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INHIBITION OF CATION COTRANSPORT BY CHOLESTEROL ENRICHMENT OF HUMAN RED CELL MEMBRANES

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SUMMARY

1. Human red cells were enriched with cholesterol by incubation with lipid dispersions having a high cholesterol : phospholipid mol ratio and the kinetics of the furosemide-sensitive cotransport system for Na^+ and K^+ were measured.

2. Influxes of both K^+ and Na^+ through this system were inhibited by 70 and 76 % in cholesterol-rich cells (cholesterol : phospholipid mol ratio 1.80) and the K_m of the furosemide-sensitive flux components for both K^+ and Na^+ was decreased.

3. Effluxes of both K^+ and Na^+ are inhibited by furosemide and the magnitudes of these furosemide-sensitive components are markedly decreased in cholesterol-rich cells.

4. The inhibitory effect of cholesterol enrichment on this carrier-mediated transport of cations suggests that cholesterol may either alter the position of the carrier or retard its movement within a more viscous membrane micro-environment.

Cholesterol is a major component of the surface membrane of mammalian cells. Small amounts of cholesterol serve to maintain membrane phospholipids in an intermediate fluid state [1], whereas larger amounts of cholesterol progressively restrict molecular motion in the membrane lipid bilayer [2]. In human red cells the mol ratio of cholesterol to phospholipid is 0.95. Isotopic studies have shown that the cholesterol of membranes rapidly exchanges with the unesterified cholesterol of lipoproteins [3]. Moreover, a dynamic equilibrium exists between the cholesterol of membranes and the cholesterol of lipoproteins in the surrounding plasma [4, 5]. This is illustrated in man by the acquisition of cholesterol by red cells in the presence of pathologic sera with a high cholesterol : phospholipid mol ratio obtained from patients with spur cell anemia [6]. Red cell membrane cholesterol is also influenced by lipid dispersions with varying ratios of cholesterol to phospholipid, and red cells may be either enriched or depleted of cholesterol by incubation with lipid dispersions with cholesterol : phospholipid mol ratios of greater than or less than 1.0, respectively [5l, 7].

Both human and duck red cells possess a cotransport system which mediates the equimolar influxes of Na^+ plus K^+ by a mechanism which appears to be carrier-mediated and can be inhibited by furosemide [8–10]. The effect of membrane cholesterol enrichment on carrier-mediated transport has not previously been reported. In the present study we have enriched red cells to a cholesterol : phospholipid mol ratio of 1.80 and have examined the kinetics of this cotransport system.

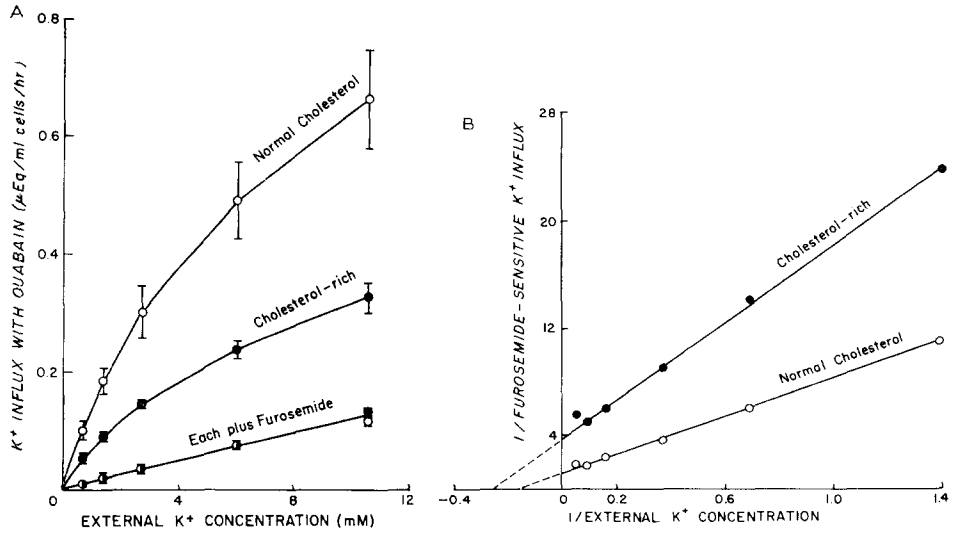

Fig. 1(A) Dependence of K^+ influx on external K^+ concentration. Incubation media contained KCl as shown plus (mM) NaCl 145, imidazole chloride 20 pH 7.5, glucose 5, ouabain 0.1, either in the presence or in the absence of 1 mM furosemide. Measurements on normal or cholesterol-rich cells were in paired incubations for 1 h at 37 °C. Vertical bars show ± 1 S.E. for three separate normal donors. (B) Michaelis-Menten plot between reciprocals of the furosemide-sensitive K^+ influx and the external K^+ concentration. Lines fitted by method of least squares.

