Effect of cholesterol on phosphate uptake by human red blood cells

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1. INTRODUCTION

Cholesterol is a major component of the plasma membrane of mammalian cells [1], including human red blood cells which have a cholesterol:phospholipid molar ratio of 0.8 [2]. Cholesterol modulates the fluidity of the membrane by ordering liquid-crystalline state phospholipids and by disordering gel state phospholipid [3–8]. In model and biological membranes in the liquid-crystalline state, cholesterol reduces the non-electrolyte permeability of the lipid bilayer [9–11]. Cholesterol also affects the activity of membrane-associated enzymes. Enrichment of red bloods with cholesterol by incubation with cholesterol-containing liposomes [12,13] results in inhibition of Na^+,K^+ co-transport [14]. The Na^+/K^+ ATPase activity was enhanced by cholesterol depletion [15], and inhibited by cholesterol enrichment [16]. The membrane cholesterol content of erythrocytes has been shown to affect anion transport, as measured by sulfate exchange [17] and by pyruvate-chloride exchange [18]. Cholesterol has also been suggested to affect the stability [3,19–21], and microviscosity [13,22] of the red cell membrane and the exposure and distribution of its membrane proteins [22,23].

In the experiments reported here, the effect of cholesterol on anion transport, as measured by phosphate uptake into human red blood cells was studied. Membrane cholesterol levels were altered by incubation of erythrocytes with liposomes containing various amounts of cholesterol [12,13] or by converting the membrane cholesterol to cholest-4-en-3one by exposing cells to cholesterol oxidase [20,21]. The effect of cholestenone on anion transport was also determined by enriching cells with this cholesterol derivative. The temperature-dependence of phosphate uptake into erythrocytes containing various levels of cholesterol was determined. It was found that anion transport was inhibited by cholesterol enrichment. However, cholestenone had very similar effects. This suggests that there is not a specific interaction between cholesterol and Band 3, the anion transport protein.

2. MATERIALS AND METHODS

Outdated human red blood cells were kindly provided by the Canadian Red Cross (Edmonton, Alberta). Cholesterol, egg phosphatidylcholine, cholestenone and penicillin were obtained from Sigma. Cholesterol oxidase was a product from Boehringer-Mannheim. [^{32}P]phosphate was obtained from New England Nuclear.

Cells were washed 3-times in buffer A, 28.5 mM sodium citrate, 205,3 mM sucrose (pH 7.4) and suspended (25% hematocrit) in buffer B 150 mM NaCl, 5 mM KCl, 6.3 mM sodium phosphate, 0.5 mM $CaCl_2$, 10 mM glucose, penicillin, 1000 μM/ml; pH 7.4. Liposomal suspensions were made as in [17]. Enrichment suspensions, containing 150 mg egg phosphatidyl choline and 190 mg cholesterol (C/P = 2.5) dissolved in benzene, were

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lyophilized to dryness, suspended in 10 ml buffer B and sonicated for 1 h at 4°C. The sonicated mixture was centrifuged with albumin added to the solution (5 mg/ml) at 35000 × *g* for 30 min at 4°C to remove vanadium particles. The supernatant was diluted with buffer B (containing 5 mg/ml albumin) to a final concentration of about 6 mg lipid/ml. Depletion suspensions contained 350 mg egg phosphatidyl choline and were prepared as above; cholestenone enrichment suspensions contained 50–100 mg cholestenone, 0–100 mg cholesterol, and 100 mg egg PC. Cells were incubated with an equal volume of liposomal suspension at 37°C in a shaking water bath for 5–10 h (depletion), 18–24 h (enrichment), or 1–20 h (cholestenone enrichment).

2.1. *Treatment with cholesterol oxidase*

Control and enriched cells were exposed to cholesterol oxidase as in [20,21]. Cells were washed in 12 vol. buffer C, 0.5 mM sodium phosphate, 310 mM sucrose (pH 7.5) at 4°C, suspended in buffer C (10% hematocrit), and equilibrated at 37°C in a shaking water bath. Enzyme was added (1–10 units/ml) and cells were incubated for 60 min. At the end of the incubation, cells were cooled on ice and washed twice with buffer A.

2.2. *Phosphate uptake assay*

Washed cells were diluted to 50% hematocrit with buffer A and equilibrated at 30°C in a shaking water bath for 5 min. To start the assay, 25 μl of buffer A containing [^{32}P]phosphate (0.02 Ci/μl) was added to cells, the suspension was quickly vortexed and returned to water bath. At 5, 10, 15, 20, 30, 45, 60, 90 and 120 min, cells were vortexed and a 0.125-ml aliquot taken. Aliquots were transferred to a 1 ml Eppendorf microfuge tube, centrifuged for 2 min in an Eppendorf microfuge, and two 25 μl aliquots of the supernatant taken. The supernatant aliquots were added to scintillation vials and counted in 5 ml Aquasol.

