B containing 145 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM K_2HPO_4, 10 mM glucose, 10 mM HEPES-tris (pH 7.4). After centrifugation erythrocyte pellets were resuspended in 200 μl of medium B containing 1 μCi/ml $^{86}RbCl$ (for measuring the total $^{86}Rb^+$ influx) and in the same medium containing 1mM ouabain (for measuring the ouabain-insensitive $^{86}Rb^+$ influx). After 30 minutes incubation, 1 ml of ice-cold 160 mM choline chloride was added and the cells were then washed twice with the same solution. Triton X-100 (0.5%, 0.5 ml) and trichloroacetic acid (10%, 0.5 ml) were added to the erythrocytes. After centrifugation 0.8 ml of the supernatant was mixed with 5 ml of the liquid scintillation cocktail. Radioactivity was measured using a liquid scintillation counter (Rack Beta-II, LKB Wallac, Finland). The activity of the Na^+,K^+-pump was calculated as the ouabain-inhibited component of $^{86}Rb^+$ influx.

Intracellular cation concentrations

Two hundred μl of packed erythrocytes were washed 5 times in 2 ml of 160 mM choline chloride and transferred into a vial containing 6 ml of 0.1 M HCl. The vials were shaken thoroughly and centrifuged (3000 ***g***, 10 min). An aliquot of the supernatant was diluted with distilled water; concentrations of sodium, potassium and magnesium were measured using an atomic absorption spectrophotometer (AAS-3, Karl Zeiss, Germany) after appropriate calibration. The intracellular water content was determined as previously described (Freedman & Hoffman 1979).

Membrane cholesterol

Erythrocyte ghosts were prepared by the method of hyposmotic haemolysis (Dodge *et al.* 1963). The cells were lysed in tenfold volume of 5 mM Na^+-phosphate buffer (pH 7.4) for 10 min at 0°C. After centrifugation (2000 ***g***, 10 min) the supernatant was transferred into another vial and centrifuged at 20 000 ***g*** for 20 min. The ghosts were washed twice in phosphate buffer and resuspended in distilled water. Lipids were extracted using n-hexane – isopropanol mixture (3 : 2 v/v). The total cholesterol content was determined by the method previously described (Courchaine *et al.* 1959).

Table 1. Na^+,K^+-pump, intracellular cations and membrane cholesterol in erythrocytes of cholesterol-fed and control rabbits

	Plasma cholesterol (mg/100 ml)	Na^+,K^+-pump (mmol K^+/l cells/h)	$[Na^+]_i$	$[K^+]_i$	$[Mg^{2+}]_i$	Membrane cholesterol (mg/mg of protein)
			(mmol/l of intracellular water)			
Control rabbits ($n = 3$)	55 ± 13	0.81 ± 0.04	21.21 ± 0.63	170.3 ± 3.4	4.81 ± 0.18	0.078 ± 0.011
Cholesterol fed rabbits ($n = 8$)	643 ± 62**	1.62 ± 0.13*	16.79 ± 1.21	164.6 ± 4.2	4.52 ± 0.20	0.075 ± 0.006

Results are presented as mean ± S.E.
* $P < 0.01$.
** $P < 0.001$.

Membrane protein was measured using the Coomassie Brilliant Blue dye-binding assay (Gogstad & Krutnes 1982).

Plasma cholesterol

For estimation of plasma cholesterol clotted blood was collected and serum serparated. The total cholesterol content was measured by the method of Courchaine *et al.* (1959).

Statistical analysis

Results were expressed as means ± SE. All comparisons were made by Student's *t*-test. Correlation analysis was possible because of the comparatively high variability of the plasma cholesterol level within the experimental group. A linear regression procedure was used for correlation analysis; the correlation factor significance level (*P*) was determined by Fischer's *Z*-method.

Results

The results of our study are summarized in Table 1.

Cholesterol feeding caused a 12-fold increase in the plasma cholesterol level in rabbits of the experimental group as compared to control animals.

The mean Na^+,K^+-pump activity in erythrocytes of rabbits fed cholesterol was double that in erythrocytes of control rabbits. The pump activity showed significant positive correlation with plasma cholesterol ($r = 0.885$, $P < 0.01$) (Figure 1).

The mean sodium concentration in erythrocytes of rabbits with high plasma cholesterol was 21% lower than that of control animals. Although this difference did not reach statistical significance ($0.05 < P < 0.1$), intracellular sodium correlated negatively with plasma cholesterol ($r = -0.752$, $P < 0.05$) and with the Na^+,K^+-pump activity ($r = -0.779$, $P < 0.02$) (Figure 2). We failed to observe any significant differences in the red cell K^+ and Mg^{2+} concentrations between the two groups (Table 1).