The effect of furosemide on the ouabain-insensitive K^+ influx was studied for red cells suspended in saline media containing 0.6–11.0 mM KCl. In red cells with a normal cholesterol content, the K^+ influx increased with a hyperbolic dependence on external K^+ concentration (Fig. 1A). Furosemide markedly inhibited K^+ influx, and the small residual flux showed a linear dependence on external K^+ concentration. The component of K^+ influx inhibitable by furosemide was subjected to kinetic analysis which revealed a linear relation between the reciprocals of each flux component and the external K^+ concentration (Fig. 1B). Enrichment of red cells with cholesterol to a cholesterol : phospholipid mol ratio of 1.8 inhibited their K^+ influx (Fig. 1A). Furosemide further inhibited the K^+ influx of cholesterol-rich red cells, although the decrement caused by furosemide was clearly less in cholesterol-rich cells than normal. The small residual flux left by furosemide was identical in magnitude for the high cholesterol and the normal cholesterol cells. Fig. 1B shows that the mean furosemide-sensitive component from three separate experiments was well described by Michaelis-Menten kinetics and that both the V and the K_m of the cotransport system were decreased in cholesterol-rich cells (Table I).

TABLE I

KINETIC PARAMETERS OF FUROSEMIDE-SENSITIVE FLUX COMPONENTS

Fluxes are measured in μequiv./ml cells per h and K_m in millimolar concentration. Values are the mean of three separate paired experiments.

	Furosemide-sensitive K^+ influx		Furosemide-sensitive Na^+ influx	
	V	K_m	V	K_m
Normal-cholesterol cells	0.82	6.2	0.71	36
High-cholesterol cells	0.25	3.8	0.17	21

The magnitude of the cation cotransport system was also estimated from the inhibition of Na^+ influx by furosemide, since this diuretic has equal effects on Na^+ and K^+ influxes for cells suspended in high-Na^+, low-K^+ media [8]. When red cells were incubated in media containing between 10 and 100 mM NaCl, the Na^+ influx showed a saturating dependence on the external Na^+ concentration (Fig. 2A). Furosemide inhibited Na^+ influx so that the residual flux in the presence of inhibitor showed an almost linear dependence on external Na^+ concentration. The Na^+ influx into cholesterol-rich cells was lower than into cells with normal cholesterol and furosemide had only a slight inhibitory effect (Fig. 2A). However, the residual flux in the presence of furosemide was very similar to the corresponding residual flux found for the cells with a normal cholesterol content. Clearly cholesterol loading inhibited Na^+ influx by reducing the magnitude of the furosemide-sensitive component. For both sets of cells the mean furosemide-sensitive components from three separate experiments were analyzed by Michaelis-Menten kinetics (Fig. 2B) which showed that cholesterol loading decreased both the V and the K_m. The kinetic parameters of the cotransport system are summarized in Table I, which shows that cholesterol enrichment decreased the V of the system by 70 or 76 % as judged by either K^+ or Na^+ influx. In addition the K_m of the cotransport system was decreased from 6.2 to 3.8 mM for K^+ and from 36 to 21 mM for Na^+.

The effect of cholesterol loading on the ouabain-insensitive effluxes of Na^+ and K^+ from red cells was also studied. Both these fluxes are inhibited by furosemide, although the relation of the furosemide-sensitive components to the inward cotransport system is not well defined [8]. The ouabain-insensitive Na^+ efflux from cells with normal cholesterol content was inhibited 0.46 μequiv./ml cells per h by furosemide and this compound also reduced K^+ efflux by 0.44 μequiv./ml cells per h. Furosemide thus inhibited an equal component of both Na^+ and K^+ efflux from normal cells. In cells with a cholesterol : phospholipid mol ratio of 1.8 the furosemide-sensitive components were depressed, since the diuretic decreased Na^+ and K^+ effluxes by only 0.05 and 0.10 μequiv./ml cells per h, respectively (Table II). This result indicated that the furosemide-sensitive cation effluxes and influxes responded in the same way to cholesterol loading of the cell.

