Cellular Cholesterol Efflux Mediated by Cyclodextrins

DEMONSTRATION OF KINETIC POOLS AND MECHANISM OF EFFLUX*

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The efflux of cholesterol from cells in culture to cyclodextrin acceptors has been reported to be substantially more rapid than efflux induced by other known acceptors of cholesterol (Kilsdonk, E. P. C., Yancey, P., Stoudt, G., Bangerter, F. W., Johnson, W. J., Phillips, M. C., and Rothblat, G. H. (1995) J. Biol. Chem. 270, 17250–17256). In this study, we compared the kinetics of cholesterol efflux from cells with 2-hydroxypropyl-β-cyclodextrins and with discoidal high density lipoprotein (HDL) particles to probe the mechanisms governing the remarkably rapid rates of cyclodextrin-mediated efflux. The rate of cholesterol efflux was enhanced by shaking cells growing in a monolayer and further enhanced by placing cells in suspension to achieve maximal efflux rates. The extent of efflux was dependent on cyclodextrin concentration, and maximal efflux was observed at concentrations >50 mM. For several cell types, biexponential kinetics of cellular cholesterol efflux were observed, indicating the existence of two kinetic pools of cholesterol: a fast pool (half-time (t½) = 19–23 s) and a slow pool with t½ of 15–30 min. Two distinct kinetic pools of cholesterol were also observed with model membranes (large unilamellar cholesterol-containing vesicles), implying that the cellular pools are in the plasma membrane. Cellular cholesterol content was altered by incubating cells with solutions of cyclodextrins complexed with increasing levels of cholesterol. The number of kinetic pools was unaffected by raising the cellular cholesterol content, but the size of the fast pool increased. After depleting cells of the fast pool of cholesterol, this pool was completely restored after a 40-min recovery period. The temperature dependence of cyclodextrin-mediated cholesterol efflux from cells and model membranes was compared; the activation energies were 7 kcal/mol and 2 kcal/mol, respectively. The equivalent activation energy observed with apo-HDL-phospholipid acceptor particles was 20 kcal/mol. It seems that cyclodextrin molecules are substantially more efficient than phospholipid acceptors, because cholesterol molecules desorbing from a membrane surface can diffuse directly into the hydrophobic core of a cyclodextrin molecule without having to desorb completely into the aqueous phase before being sequestered by the acceptor.

The first step in reverse cholesterol transport is the efflux of cellular cholesterol molecules to extracellular acceptors (1–3). This initial step is thought to be mediated by high density lipoproteins (HDL)1 or by specific subpopulations of HDL (1–3). It is generally accepted that cholesterol efflux occurs by an aqueous diffusion mechanism whereby the cholesterol molecules desorb from the plasma membrane into the aqueous phase, diffuse, and are solubilized by an acceptor particle (2, 4).

β-Cyclodextrins are cyclic heptasaccharides consisting of β(1–4)-glucopyranose units (5). These water-soluble compounds contain a hydrophobic core capable of solubilizing nonpolar substances (5, 6). Thus, cyclodextrins have been used as vehicles to deliver hydrophobic drugs (5, 6). The β-cyclodextrins (7 glucose units), when compared with α (6 glucose units) and γ (8 glucose units) cyclodextrins, have the highest affinity for encapsulating sterols, in particular cholesterol (7). Chemical modifications of the hydroxyl groups of cyclodextrins often enhance both their solubility in water and their ability to dissolve hydrophobic compounds (7–9). Recent studies from our laboratory compared the abilities of β-cyclodextrins, 2-hydroxypropyl-β-cyclodextrins (20HβpCDs), and methyl-β-cyclodextrins (MβCD) to promote cholesterol efflux from mouse L-cell fibroblasts (10). The order of efficiency in accepting cholesterol was found to be MβCD > 20HβpCD > β-cyclodextrin. These studies also showed that there was an initial rapid efflux of the cholesterol from mouse L-cell fibroblasts to 20HβpCD with 50–90% of the initial cellular cholesterol being released during the first 0.5–1 h of an 8-h incubation. This is in contrast to that observed when cells are incubated with HDL3, where cholesterol was released at a roughly constant rate throughout the incubation, with 40% being released at the end of 8 h. In addition, earlier studies from this laboratory showed that the rate of cholesterol release to various acceptors that contain phospholipid, including apo AI discs and HDL3, differs depending on the cell type used with the order of the rates being FuSAH hepatoma > mouse L-cell fibroblasts > human skin fibroblasts (2, 4). In contrast, our recent studies comparing cyclodextrins and HDL3 as acceptors showed that this difference in the maximal cholesterol efflux