2.3. *Effect of temperature on phosphate uptake*

Phosphate uptake assays were performed for control, enriched and depleted cells for a range of temperatures (10–38°C) as described above. Cell suspensions were sampled at various time intervals after addition of [^{32}P]phosphate, depending on assay temperature, according to the following schedule: 1,2 and 3 h for cells at 10–20°C; 30, 60 and 120 min for cells at 22 and 24°C; 15, 30 and 60 min for cells at 26 and 28°C; 5, 10 and 20 min for cells at 30 and 32°C; 2, 4, 8 and 10 min for cells at 34 and 36°C; and 1, 2, 4 and 6 min for cells at 38°C.

2.4. *Chemical assays*

Cell membranes were purified by lysis of cells in 5 vol. ice-cold 5P8 buffer (5 mM sodium phosphate, pH 8), and repeated centrifugation at 15000 rev./min for 20 min at 4°C [24]. Membranes were analyzed for protein as in [25]. Membrane lipids were extracted by adding 1 vol. membranes to 5 vol. methanol while vortexing. The mixture was left on ice for 20 min, then an equal volume of chloroform was added while vortexing solution. Mixture was again left on ice for 20 min, then centrifuged at 2000 rev./min for 5 min at 4°C. The supernatant was decanted into a large test tube and the extraction process repeated with 2 vol. of each solvent with the resuspended pellet. The pooled supernatant was adjusted so that final proportions of chloroform:methanol:water were 10:5:1. The solution was vortexed and centrifuged at 5000 rev./min for 10 min at 4°C and the upper phase was removed. The organic phase was evaporated to dryness under a stream of nitrogen and lipids were resuspended in chloroform. Equal volumes of lipid were dispensed into test tubes for the lipid analyses. Cholesterol was analyzed as in [26] except that absorbance was read at 620 nm. Phospholipid content was determined by a modification of the method in [27]. The lipid sample was evaporated under a stream of nitrogen and resuspended in 0.5 ml chloroform which was placed in a boiling water bath until dry. To the test tube were added 0.4 ml chloroform and 0.2 ml chromogenic solution. The tube was vortexed and placed in boiling water for 2–3 min. When the tube was cool, 3 ml of chloroform containing 3% methanol was added, the mixture was vortexed and centrifuged on a table top centrifuge for 5 min. Absorbance was read at 716 nm. Cholestenone was analyzed by the modification in [28] of the method in [29]. Membrane protein was analyzed by SDS–polyacrylamide gel electrophoresis [30] and quantitated by scanning the gels and measuring the relative peak areas.

3. RESULTS AND DISCUSSION

The cholesterol:phospholipid ratio in the red cell membrane could be varied from 0.4 to 1.5 (fig.1) by incubating the cells with phosphatidylcholine liposomes containing various amounts of cholesterol. The level of cholesterol in the membrane was found to affect phosphate uptake into chloride-loaded cells (fig.1). Transport was inhibited at high cholesterol levels and stimulated at low cholesterol levels. A straight line, fitted by linear regression analysis, with a correlation coefficient of 0.75 could be drawn through the data points. Non-linear lines could also be fitted to the data. The effects of cholesterol on phosphate transport are fully reversible (table 1). Cells enriched with cholesterol could be depleted of cholesterol with an increase in transport. The protein profiles of normal cholesterol-enriched or depleted membranes were very similar, showing that little extraction or proteolytic degradation of membrane proteins occurred during the prolonged incubations (not shown).

The activity of some membrane-bound enzymes is sensitive to the phase state of the membrane lipid [31]. Studies of the temperature dependence of the transport or enzymatic activities of membrane proteins have produced curved or multiphasic Arrhenius plots [31]. The temperature at which a change in activation energy occurs can sometimes be related to phase changes in the lipid, although in other cases no correlation with a lipid transition can be established. No cooperative phase transition could be detected in red cell membranes or total lipids by differential scanning calorimetry [4]. ^{2}H-NMR [32], ^{31}P-NMR [33] and fluorescence polarization studies using the extrinsic probe, 1,6-diphenyl-1,3,5-hexatriene [33], have indicated that the lipid of the erythrocyte membrane is in the liquid–crystalline phase at all temperatures above −5°C, with no evidence for lateral phase separations. A broad phase transition extending from −20 to +10°C could be detected in erythrocyte membranes by Raman spectroscopy and a sharp transition was detected at 17°C using β-carotene as an extrinsic probe [34]. In [35] a small viscosity change was detected at 18–19°C. X-ray diffraction [23,36] showed that the membrane contained no gel state lipid above −20°C, although lowering the cholesterol phospholipid ratio to 0.5 raised the upper boundary of a phase transition from <0–20°C [23,26]. Membranes depleted of cholesterol by ether extraction also showed a transition at 20°C by ^{31}P-NMR [33]. Normal red cell membranes therefore do not appear to undergo a sharp phase transition above 0°C. Reducing the cholesterol level of the membrane moves the upper boundary of a broad phase transition to about 20°C.

