A STUDY OF CHOLESTEROL TRANSFERS BETWEEN ERYTHROCYTES AND LIPID VESICLES

Possible involvement of interparticular collisions

F. GIRAUD+ and M. CLARET*

Laboratoires de Physiologie de la Nutrition et de Physiologie comparée, 91405 Orsay Cedex, France

Received 24 April 1979

concentrations, complete exchange of erythrocyte cholesterol with a single rate constant was observed. The same rate constants were found for both exchange and depletion. But, the value of the rate constant for both processes was markedly dependent on the vesicle concentration. These results are explained assuming that cholesterol transfers occur through collisions between cells and vesicles.

1. Introduction

Both the exchange of cholesterol between erythrocytes and plasma lipoproteins, and the depletion of erythrocyte cholesterol in the presence of plasma of lowered cholesterol content have been extensively studied over the last 25 years ([1–11], reviewed [12]). The results obtained are sometimes different from one study to the other. For instance it has been reported that only a fraction of total cholesterol is exchangeable in pig and rat erythrocytes [6,8]. However in the other studies, it is generally shown that all erythrocyte cholesterol is available for exchange [1,3–5,7,9] and that this exchange proceeds with a single rate constant, i.e., that all cholesterol molecules behave as a single kinetic pool [1,5,9]. Another interesting characteristic is the fact that cholesterol can be partially removed from the erythrocyte membrane and that this second process, depletion, exhibits the same rate constant as does exchange [10].

Here we have studied exchange and depletion of human erythrocyte cholesterol using unilamellar lipid vesicles, a method first proposed [13] and further used [14–16]. Over a wide range of vesicle

Abbreviations: PC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; C, cholesterol; *C/P*, cholesterol/phospholipid molar ratio; PBS, phosphate buffer saline

+ Formerly F. D'Hollander, present address: Dept. Biochemistry, Royal Free Hospital School of Medicine, London WC1

* Present address: Dept. Pharmacology, University College London WC1, England

2. Materials and methods

7(*n*)-[^{3}H]Cholesterol was purchased from CEA (France), [*choline-methyl*-^{14}C]dipalmitoylphosphatidylcholine and glycerol-tri 9–10 (*n*)-[^{3}H]oleate from the Radiochemical Centre (Amersham). Egg phosphatidylcholine (Fraction VE) and cholesterol were obtained from Sigma. Unilamellar egg PC and PC-C vesicles were prepared in PBS (for composition see [16]) by probe sonication and ultracentrifugation as in [16]. Human erythrocytes obtained from fresh blood under heparin, were washed 3 times with PBS before use. Cell-vesicle incubations were at 37°C under gentle agitation. Cholesterol exchange experiments were done with PC-C vesicles labelled with [^{3}H]cholesterol and [^{14}C]DPPC (*C/P* = 0.9) at 0.1–6 mM PC, incubated for 24 h with erythrocytes at $3–30 \times 10^7$ cells $.ml^{-1}$. At different time intervals, samples were centrifuged and aliquots of the supernatant (vesicles) and of the erythrocytes, after 3 washes, were taken for scintillation counting. [^{14}C]DPPC was used as a marker of the vesicles to

 Elsevier/North-Holland Biomedical Press

measure their uptake by the cells and to make the corresponding correction on the cell radioactivity, and to measure the vesicle recovery in the supernatant. In two experiments carried out at 6 vesicle concentrations, the recovery of [^{14}C]DPPC was compared to that of [^{3}H]Triolein (the usual non-exchangeable marker of the vesicles). Cholesterol depletion experiments were done with PC vesicles (0.4–11 mM PC, 50–170 × 10^7 cells .ml^{-1}) for 2–7 h so that the depletion was < 40% and the extent of hemolysis was very low. At different time intervals, samples were centrifuged and erythrocytes were washed 3 times. Their cholesterol content was measured as previously [16] and expressed as their *C/P* assuming that their phospholipid content did not change [14,15].

