Biochimica Biophysica Acta, 356(1974) 125—130

BBA Report

BBA 71204

CHANGES OF MEMBRANE PERMEABILITY DUE TO EXTENSIVE CHOLESTEROL DEPLETION IN MAMMALIAN ERYTHROCYTES

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(Received May 14th, 1974)

Summary

55 % of the total membrane cholesterol could be removed from porcine, bovine and human erythrocytes by incubating the cells in suspensions of lecithin liposomes. Up to 30 % depletion, membrane permeability remained unaltered; more extensive depletion induced a marked increase of the transfer rates of nonelectrolytes and of organic acids penetrating by nonionic diffusion. This biphasic response of permeability to cholesterol depletion, which has not been observed in artificial lipid membranes, may be related to the heterogeneity of the erythrocyte membrane lipids or to a pool of cholesterol not interacting with the phospholipids.

Cholesterol has recently been shown to influence the permeability of phospholipid bilayer membranes by modifying the fluidity of the hydrophobic core in a dual way (cf. refs 1 and 2). Hydrocarbon phases in the gel state become more fluid and permeable [3,4], liquid—crystalline phases less fluid [2—6] in the presence of the sterol, which thus seems to be able to convey a state of "intermediate fluidity" [1,7] to phospholipid membranes. The molecular events underlying this phenomenon have not yet been fully elucidated [7—11].

In spite of the wide distribution of sterols in plasma membranes there is little unequivocal evidence as yet that cholesterol also acts as a modifier of permeability in these complex systems, a lack of evidence mainly due to experimental difficulties in altering the membrane cholesterol content of intact cells. So far, only the microorganism *Acholeplasma laidlawii* and the mammalian erythrocyte have proven suitable for this purpose. In *Acholeplasma*,

TABLE I

LACK OF INFLUENCE OF 30 % DEPLETION OF MEMBRANE CHOLESTEROL ON NON-ELECTROLYTE AND ANION TRANSFER IN HUMAN ERYTHROCYTES

Cells depleted by Murphy's technique [19] Erythritol fluxes were measured in the presence of 100 mM glucose in order to inhibit the mediated component of erythritol permeability [31,32] For further details see text.

	Rate coefficient of efflux ($k \times 10^2$ min^{-1}) ± S.D		n
	Control (0 728 μmoles/ μmole Hemoglobin)*	30 % Depleted (0 506 μmoles/ μmole Hemoglobin)*	
Sulfate, 30 °C	1 36	1 38	1
Glycolate, 5 °C	2 11 ± 0 23	2.12 ± 0.22	3
Lactate, 25 °C	6 04 ± 0 29	5 40 ± 0.46	4
Erythritol, 35 °C	4 96 ± 0.43	5 17 ± 0 33	5

*Cholesterol

marked effects of cholesterol on membrane permeability could be shown [12,13]. Studies on erythrocytes have provided positive [14—16] as well as negative [17,18] results. In a recent study [18] we have demonstrated that the removal of 35 % of the membrane cholesterol from pig erythrocytes by the technique of Murphy [19] does not induce notable changes of anion and nonelectrolyte permeability. The same proved to be true for human erythrocytes (Table I).

Murphy's technique [19], which uses cholesterol-depleted serum for removing cholesterol from the erythrocyte membrane, has a number of principal and technical disadvantages. In an attempt to remove larger amount of cholesterol from intact erythrocytes we have therefore applied the technique of Bruckdorfer et al. [14] which involves phospholipid vesicles.

Human, porcine and bovine erythrocytes were depleted of cholesterol by 14—42 h incubation (37 °C, hematocrit 10 %) in liposome suspensions. These suspensions were prepared by sonicating (Branson sonifier B 12, 100 W, 0 °C) 200 mg % of egg lecithin (BDH product no. 29053, or Merck art. no. 5331, used without further purification) in an N_2-saturated medium of the following composition (mM): NaCl 140; Na_2HPO_4/NaH_2PO_4 12.5; sucrose 45; penicillin 8 mg/100 ml; streptomycin 20 mg/100 ml; pH 7.35—7.4. Erythrocytes incubated in these liposome suspensions could be depleted of about 50—55 % of their cholesterol as determined [20] in chloroform—isopropanol extracts [21] of the washed cells. Characteristic data are given in Table II*. Controls incubated in liposome-free media did not lose cholesterol. Total lipid phosphorus, determined [22] after removal of water soluble phosphorus [23], remained essentially unaltered.