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1 The abbreviations used are: HDL, high density lipoprotein; CS, calf serum; MβCD, methyl-β-cyclodextrin; MEM, minimum essential medium; 20HβpCD, 2-hydroxy-propyl-β-cyclodextrin; PC, phosphatidylcholine; DPPC, 1,2-dipalmitoyl phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; CHE, cholesteryl hexadecyl ether; LUV, large unilamellar vesicle.
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among cell types is largely lost when cyclodextrins are used as acceptors. These studies also have shown that the maximal rate constant for cholesterol efflux in the presence of cyclodextrin was 3.5–75-fold greater than those observed when HDL was used as an acceptor for cholesterol (10). Estimates for the half-times of cholesterol release from the three types of cells to cyclodextrins were found to be in the range of 12–14 min (10), in contrast to the 2–18 h half-times for efflux to HDL3 (2, 4).

While these half-times for cholesterol efflux to cyclodextrins are indeed low, there have been reports of even shorter half-times when cyclodextrins are used as acceptors of cholesterol from erythrocytes (7). A variety of factors could explain the differences in half-times between tissue culture cells and erythrocytes, including the fact that the tissue culture cells were studied as monolayers, whereas erythrocytes were used in suspension. Since both stirring and maintenance of cells in suspension enhance efflux (11), these factors could explain the differences in efflux between erythrocytes and cells in culture.

The first aim of the present studies was to determine if the mechanism(s) of cholesterol efflux from cells to cyclodextrins differs from that to HDL or apo-HDL-cholesterol complexes. The second aim was to explain the discrepancy in efflux half-times between erythrocytes and monolayers of cells in culture. This involved comparing the kinetics of cyclodextrin-mediated cholesterol release from cell monolayers and from the same cells in suspension. The ability of cyclodextrins to rapidly remove cholesterol from cells was used to define kinetic pools of cholesterol that are not readily detected when less efficient acceptor particles (e.g. HDL) are utilized.

**EXPERIMENTAL PROCEDURES**

**Materials—** Tissue culture flasks and plates were obtained from Falcon (Lincoln, N.J.) and Corning Glass Works (Corning, N.Y.). Culture media and trypsin were obtained from Life Technologies, Inc. Bovine serum albumin (essentially fatty acid-free), heat-inactivated fetal bovine serum, DEAE-Sephadex, cholesteryl methyl ether, and unesterified cholesteryl esters were purchased from Sigma. 1,2-dipalmitoylphosphatidylcholine (DPPC), 1-palmitoil-2-oleyl phosphatidylcholine (POPC), 1-palmitoil-2-oleyl phosphatidylethanolamine (POPE), and 1-palmitoil-2-oleyl phosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesteryl oleate, [3H]cholesterol (51 Ci/mmol), [14C]cholesterol (58 mCi/mmol), [3H]D-2OHp, and [3H]UDP, were purchased from DuPont NEN. All organic solvents were purchased from J. T. Baker Chemical Co. HEPES and were supplemented with 2 mM glutamine and 50 mM NaCl.

**Cell Culture—** Cells (mouse L-cell and GM 3468A human skin fibroblast line cultured in minimal essential medium (MEM) containing 10% fetal bovine serum or 5% fetal bovine serum (Fu5AH cells). All media were buffered with sodium bicarbonate (24 mM) and 50 mM HEPES and were supplemented with 2 mM glutamine and 50 μg/ml gentamicin. All incubations were done in a humidified incubator at 37°C with 5% CO2.

**Labeling and Equilibration of Cell Cholesterol—** Cells were plated 3–7 days before cholesterol efflux experiments. Cells were labeled by incubation for 1–2 days with medium containing 1–2 μCi/ml of either [3H]cholesterol or [14C]cholesterol (0.1% ethanol final concentration) and 5% fetal bovine serum. After radiolabeling, the cellular pools of cholesterol were equilibrated by incubation for 24 h in MEM containing 0.2% bovine serum albumin. Compound 38-035 was included in both the labeling and equilibration media (1 μg/ml, 0.1% dimethyl sulfoxide final concentration) to prevent the esterification of cholesterol by acyl-CoA:cholesterol-acyltransferase (16).