The rate contant for cholesterol depletion (k_{cv}) was calculated according to the model proposed [10] in which the unidirectional flux of cholesterol from cell to vesicles is proportional to the cell $(C/P)_t$ until a *C/P* equilibrium is reached ($(C/P)_\infty$):

$(C/P)_t = (C/P)_0$ at time 0

and

$(C/P)_t = (C/P)_\infty$ at time ∞

The classical formulae for such a two-closed compartment system were used to calculate the apparent rate constant ($k_{cv} + k_{vc}$) the sum of the actual rate constant in each direction (cells to vesicles, k_{cv}; vesicles to cells, k_{vc}):

$$\text{Log}\,[(C/P)_t - (C/P)_\infty] = \text{Log}\,[(C/P)_0 - (C/P)_\infty] - (k_{cv} + K_{vc})\,t \quad (1)$$

$(C/P)_\infty$ is calculated from: $(C/P)_\infty =$

$$(C/P)_0 \,.\, P_c/(P_c + P_v) \quad (2)$$

in which P_c and P_v are, respectively, the phospholipid content of cells and vesicles, and k_{cv} is given by:

$$k_{cv} = (k_{cv} + k_{vc}) \,.\, [1 - (C/P)_\infty/(C/P)_0] \quad (3)$$

The rate constant for cholesterol exchange was calculated in similar way but with the simplification that the cholesterol specific activity of the vesicles (a_v) remains constant during the incubation (see fig.1b). This was due to the fact that the vesicles/cell cholesterol pool ratio was always between 50 and 100:

$$\text{Log}\,(a_v - a_{ct}) = \text{Log}\,a_v - k_{cv}\,t \quad (4)$$

where a_{ct} is the cholesterol specific activity of the cells at time t.

3. Results

3.1. *Vesicle uptake by erythrocytes: kinetics and concentration dependence*

Figure 1a shows the time-course of DPPC and cholesterol uptake by the cells, when incubated with ([^{14}C]DPPC + [^{3}H]cholesterol) PC-C vesicles. The vesicle uptake, estimated from the DPPC uptake, increased along a near saturable function. The time required to reach the maximal value, and the value itself, were dependent on the vesicle concentration. This is illustrated by the results of the recovery experiments shown in table 1. At low vesicle concentrations, the vesicle recover in the supernatant (*R*) was not changed between 15 min and 24 h. At high concentrations of vesicles, $R_{24\,h}$ was significantly lower than $R_{15\,min}$ indicating that more vesicles become cell-associated with time. The uptake also increased with the vesicle concentration since ~10% of the vesicles were taken by the cells at all concentrations tested. Thus, in all conditions the vesicle concentration at the cell surface was always higher than that in the medium. In two further experiments carried out with [^{3}H]Triolein + [^{14}C]DPPC PC-C vesicles at 6 concentrations, the recovery of [^{14}C]DPPC was compared to that of [^{3}H]Triolein (non-exchangeable lipid). The ^{14}C/^{3}H ratio did not change during the course of incubation (see values in parentheses in table 1) suggesting that under our experimental conditions, the exchange of [^{14}C]DPPC with the cell phopholipids is negligible compared to the uptake of vesicles by the cells and obviously with the exchange of cholesterol.

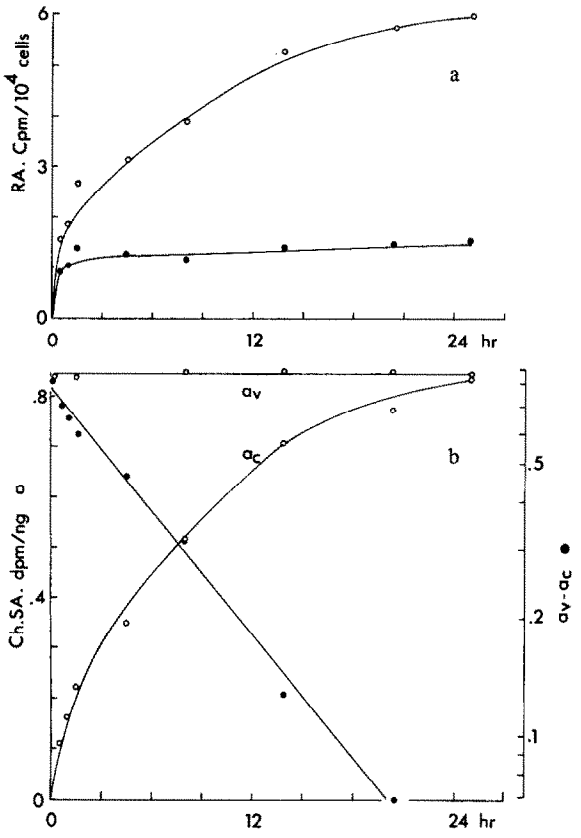

Fig.1. Exchange experiment: erythrocytes (8×10^7 cells .ml^{-1} were incubated with ([^{14}C]DPPC + [^{3}H]C) PC-C vesicles (1.9 mM PC, $C/P = 0.9$, $^3H/^{14}C = 1$). At different time intervals, samples were centrifuged at 12 000 × g for 1.5 min. Aliquots of supernatant were taken for counting. The cell pellet was washed 3 times with PBS and counted. (a) (○) ^{3}H radioactivity of the pellet; (●) ^{14}C radioactivity of the pellet. (b) a_v, Vesicle cholesterol specific activity; a_c cell cholesterol specific activity. The plot Log $(a_v - a_c)$ as a function of the time is used to calculate $(k_{cv} + k_{vc})$ and then k_{cv} (see section 2).