We have previously reported that cholesterol enrichment of human red cells causes no change in active (ouabain-sensitive) Na^+ efflux or K^+ influx [5]. However, Kroes and Ostwald [11] observed a decrease in both the active and the passive efflux of Na^+ from guinea pig red cells enriched with cholesterol by dietary means. In the

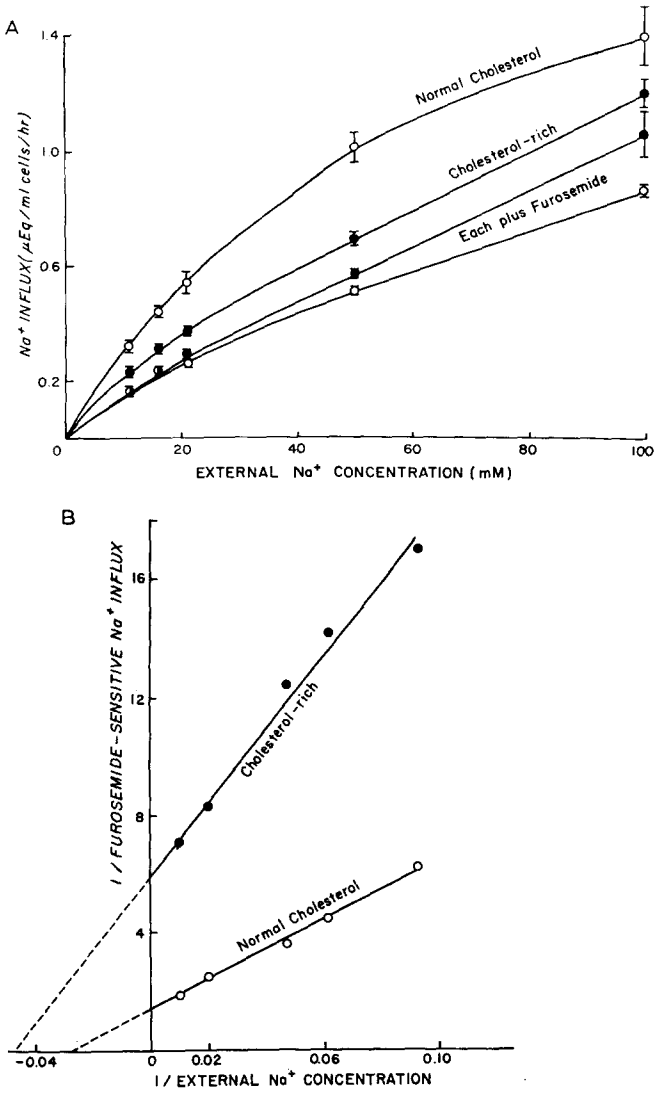

Fig. 2.(A) Dependence of Na^+ influx on external Na^+ concentration. All media contained NaCl as shown plus (mM) KCl 50, imidazole chloride 20 pH 7.5, glucose 5, ouabain 0.1 and sufficient choline chloride to maintain isotonicity. Normal or cholesterol-rich cells were incubated in paired flasks for 10 and 20 min at 37 °C either in the presence or in the absence of 1 mM furosemide. Vertical bars show ± 1 S.E. for the same three donors as in Fig. 1. (B) Double reciprocal plot of furosemide-sensitive Na^+ influx and external Na^+ concentration.

present study inhibition of ion transport was confined to the furosemide-sensitive system. This system has been characterized in both human and duck red cells and shows features of a carrier-mediated process [12]. Na^+ influx into cells is stimulated by K^+, while, conversely, the K^+ influx is stimulated by Na^+, and this synergism is abolished by furosemide. The influxes of both Na^+ and of K^+ in the presence of the other ion show saturation kinetics typical of a carrier-mediated process. Moreover,

TABLE II

EFFECT OF FUROSEMIDE ON CATION EFFLUX

Red cells were loaded with $^{22}Na^+$ or $^{42}K^+$ by preincubation with isotope for 4 h at 37 °C. Cells were washed 5 times in 0.11 M $MgCl_2$ and added to make 5–7 % hematocrit in media of composition 145 mM NaCl, 5 mM KCl, 20 mM imidazole chloride, pH 7.5, 0.1 mM ouabain, either in the presence or in the absence of 1 mM furosemide. Values given are in μequiv./ml cells per h.