**Efflux of Labeled Cholesterol from Cell Monolayers and Cells in Suspension—** In experiments comparing the efflux of cholesterol from cell monolayers with cells in suspension, the cells were plated in 35-mm dishes with 1 ml of MEM containing [3H]cholesterol, and equilibrated as described above. The cell monolayers were then washed 3 times with MEM containing HEPES (50 mM) and switched to the same medium containing the various cholesterol acceptors. Prior to the efflux phase of the experiments, a set of cells was harvested to determine the initial cellular [3H]cholesterol content. Briefly, the cell monolayers were rinsed 3 times with phosphate-buffered saline containing 1 mM CaCl2 and 0.5 mM MgSO4, and the lipids were extracted by incubation with isopropanol alcohol (17). The cell lipids were then dried under N2, and solubilized in toluene, and an aliquot was taken for liquid scintillation counting.

Two methods were used to obtain cells for suspension cultures. In the first method, cell monolayers were first rinsed 2 times with phosphate-buffered saline and incubated at 37°C for 2–3 min in MEM containing 0.05% trypsin and 0.53 mM EDTA, and then 1% bovine serum albumin/MEM was added to inhibit trypsin. The cells were then pooled and pelleted by centrifugation at 1000 rpm for 10 min. The cells were resuspended in MEM containing HEPES (50 mM) and washed 3 times using the same medium by alternate pelleting and resuspension. In other experiments, Fu5AH or L-cells were removed from the dishes by incubation at 37°C for 5 min with cation-free phosphate-buffered saline containing 1 mM EDTA. No significant differences were observed in the rate of cholesterol efflux from either type of cell when comparing the two methods (data not shown). However, the size of the kinetic cholesterol efflux pools for L-cells was somewhat different between cells obtained with EDTA or without EDTA (data not shown). Cells obtained with EDTA treatment tended to clump and may explain variations in some kinetic parameters. Thus, in all experiments presented in this paper, cells were placed in suspension culture by incubation with trypsin. For the efflux phase of the experiment, the cells were resuspended in an appropriate volume of MEM (50 mM HEPES), such that 1 ml of suspension contained the same amount of cellular [3H]cholesterol as was present in the wells containing the starting cell monolayers, and to initiate efflux, 1 ml of MEM containing the desired concentration of the various cholesterol acceptors was added to the cells in suspension. To examine the effects of shaking on the efflux of cellular cholesterol, parallel sets of cell monolayers and suspension cultures were shaken at 60 rpm using a minirotor shaker (Bellco Glass, Vineland, NJ). In experiments where only suspension cultures were used, the cells were first seeded in 100-mm plates, radiolabeled, and then equilibrated as described above. Typically, cells from 15–20 100-mm plates were pooled for an experiment, and the amount of cell cholesterol used in the efflux experiments ranged from 8 to 30 μg/culture.

In some experiments, instead of suspension cultures, aliquots were taken at various time points and filtered through a 0.45-μm Multiscreen (96 screens) filtration plates (Millipore Corp.). Aliquots of the filtrate were then taken for liquid scintillation counting. In experiments where the mass of cell cholesterol was determined, the cells were separated from medium containing the acceptors by filtration through a Millipore filter with a diameter of 25 mm and a pore size of 0.45 μm. The filters were then washed 3 times with warm (37°C) phosphate-buffered saline and dried in an oven at 110°C for 15 min. The cell lipids were then extracted by incubating the filters in 2 ml of...
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isopropyl alcohol at room temperature overnight, and the cholesterol content was quantified by the procedure of Ishikawa et al. (18) as modified by Kianseck et al. (19) using cholesteryl methyl ether as an internal standard. Cell protein was quantified by incubating the filters for 3 h in a solution containing 0.1 N NaOH and 1% SDS, and the protein content was measured using the method of Lowry (20) as modified by Markwell et al. (21).