Cholesterol-depleted and control cells were subsequently washed 3 times (NaCl 140 mM; Na_2HPO_4/NaH_2PO_4 12.5 mM; sucrose 45 mM, pH 7.35, 20 °C)

*The time course of cholesterol depletion and the extent tolerated by the cells without hemolysis varied from experiment to experiment. Complete hemolysis occurred above 60 % depletion.

TABLE II

CHANGES OF THE MEMBRANE CHOLESTEROL CONTENT OF PIG ERYTHROCYTES DURING INCUBATION WITH LECITHIN LIPOSOMES

Time of depletion (h)	Cholesterol*	Depletion (%)	Lipid phosphorus*	Cholesterol / Phospholipid	Mole % Cholesterol**
0	0.902	0	1.087	0.83	45
14	0.654	−28	1.004	0.65	39
30	0.533	−41	0.974	0.55	35
42	0.402	−56	0.972	0.41	29

*μmoles/μmole hemoglobin.

**Calculated as $\frac{\text{Cholesterol}}{(\text{Cholesterol} + \text{phospholipid})} \times 100$.

and the fluxes of nonelectrolytes (erythritol, glycerol) and of anions penetrating by nonionic diffusion (acetate, propionate) measured by tracer techniques [24].

In agreement with the results [18] obtained using Murphy's technique, cholesterol depletion of pig erythrocytes up to approximately 30 % did not alter non-mediated erythritol transfer nor the nonionic diffusion of acetate*, two processes supposed to involve the lipid phase of the membrane. The removal of larger quantities of cholesterol, however, induced a pronounced enhancement of erythritol and acetate permeability (Fig. 1). Transfer rates increased markedly with the extent of depletion. At 55 % almost twice the control value was reached. A similar increase in permeability was demonstrated for glycerol (Table III). Moreover, cholesterol depletion of human and bovine erythrocytes also induced an enhancement of the non-mediated transfer of nonelectrolytes and the nonionic diffusion of organic acids. These results, which could be substantiated using the Murphy [19] technique of cholesterol depletion, provide direct evidence that cholesterol in the mammalian erythrocyte membrane lowers the passive permeability of solutes assumed to penetrate via the lipid part of the membrane.

Principally, the influence of cholesterol on the erythrocyte membrane thus agrees with its effect on artificial phospholipid bilayers in the liquid—crystalline state [2—5]. This agreement supports the concept that the lipids of the erythrocyte membrane are arranged as a bilayer. Some details, however, of the permeability changes observed in erythrocytes are difficult to reconcile with results obtained on artificial bilayers.

Firstly, cholesterol depletion enhances the permeability of porcine, bovine and human erythrocytes to the same extent, in spite of considerable differences in the lipid composition of their membranes as indicated by the sphingomyelin/lecithin ratio (man and pig 1:1, ox 12:1 [26]) and the double bond index of the hydrocarbon chains (man 1.43, pig 0.9, ox 0.8; calculated

*Acetate and propionate also penetrate the erythrocyte membrane by ionic diffusion to some extent. In the present experiments this process was blocked by phenopyrazone [25].