	Na^+ efflux with ouabain	Further inhibition by furosemide	K^+ efflux with ouabain	Further inhibition by furosemide
Normal-cholesterol cells	0.79	0.46	1.40	0.44
High-cholesterol cells	0.44	0.05	1.40	0.10

the cosubstrates, Na^+ and K^+, stimulate the net uptake of each other into red cells containing Li^+ as the sole internal cation [8]. Finally, a stoichiometry of 1 : 1 is found between the furosemide-sensitive components of both Na^+ and K^+ influx, although in common with other cotransport systems it appears that the specificities are not absolute and that other ions can substitute for K^+ [9, 10]. Table I shows that stoichiometry was observed in this study even when the cotransport process was inhibited by cholesterol enrichment. Cholesterol addition to the red cell seems a result of its direct transfer from lipid dispersion to membrane rather than from fusion or adsorption of whole vesicles with the membrane. Scanning electron micrographs of cholesterol-enriched cells show no sign of surface vesicles [5] and moreover there is no increment in red cell phospholipid concomitant with the cholesterol gain and both these observations favor some selective transfer of cholesterol at the molecular level. The mechanism by which excess cholesterol inhibits the rate of cation cotransport may relate to a direct association of cholesterol with the carrier system. However, it seems more likely that the addition of cholesterol to the lipid bilayer either influences the position of the carrier within the membrane or decreases its ability to move within a more viscous microenvironment.

EXPERIMENTAL

Analytical methods

Serum and sonicated lipids were extracted with acetone/ethanol (1 : 1) for measurement of cholesterol [13] and lipid phosphorus [14]. Phospholipid values were calculated by multiplying lipid phosphorus values by 25. Individual phospholipids were quantitated by thin-layer chromatography with chloroform/methanol/glacial acetic acid/water (25 : 15 : 4 : 2) [15]. For measurement of red cell lipids, the cells were washed three times in 0.15 M NaCl and extracted with isopropanol and chloroform [16] for measurement of cholesterol and lipid phosphorus. Red cell ghosts were prepared by hypotonic hemolysis and washing in 5 mM sodium phosphate buffer, pH 7.4 [17].

Preparation of lipid dispersions

Lipid dispersions were prepared by sonicating 40 mg of dipalmitoyl phosphatidylcholine (General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio) with 80 mg of cholesterol (Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 0.155 M

NaCl at 70 W for 60 min at 0 °C in a sonifier (Branson Instruments Co., Stanford, Conn.) with a standard tip, as described previously [5]. Purity of the phosphatidylcholine was verified by thin-layer chromatography [15] in which more than 95 % of the phospholipid both before and after sonication ran as phosphatidylcholine, 1–2 % was recovered in the area of phosphatidylserine and the remainder was recovered in the region of lysolecithin. By maintaining the temperature at 0 °C the amount of lysophosphatide (approx. 3 %) was the same before and after sonication. Purity of cholesterol was checked by gas-liquid chromatography in which it gave a single peak. After sonication, the dispersions were mixed with 0.4 vol. of normal compatible serum, previously heated to 56 °C for 30 min, and centrifuged at 21 800 $\times g$ for 30 min to sediment undispersed lipid. Human serum albumin (Sigma Chemical Co.) at a concentration of 2.0 g/100 ml in 0.155 M NaCl could be substituted for heated serum with an identical result for the amount of lipid transferred to cells. No experiments were performed on cells incubated with lipid dispersions without addition of the heated serum. The cholesterol : phospholipid mol ratio of the final mixtures of dispersed lipids was 1.85.

Incubation of cell suspensions

Red cells were separated from freshly drawn blood, thrice washed in Hank's balanced salt solution, and incubated for 16 h at 37 °C at a cell concentration of 5 % in the serum/sonicate mixture or serum/saline control, each diluted 1 : 1 with Hank's containing penicillin, 100 units/ml. Adenosine (10 mM) was added to cell suspensions 1 h before the end of incubation to ensure a normal content of cell ATP [18]. Red cell cholesterol was approximately doubled after incubation with heated serum/lipid sonicate mixture since the initial value of 13.3–14.5 $\mu g/10^8$ cells increased to 26.0–30.0 $\mu g/10^8$ cells. Red cell cholesterol did not change after incubation with the heated serum/saline control. There was no change in the net phospholipid content of red cells in either condition and both pre- and post-incubation values remained in the range 30.6–32.0 $\mu g/10^8$ cells. In addition, thin-layer chromatography showed that there was no change in the relative proportion of the red cell phospholipid classes after incubation with the heated serum/lipid sonicate mixture. Thus in each experiment from one donor there were paired flasks containing cells with final cholesterol : phospholipid mol ratios of 1.80 and 0.95, respectively. In both cases the cholesterol : phospholipid mol ratio of ghosts prepared by hypotonic hemolysis was identical to that of the intact cells, so that the added cholesterol was localized to the membrane.

Cation fluxes

The paired sets of red cells were thrice washed in 0.11 M $MgCl_2$ and added to media containing $^{42}K^+$ or $^{22}Na^+$. The influx and efflux of K^+ and Na^+ were measured as described previously [8]. In all flux experiments ouabain (0.1 mM) was added to eliminate any flux mediated by the active cation pump. Furosemide was always added in a final concentration of 1 mM.

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