The extent of cholesterol efflux was calculated from the fraction of initial labeled cholesterol remaining in the cells. For time course experiments (as noted in the figure legends), the data were either fitted to a single exponential decay equation or described in terms of parallel cholesterol efflux from two independent pools fitted using a biexponential equation by non-linear regression, as described previously (22). The cyclodextrin acceptor was present in large excess, so unidirectional efflux of radiolabeled cholesterol was assumed. Efflux from a single pool was described by the equation \( Y = Y_0 e^{-kt} \), where \( Y \) is the fraction of radiolabeled cholesterol remaining in the cell at time \( t \). \( A \) is the size of the cellular cholesterol pool (in this case \( 1 \)) that is available for efflux with an apparent rate constant \( k_e \). Efflux from two independent pools was described by the equation \( Y = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \). In this case, \( A \) and \( B \) are the fractional sizes of pool \( 1 \) and pool \( 2 \), respectively. The apparent rate constants for efflux from pools \( 1 \) and \( 2 \) are \( k_1 \) and \( k_2 \), respectively. As a first approximation, exchange of cholesterol between pools \( 1 \) and \( 2 \) is ignored in this analysis. The rate constants are apparent values because they are dependent on the concentration of cyclodextrin acceptor. The apparent half-times were calculated as \( t_{1/2} = \ln 2/k_e \).

Efflux of Labeled LUV Cholesterol—To measure the kinetics of cholesterol efflux from model membranes, donor vesicles (DPPC/POPG/cholesterol, 80:15:5 molar ratio) were made primarily with DPPC, a gel-state phospholipid at 37 °C, in order to slow the rate of sterol efflux. Donor vesicles also contained POPG, which imparts a negative charge on the vesicles and facilitates donor and acceptor separation by ion exchange procedures. Typically, donor LUVs were radiolabeled with 2-5 µCi of \(^{3}H\)cholesterol/ml of vesicle preparation. For the efflux phase, the LUV donor vesicles were incubated with \(^{3}H\) cholesterol in buffer containing 150 mM NaCl and 10 mM Tris (pH 7.4) for 30 s to 2 h at 37 °C. Donor LUVs were separated from 2OHpCD acceptors by an ion exchange–filtration separation procedure. Miniature ion exchange columns were prepared in a Millipore multiscreen vacuum filter unit. Briefly, two consecutive 250-µl aliquots of a slurry of DEAE-Phosphaq (Pharmacia; hydrated in Tris-buffered saline to approximately 1:1 v/v) were placed into each well of the filter unit to give a final bed volume of approximately 200 µl of ion exchange resin/well. Two filters were washed with 75 mM NaCl, 10 mM Tris, pH 7.4 (wash/elution buffer) were carried out prior to the experiments. For each time point, the aliquot of the donor/acceptor mixture was placed into the filtration wells under vacuum for rapid separation and then quickly washed with 300 µl of wash/elution buffer. The recovery of acceptors was monitored by following trace amounts of radiolabeled \(^{3}H\)cholesterol initially present in the donor/acceptor mixture and was typically 80 ± 3%. The extent of donor particle spill over into filtrate was determined by incubating donor vesicles with Tris-buffered saline and was found to be < 0.5%. Efflux was calculated as the fraction of the initial LUV \(^{3}H\)cholesterol recovered with the acceptors in the filtrate and was corrected for the percentage of loss of acceptors during the separation procedure.

Loading of the 2OHpCDs with Free Cholesterol—The cholesterol content of the cells used in the efflux experiments was varied by using cyclodextrins containing unlabeled cholesterol. A stock solution of 2OHpCD preloaded with cholesterol was prepared at a 18.1 molar ratio of 2OHpCD to cholesterol. Briefly, the desired amount of cholesterol was dried under \( N_2 \) and then under high vacuum, and an appropriate volume of MEM (50 mM HEPES) containing 2OHpCD (100 mM) was added to the dried lipid film. The mixture was incubated at 37 °C for 48 h in a shaking water bath and, prior to use, was filtered through a 0.22-µm Millipore filter. To achieve cyclodextrin solutions with lower amounts of cholesterol, the stock 18.1 mixture was diluted with a 100 mM cyclodextrin solution to yield a series of 100 mM cyclodextrin solutions with differing cyclodextrin:cholesterol ratios. This approach prevented the precipitation of cholesterol known to occur upon dilution of cyclodextrin-cholesterol complexes with aqueous solutions (23). Also, to avoid this possibility during experiments, concentrated cell suspensions were added in a volume of MEM that was \( V_0 \) of the volume of the cyclodextrin:cholesterol solution.