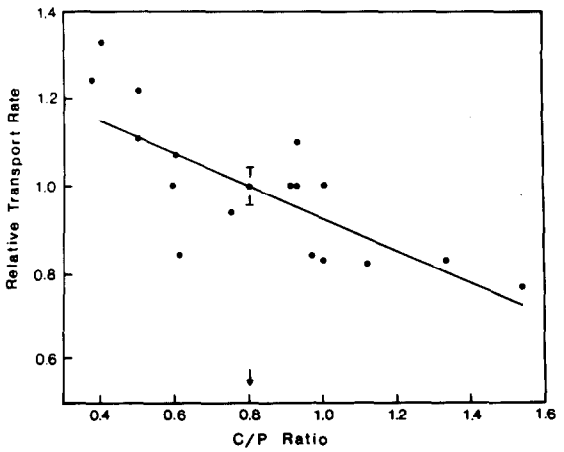

Fig.1. Effect of the cholesterol content of the membrane on phosphate uptake into red blood cells. C/P: cholesterol/phospholipid molar ratio. The cholesterol content of the membrane was modified as described in section 2.

Table 1

Reversibility of the effect of cholesterol on phosphate uptake

Treatment	Cholesterol/ phospholipid ratio	$t_{1/2}$ (min)
Control cells	0.8	14.0
Cholesterol-enriched	1.5	17.5
Cholesterol-depleted	0.5	10.0
Cholesterol-enriched/ depleted	0.9	14.0
Cholesterol-depleted/ enriched	0.8	13.5

The effect of changing the cholesterol level of the membrane on the temperature-dependence of phosphate transport was determined. As shown in fig.2, Arrhenius plots of phosphate uptake in nor-

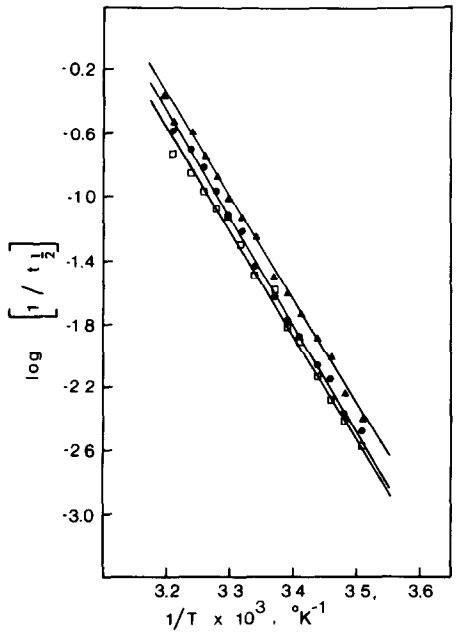

Fig.2. Arrhenius plot of phosphate uptake into red blood cells. (●) control cells, C/P = 0.8; (▲) cholesterol-depleted cells, C/P = 0.5; (□) cholesterol-enriched cells, C/P = 1.4.

mal, cholesterol-enriched and cholesterol-depleted membranes produced straight, parallel lines from 10–40°C. An activation energy of 27.2 kcal/mol was calculated. The authors of a study of the temperature-dependence of chloride-bicarbonate exchange in human erythrocyte suggested that a break in the Arrhenius plot occurred at 20°C [37]. The activation energy is 12 kcal/mol above this temperature, and 20 kcal/mol below this temperature. A similar change in activation energy was suggested for chloride exchange [38] where the apparent activation energy decreased from 30 kcal/mol to 20 kcal/mol above a temperature of 15°C. A close examination of the data in these references [37,38] reveals that the Arrhenius plots could also be fitted to curvilinear lines without sharp breaks. No such curve or break in the Arrhenius plot was observed for phosphate uptake even with cholesterol-depleted membranes suggesting that the phosphate transport is unaffected by the change in the rate-limiting step that affected chloride and bicarbonate transport. This may be due to the fact that phosphate is transported 10^5-times more slowly than chloride [39]. No change in the activation energy from 0–37°C was observed for other slowly transported ions such as I^- or sulfate [39–43].