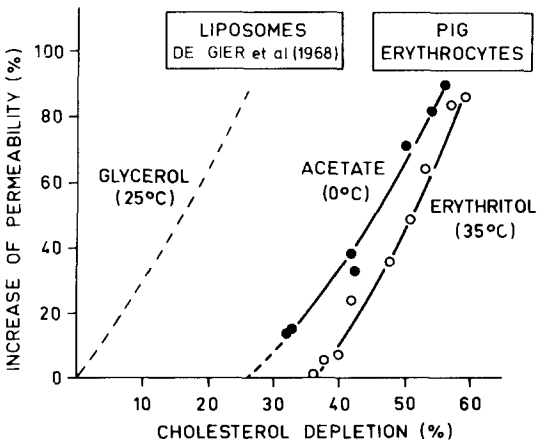

Fig 1 Influence of cholesterol depletion on the permeability of pig erythrocytes to erythritol and acetate Incubation media for erythritol experiments contained (mM) NaCl 140, Na_2HPO_4/NaH_2PO_4 5 5, glucose 7.5, sucrose 45, erythritol 30, pH 7.35, 35 °C, for acetate experiments (mM) NaCl 110, Na_2HPO_4/NaH_2PO_4 25, sucrose 40, acetate 10, phenopyrazone 9; pH 8 2, 0 °C Relative permeabilities were calculated from the first order rate coefficients of tracer efflux [24]. Permeability data for liposomes (taken from de Gier et al [5], Fig 5a) refer to systems containing egg lecithin, cholesterol and 4 mole % phosphatide acid, 0 % cholesterol depletion being equivalent to 50 mole % cholesterol

TABLE III

PERMEABILITY CHANGES INDUCED BY CHOLESTEROL DEPLETION IN MAMMALIAN ERYTHROCYTES

For details of the experimental procedure see text and legend to Fig. 1. Erythritol fluxes in human erythrocytes were measured in the presence of 100 mM glucose in order to inhibit the mediated component of erythritol transfer [31,32].

Species	Cholesterol depletion	Permeant	Increase of permeability (1 0 = control)
Pig	−46 %	Glycerol (15 °C)	1 56
Man	−45 %	Erythritol (35 °C)	1 54
Ox	−40 %	Glycerol (15 °C)	1.52
	−37 %	Acetate* (0 °C)	1.48
	−37 %	Propionate* (0 °C)	1 52

*In the presence of 9 mM phenopyrazone (cf footnote p 2)

from data in ref. 27). This identical response of the permeabilities to cholesterol depletion seems difficult to reconcile with spectroscopic evidence indicating that, in the temperature range of our efflux experiments (0—35 °C), cholesterol reduces the fluidity of unsaturated lecithins [1,3] but increases that of the more saturated sphingomyelins [3,28] and acts differently on the permeability of lecithin bilayers varying in the degree of unsaturation [2,5,29]. Inoue, on the other hand, very recently reported [4] that in

cholesterol/phospholipid liposomes containing saturated lecithins or sphingomyelin, in unsaturated lecithin/cholesterol systems [5], cholesterol depletion enhances glucose permeability, at temperatures below 37 °C, in the range of high ratios of cholesterol/phospholipid. Our data on ox erythrocytes may be understood on the basis of this finding.

Secondly, the permeability of the erythrocyte membrane only increases when more than 25—35 % of the cholesterol has been removed, i.e. when the cholesterol/phospholipid ratio has decreased below 0.7. In contrast, the permeability of egg lecithin/cholesterol liposomes, according to the results of de Gier et al. [5] and others [29], already rises when the ratio is diminished only slightly below equimolarity (cf. hatched line in Fig. 1). This lack of permeability changes up to about 30 % of cholesterol depletion may indicate that part of the cholesterol in the erythrocyte membrane is either not located in the direct neighborhood of the phospholipids or arranged in a particular phospholipid environment such that the fluidity of the membrane remains unaltered upon depletion of this pool of cholesterol. The peculiar response of the erythrocyte membrane to cholesterol depletion could thus be related to the marked heterogeneity [30], or the transverse asymmetry [26], of its phospholipids, two properties not to be expected in the homogeneous artificial membrane systems used for most previous studies of the permeability effects of cholesterol. The elucidation of the molecular basis of the effect of cholesterol on the erythrocyte membrane will therefore require systematic studies on model systems prepared from heterogeneous phospholipids with special regard to the concentration dependency of cholesterol-induced permeability changes in the range of high molar cholesterol/phospholipid ratios, which so far has received only little attention [4,29].

Supported by the Deutsche Forschungsgemeinschaft (SFB 160). The technical assistance of Mrs A. Daniels, I. Flotho and I. Rickert is gratefully acknowledged. The authors are indebted to Dr C.W.M. Haest for helpful discussions.

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