**RESULTS**

The Effects of Shaking on Cholesterol Efflux to Cyclodextrins from Monolayer and Suspension Cultures—Time courses of cholesterol efflux to 10 mM 2OHpCD from both mouse L-cell monolayers and suspension cultures are shown in Fig. 1. The release of cholesterol was significantly greater from the suspension cultures compared to monolayer cultures, and using suspension cultures had a greater impact on efflux than shaking the cells. This effect was most apparent at early times. For example, after 30 s of efflux, stationary monolayer cultures released 1% of their initial \(^{3}H\)cholesterol, whereas stationary suspension cultures released 10%. When mouse L-cell monolayers and suspension cultures were shaken, they released 2 and 16% of their \(^{3}H\)cholesterol in 30 s. Similar results were observed in experiments using Fu5AH hepatoma cells and human skin fibroblasts (data not shown). Based on these results, we used suspension cultures that were shaken for all subsequent experiments in order to obtain the maximum possible efflux to cyclodextrin acceptors.

Dependence of Cyclodextrin-mediated Cholesterol Efflux on the Concentration of both Donors and Acceptors—The dose–response curves for efflux of cholesterol from Fu5AH cells and mouse L-cells in suspension to 2OHpCD after 30 s, 10 min, and 20 min of incubation are shown in Fig. 2, A and B. At all three times of incubation, and at low concentrations of 2OHpCD, the cholesterol efflux from both Fu5AH cells and mouse L-cells was proportional to the amount of cyclodextrin present in the medium. Saturation of cholesterol efflux from both cell types was observed at high concentrations of 2OHpCD (>50 mM for Fu5AH cells and >75 mM for mouse L-cells). Under this condition the Fu5AH cells released 41, 66, and 81% of their \(^{3}H\)cholesterol at 30 s, 10 min, and 20 min of incubation, respectively (Fig. 2A). There was release of 30, 51, and 60% of their \(^{3}H\)cholesterol from the mouse L-cells after 30 s, 10 min, and 20 min of incubation (Fig. 2B). Similarly, the amount of cholesterol efflux was proportional to the number of cells when a constant amount of 2OHpCD (10 mM) was incubated for 30 s with increasing numbers of Fu5AH cells (data not shown), indicating that low concentrations of acceptor, cholesterol efflux follows second-order kinetics. In addition, similar kinetics were observed when Fu5AH cells in suspension were incu-
bated for 30 min with increasing concentrations of apo-HDL-PC and when a constant amount of apo-HDL-PC was incubated with increasing numbers of Fu5AH cells (data not shown).

The Kinetics of Cyclodextrin-mediated Cholesterol Efflux from Cells and LUVs—The time courses of cholesterol efflux from Fu5AH cells, mouse L-cells, and human skin fibroblasts incubated with concentrations of 2OHpCD where cholesterol efflux is saturated are shown in Fig. 3, A, B, and C. Two kinetic pools were observed for cholesterol efflux from all cell types. The half-times of cholesterol release from the fast pool were 19, 20, and 21 s from the Fu5AH cells, L-cells, and skin fibroblasts, respectively. Efflux of cholesterol from the slow pool occurred with half-times of 15 min for the Fu5AH cells, 28 min for the mouse L-cells, and 35 min for the fibroblasts. In a series of experiments the size of the fast and slow pools ranged from approximately 20 to 60%, with Fu5AH cells having the largest fast pool and skin fibroblasts the smallest.

To compare the kinetics of cyclodextrin-mediated cholesterol efflux from artificial membranes we examined the time course of the efflux of cholesterol from DPPC/POPG/cholesterol (80:15:5) donor LUV to 2OHpCD (Fig. 4). As with the cells, there were two pools of cholesterol exhibiting different efflux kinetics. The fast pool comprised 78% of the cholesterol, and this pool exhibited a half-time of 28 s. The slow pool (22%) had a half-time of 219 min. Similar half-times and pool sizes were obtained when the ratio of LUV to cyclodextrin was varied by keeping the cyclodextrin concentration constant (100 mM) and incubating with either one-half or double the concentration of LUV used in Fig. 4 (data not shown). The extent of phospholipid release over a period of 2 h was found to be less than 3%, whereas over 75% of the cholesterol was released within 5 min. Moreover, by gel filtration chromatography using a 10 × 1.5-cm Bio-Gel A-1.5m column to separate donor LUV from 2OHpCD, we found no detectable binding or entrapment of cyclodextrins by LUV during a 2-h incubation at 37 °C (data not shown).