3.2. *Cholesterol transfers from erythrocytes to vesicle depletion and exchange. Kinetics and concentration dependence*

Incubation of erythrocytes with PC-C vesicles (molar ratio $C/P = 0.9$) results in an exchange without any net movement of cholesterol between cells and vesicles. Incubation with PC vesicles ($C/P = 0$) causes a net flux of cholesterol from the cells to the vesicles until an equal C/P is reached in the two membranes (see section 2). In exchange experiments, the cholesterol specific activities of the cells (a_c) and of the vesicles (a_v) were equal after 24 h incubation (fig.1b). The plot of Log $(a_v - a_c)$ as a function of time gave a straight line (fig.1b). These results show that at physiological and constant C/P, all cholesterol of the cell membrane is exchangeable with that of artificial membranes as a single pool. Similarly in depletion experiments (up to 40%) the plot of the Log $[(C/P)_t - (C/P)_\infty]$ as a function of time gave straight lines (results not shown). Therefore both processes, exchange and depletion (at least for the first 40% of depletion) affect the same kinetically homogeneous pool of membrane cholesterol in erythrocytes as already demonstrated in the same cells incubated with plasma [10]. These experiments were carried out over a very large range of vesicle concentrations, from 0.1–6 mM PC for exchange and 0.4–11 mM PC for depletion. Again, at all concentrations tested, total exchange and monoexponential kinetics were observed. But the value of the actual rate constant of the cholesterol efflux from erythrocyte (k_{cv}) was found to be dependent on the vesicle concentration [V]. Figure 2 shows the effect of a 110-fold increase in the vesicle concentration on K_{cv}: the $k_{cv} - [V]$ function first

Table 1
Vesicle recovery: time and concentration dependence

[V] mM PC ^{14}C/^{3}H	15 min R ^{14}C/^{3}H	24 h R ^{14}C/^{3}H
0.06 (1.13)	0.94 (1.09)	0.93 (1.10)
0.12 (1.19)	0.96 (1.13)	0.93 (1.17)
0.54 (1.20)	0.98 (1.13)	0.89 (1.16)
1.10 (1.15)	0.99 (1.14)	0.88 (1.13)
2.80 (1.14)	0.99 (1.15)	0.91 (1.18)
5.55 (1.14)	0.99 (1.17)	0.90 (1.17)

Erythrocytes (10×10^7 cells .ml^{-1} were incubated with ([^{14}C]-DPPC) PC-C vesicles (1 expt) or ([^{14}C]DPPC + [^{3}H]Triolein) PC-C vesicles (2 expt) at different concentrations [V]. At the time indicated, samples were centrifuged at 12 000 × g for 1.5 min and the concentration of radioactive vesicles was measured in the supernatant [V']. The ratio [V']/[V] is defined as the recovery (R). R values are means of triplicates in 3 different experiments. SD on R were always < 0.015 (not shown). Values in parentheses (mean of triplicates in 2 different experiments) are the ^{14}C/^{3}H ratios of the vesicle suspensions. Their constancy during the incubation suggests that in our experimental conditions the possible exchange of [^{14}C]DPPC with cell phospholipids is negligible as compared to the uptake of the vesicles by the cells

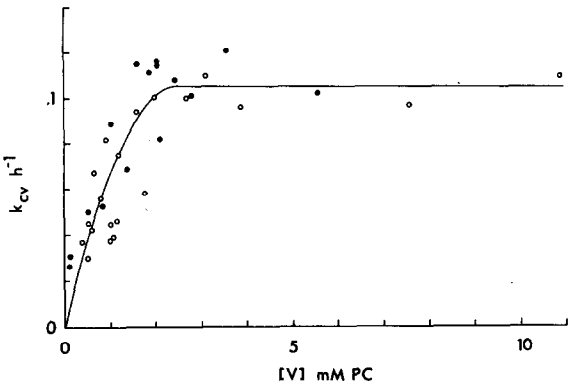

Fig.2. Rate constant of cholesterol transfers (k_{cv}) from erythrocytes to vesicles as a function of the vesicle concentration [V]. (•) Cholesterol exchange (PC-C vesicles, C/P = 0.9), (○) cholesterol depletion (PC vesicles). The rate constants for cholesterol depletion concern experiments in which the maximal depletion was < 40% of the initial cholesterol content. Each point represents an individual experiment. k_{cv} for exchange and depletion was calculated as outlined in section 2. In one depletion experiment the rate constant (0.3 h^{-1}) was higher than the mean value of the plateau. The point is not shown on the figure. It does not significantly modify the mean plateau value calculated on 13 exp.