Cholesterol has been shown to expand the red cell surface area. Cholesterol enters the outer leaflet of the bilayer preferentially [8,44] but can cross the bilayer quite rapidly [20,21]. The enrichment of cholesterol in the membrane increases lipid order, but may also affect the exposure of membrane proteins including Band 3, the anion transport protein [22]. Cholesterol has been suggested to bind directly to Band 3 [46]. The protein was prepared in this study by extraction with 92% acetic acid and the protein is highly aggregated [47]. This may lead to trapping of lipids, including cholesterol. Varying the cholesterol content of the erythrocyte membrane, on the other hand, had no effect on the rotational diffusion of Band 3 [48]. In order to study the specificity of the effect of cholesterol on Band 3 and anion transport, the effect of a cholesterol derivative, cholestenone, on anion transport was determined.

Under appropriate conditions, cholesterol oxidase converts the total cholesterol content of red cell membranes to cholestenone [20,21]. The effect of this conversion on phosphate uptake was determined. As shown in table 2, conversion of cholesterol to cholestenone had a slight stimulating effect on phosphate uptake. This shows that there is not a strict requirement for cholesterol in anion transport and that cholestenone can substitute for cholesterol in the membrane. Red cells were also enriched with cholestenone directly and the effect on anion transport was determined (table 3). The incubation with cholestenone-containing liposomes resulted in replacement of membrane cholesterol by cholestenone and an increase in the overall steroid content of the membrane (table 3). The decrease in membrane cholesterol normally resulted in a stimulation of transport (fig.1). The incorporation of cholestenone into the membrane, however, resulted in inhibition of phosphate uptake (table 3), although the cholesterol level was lower. This suggests that cholestenone is similar to cholesterol in its inhibitory effect on transport. Since cholesterol and cholestenone had similar effects on phosphate uptake, this suggests that any interaction between the steroid and Band 3 does not require an hydroxyl group at position 3 on the A ring. This limits the interaction to one exclusive-

Table 2

Effect of cholesterol oxidase treatment of red blood cells on phosphate uptake

Buffer[a]	Cholesterol oxidase (IU/ml)	% Cholesterol oxidized	$t_{1/2}$ (min)
Phosphate-buffered saline, pH 7.4	0	0	12.0
0.5 mM Sodium phosphate, 310 mM sucrose, pH 7.4	0	0	12.2
0.5 mM Sodium phosphate, 310 mM sucrose, pH 7.4	1	50	12.2
0.5 mM Sodium phosphate, 310 mM sucrose, pH 7.4	2	61	11.7
0.5 mM Sodium phosphate, 310 mM sucrose, pH 7.4	4	90	11.3

[a] Buffer in which the cholesterol oxidase treatment was performed

ly of a hydrophobic nature, and indicates a lack of specific interaction. The 3′-OH of cholesterol may, however, be required for the characteristic ordering effect of cholesterol on fluid phospholipid membranes [49]. Cholesterol analogues have effects on the passive permeability of red cells. For example, the rates of penetration of glycerol in red blood cells was higher in the presence of 3-keto steroids compared to 3β-hydroxy steroids [19], suggesting that 3-keto steroids such as cholestenone disorder the liquid–crystalline membrane lipids. The integrity of the membrane of vesicular stomatitis virus was not greatly altered by cholesterol oxidase treatment [50].

Table 3

Effect of cholestenone enrichment on phosphate uptake

Cholesterol : cholestenone : phospholipid (Molar ratio)			Relative transport rate
Liposomes		Membranes	
0 :0:0		0.8 :0 :1	1.0
2.5:0:1	(5 h)[a]	1.20:0 :1	0.81
0.5:1:1	(5 h)[a]	0.62:0.54:1	0.8
0.5:1:1	(20 h)[a]	0.58:0.60:1	0.60

[a] Incubation time of cells with liposomes

Band 3 must undergo a conformation change in order to change the orientation of the anion binding site from one side of the membrane to the other. This conformational change may be readily accommodated in a membrane composed of 0.8 mol cholesterol/mol phospholipid. An increase in the cholesterol level of the membrane will increase the probability of cholesterol being a component of the lipid at the Band 3 protein–lipid interface. The rigid steroid ring structure may restrict the conformational change that occurs upon transport compared to the more flexible acyl chains of phospholipids. It is unlikely that the increased cholesterol level in the membrane influences the oligomeric structure of Band 3. Cholesterol has been shown not to affect the rotation of Band 3 in the membrane [48] suggesting that a dramatic rearrangement of the protein oligomers does not occur.

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