Cholesterol content in erythrocyte ghosts from rabbits of the two groups was statistically the same (Table 1) and did not correlate with the plasma cholesterol level.

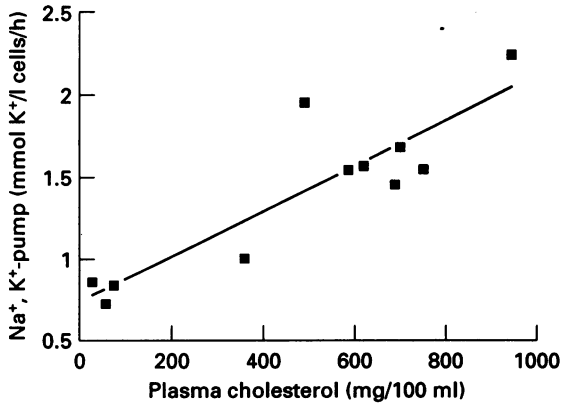

Figure 1. Correlation between Na^+,K^+-pump activity in rabbit erythrocytes and plasma cholesterol ($r = 0.885$, $P < 0.01$).

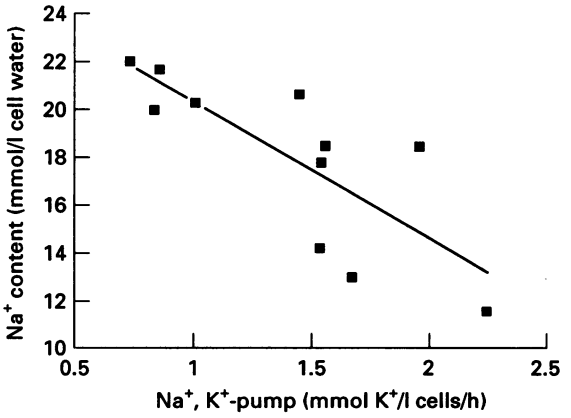

Figure 2. Correlation between intracellular Na^+ concentration and Na^+,K^+-pump activity in rabbit erythrocytes ($r = -0.779$, $P < 0.02$).

Discussion

Activation of the Na^+,K^+-ATPase in erythrocyte ghosts from hypercholesterolaemic rabbits has been briefly reported previously (Wells & Tsubone 1969); however, no data on the membrane cholesterol content were presented. In other previous studies on the Na^+,K^+-ATPase activity in red cells of cholesterol-fed rabbits (Torkhovskaya *et al.* 1980; Uysal 1986) the diet-induced increase in the plasma cholesterol level was accompanied by an increase in membrane cholesterol and a substantial inhibition of the Na^+,K^+-ATPase. These data, together with some results on other animal models and *in vitro* studies (review by Yeagle 1989) prove that high membrane cholesterol inhibits the enzyme activity, probably by reducing its ability to undergo conformational transitions.

The reason for the unchanged membrane cholesterol content in erythrocytes of cholesterol-fed rabbits in the present study is rather obscure. However, there is evidence that high content of cholesterol in erythrocytes of guinea-pigs (Ostwald & Shannon 1964), dogs (Cooper *et al.* 1980), and rabbits (Westerman *et al.* 1970) is a hallmark of haemolytic anaemia. Very high plasma cholesterol levels in rabbits did not increase the content of cholesterol in red cells when haemolytic anaemia was not present (Westerman *et al.* 1970). As cholesterol enrichment of membranes (Panasenko *et al.* 1987) and inhibition of Na^+,K^+-ATPase (Uysal 1986) have been reported to be influenced by formation of lipid peroxides, suppression of lipid peroxidation may have been the cause of the unchanged membrane cholesterol in this study.

An adequate interpretation of the Na^+,K^+-pump activation in erythrocytes of cholesterol-fed rabbits certainly needs further investigation. We can exclude activation by an increase in the intracellular substrate (Na^+) concentration, as the sodium content correlated negatively with the pump activity. Magnesium concentration in red cells was independent of the plasma cholesterol level and therefore could not affect the activity of Na^+,K^+-ATPase. It should be noted that measuring the number of ouabain-binding sites of erythrocytes membranes from hypercholesterolaemic and control animals could elucidate whether we are dealing with enzyme activation or with a greater number of enzyme molecules in erythrocytes of rabbits fed cholesterol.

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