increased with [V], then saturated when [V] ≈ 2 mM. For both exchange and depletion the points fell on the same curve.

4. Discussion

The present experiments carried out with vesicles show that all cholesterol molecules of the erythrocyte membrane are exchangeable and behave as a single kinetic pool. A similar conclusion was reached for the cholesterol exchange between erythrocytes and plasma [1,5,9]. Moreover the maximal value of cholesterol exchange rate constant found in our experiments is in good agreement with that reported when erythrocytes are incubated in plasma: ~ 0.1 h^{-1} [5,7,9,10]. These findings indicate that these exchanges do not depend on the nature of the external receptor.

It has been reported that cholesterol is present within each leaflet of the bilayer in the erythrocyte membrane, about 2/3 external and 1/3 internal [17]. Thus a transbilayer movement of cholesterol exists which proceeds at a higher rate than that corresponding to the cholesterol exchange with an external receptor. The observation of such a single pool with a rate constant of 0.1 h^{-1} (see fig.2) could thus imply that the rate constant of the transbilayer movement is markedly higher than 0.1 h^{-1}. This observation is consistent with the estimate obtained by different approaches and reported in the same membrane [18] ($k \geqslant 0.83\ h^{-1}$) and [19] ($k \geqslant 0.69\ h^{-1}$) or for the sterophenol translocation rate in PC vesicles [20] (k 0.57 h^{-1}).

The cholesterol depletions affect also an homogeneous pool at least for the first 40% removed in our conditions. However, the maximal depletions reported are ~60–70% (C/P = 0.3 – 0.2) [15]. Since all cholesterol is exchangeable at C/P = 0.9, these results indicate that there is a drastic reduction in the rate of cholesterol transfer when C/P reaches these values. This may be interpreted as the result of an increase in the activation energy of the cholesterol molecules (see below, eq. (5)) which would become more tightly bound to phospholipids when the C/P approaches 0.2–0.3. But as far as the first 40% of the molecules are concerned, the rate constant for cholesterol depletion is independent of the membrane cholesterol content. This is also deduced from the results of exchange and depletion experiments shown in fig.2. The rate constants for both processes follow the same relationship over a wide range of vesicle concentrations.

The simplest hypothesis to explain an interaction between k_{cv} and [V] (see fig.2) is to assume that the cholesterol transfers occur through collisions between cells and vesicles. Such a proposal has already been offered in other studies but was supported by little experimental evidence [21–24]. A collisional mechanism would imply the number of cholesterol molecules transferred per time unit to be proportional to the collision frequency. Accordingly the unidirectional cholesterol flux from cells to vesicles at steady-state (Φ_{cv}) would obey the Arrhenius equation [25]:

$$\Phi_{cv} = P \,.\, Z \,.\, e^{-E_a/RT} \qquad (5)$$

in which Z is the collision frequency between cells and vesicles, P a steric factor, E_a the activation energy of the cholesterol molecules and R and T have their usual meanings. Φ_{cv} and Z are both

expressed per time and volume unit. Z is itself proportional to the product of the particle concentrations, vesicles $[V]$ and cells $[C]$:

$$Z = a \,.\, [V] \,.\, [C] \quad (6)$$

a is a factor depending on the particle characteristics and on the viscosity of the medium. In our conditions a,P,R and T are constants. E_a is taken as a constant for depletions up to 40%. Thus:

$$\Phi_{cv} = \text{constant} \,.\, [V] \,.\, [C] \quad (7)$$

Φ_{cv} is also given by:

$$\Phi_{cv} = k_{cv} \,.\, [C] \,.\, q \quad (8)$$

where q, the cholesterol content of a single erythrocyte at time 0, is a constant.
Finally:

$$k_{cv} = \text{constant} \,.\, [V] \quad (9)$$

So the collision theory predicts that k_{cv} should be a linear function of $[V]$, whereas if a collision mechanism was not involved, k_{cv} should be independent of $[V]$. It is clear that k_{cv} depends on $[V]$ but according to a saturating function (fig.2). When $[V] \approx 2$ mM PC k_{cv} no longer increases. If the collision hypothesis is assumed, the saturation of the function indicates that the frequency of the efficient collisions responsible for the transfers reaches a limit value. This limitation, observed at high vesicle concentration, may be due to a steric hindrance of vesicles at the cell membrane caused by a contact time of cells with vesicles greater than the diffusion time of the vesicles in the surrounding medium. Consistent with this view, is the fact that the vesicle concentration in the microenvironment of the cell membrane is greater than that found in the medium (table 1). Such a process could occur whether or not there is a limited number of vesicle-binding sites. Cholesterol transfer was suggested [12] to occur through the transitory fusion of the membranes (erythrocytes and lipoproteins in their model) creating an hydrophobic channel, where the less polar molecules, i.e., cholesterol could diffuse more easily. In such a model the time of transitory fusion would impose the limit to the frequency of efficient collisions, i.e., to the rate of cholesterol transfers above a certain concentration of vesicles in the medium.

Acknowledgements

The authors are grateful to Dr K. R. Bruckdorfer and A. Fontaine for helpful discussions. F. G. and M. C. hold a Fellowship under the European Science exchange Programme. This work was supported by INSERM and DGRST.

References

[1] Hagerman, J. S. and Gould, R. G. (1951) Proc. Soc. Exp. Biol. Med. 78, 329–332.
[2] Murphy, J. R. (1962) J. Lab. Chin. Med. 60, 86–109.
[3] Murphy, J. R. (1962) J. Lab. Chin. Med. 60, 571–578.
[4] Bruckdorfer, K. R. and Green, C. (1967) Biochem. J. 104, 270–277.
[5] Quarfordt, S. H. and Hilderman, H. L. (1970) J. Lipid Res. 11, 528–535.
[6] Bell, F. P. and Schwartz, C. J. (1971) Biochim. Biophys. Acta 231, 553–557.
[7] D'Hollander, F. and Chevallier, F. (1972) J. Lipid Res. 13, 733–744.
[8] Malhotra, S. and Kritchevsky, D. (1975) Mech. Ageing Dev. 4, 137–145.
[9] Bjornson, L. K., Gniewkowski, C. and Kayden, H. J. (1975) J. Lipid Res. 16, 39–53.
[10] Lange, Y. and D'Alessandro, J. S. (1977) Biochemistry 16, 4339–4343.
[11] Gottlieb, M. (1977) Biochim. Biophys. Acta 466, 422–428.
[12] Bruckdorfer, K. R. and Graham, J. M. (1976) Biological Membranes (Chapman, D. and Wallach, F. F. H. eds) vol. 3, pp. 103–152, Academic Press, London.
[13] Bruckdorfer, K. R., Edward, P. A. and Green, C. (1968) Eur. J. Biochem. 4, 506–511.
[14] Grunze, M. and Deuticke, B. (1974) Biochim. Biophys. Acta 356, 125–130.
[15] Cooper, R. A., Arner, E. C., Wiley, J. S. and Shattil, S. J. (1975) J. Clin. Invest. 55, 115–126.
[16] Claret, M., Garay, R. and Giraud, F. (1978) J. Physiol. 274, 247–263.
[17] Fisher, K. A. (1976) Proc. Natl. Acad. Sci. USA 73, 173–177.
[18] Lange, Y., Cohen, C. M. and Poznansky, M. J. (1977) Proc. Natl. Acad. Sci. USA 74, 1538–1542.

[19] Kirby, C. J. and Green, C. (1977) Biochem. J. 168, 575–577.
[20] Smith, R. J. and Green, C. (1974) FEBS Lett. 42, 108–111.
[21] Gurd, F. R. N. (1960) Lipid Chemistry (Hanahan, D. J. et al. eds), p. 283, Wiley, London, New York.
[22] Haran, N. and Shporer, M. (1977) Biochim. Biophys. Acta 465, 11–18.
[23] Poznansky, M. J. and Lange, Y. (1978) Biochim. Biophys. Acta 506, 256–264.
[24] Patzer, E. J., Shaw, J. M., More, N. F., Thompson, T. E. and Wagner, R. R. (1978) Biochemistry 17, 4192–4200.
[25] Bull, H. B. (1964) An Introduction to Physical Biochemistry, p. 362, F. A. Davis Co.