Previous studies have shown that at least two kinetic pools for cholesterol efflux can be observed in a number of cells in culture (22, 24). The relationship between these pools and cell cholesterol content has not been established. The study presented in Fig. 5, A and B, examines whether the kinetics of cyclodextrin-mediated cholesterol efflux from Fu5AH cells is influenced by the content of cellular cholesterol. The kinetics of cholesterol efflux when Fu5AH cells are incubated with 2OHpCD complexed with increasing levels of free cholesterol is shown in Fig. 5A. The cells were incubated for up to 30 min with the various 2OHpCD-cholesterol solutions and, at the end of the incubations, exhibited differing cholesterol mass contents (see inset). Similar to the data shown in Fig. 3A, when the Fu5AH cells were incubated with 2OHpCD that contained no cholesterol there were two kinetic pools of cholesterol released. By the end of the 30-min incubation these cells contained 2 μg of cholesterol/mg of protein, in contrast to control cells maintained in the absence of cyclodextrin, which contained 20 μg/mg of protein. When the cells were incubated with the 2OHpCD solution that was 50% saturated with cholesterol (see "Experimental Procedures"), the cells contained 18 μg of cholesterol/mg of protein, comparable with the mass remaining in the cells that were incubated with MEM alone. Under these conditions, there is an exchange of cell and exogenous cholesterol mass, with no net mass depletion. Under these cholesterol exchange conditions, there are clearly two kinetic pools of cholesterol released from the cells (Fig. 5). There was net accumulation of cholesterol when cells were incubated with 2OHpCD that was either 65 or 100% saturated with free cholesterol; the cell contents were 27 and 50 μg of cholesterol/mg of protein, respectively. Under these conditions of net cholesterol accumulation, two distinct kinetic pools of cholesterol efflux remained. While changing cholesterol contents did not affect the number of kinetic pools, there was an effect on the size of the fast pool. The size of the fast pool is plotted in Fig. 5B against the mass of cholesterol remaining in the cells after the 30-min incubation with the various 2OHpCD-cholesterol complexes. It can be seen that raising the content of cholesterol in cells increased the size of the fast pool for cholesterol efflux.

Exchangeability of the Slow Pool of Cholesterol Efflux with the Fast Pool—The study shown in Fig. 6, A and B, examines whether the cholesterol of the slow pool is exchangeable with that in the fast pool. Fu5AH cells in suspension were divided into four sets. The first set was incubated for up to 30 min with 100 mM 2OHpCD that was 50% saturated with cholesterol (Fig. 6A). Similar to previous observations, there were two kinetic pools of [3H]cholesterol released, with half-times of 13 s and 22 min for the fast and slow pools, respectively. The size of the fast pool was 40%, and the size of slow pool was 60%. The other three sets of cells were preincubated for 2.5 min with 2OHpCD solution (50% saturated with cholesterol) to remove the [3H] cholesterol from the cell fast pool. These cells were then pelleted by centrifugation, resuspended in MEM alone, and equilibrated for 0, 20, or 40 min to allow for the movement of the [3H] cholesterol from the slow pool into the fast pool of cholesterol prior to re-exposure to cyclodextrins to establish the new kinetics of efflux. When the cholesterol pools were determined with cells that had undergone no chase period after initial exposure, 14% of the original fast pool, as monitored by [3H] cholesterol, was present. After 20 min of equilibration, the size of the fast pool had increased to 28%, and by the end of 40

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**Fig. 2.** Dependence of [3H]cholesterol efflux from Fu5AH cells and mouse L-cells on 2OHpCD concentration. Fu5AH cells (panel A) and mouse L-cells (panel B) were labeled with [3H]cholesterol, equilibrated, and put in suspension as described under "Experimental Procedures." The cells were then incubated for 30 s ( ), 10 min ( ), or 20 min ( ) at 37 °C in MEM-HEPES containing the indicated concentrations of 2OHpCD. Efflux is expressed as the percentage of [3H] cholesterol released. Values are the means ± S.D. for triplicate determinations. Where not visible, error bars are within the points.
Fig. 3. Time courses of the efflux of [3H]cholesterol from Fu5AH cells, mouse L-cells, and human skin fibroblasts in the presence of 2OHpCD. Fu5AH cells (panel A), mouse L-cells (panel B), and human skin fibroblasts (panel C) were labeled with [3H]cholesterol, equilibrated, and put in suspension as described under "Experimental Procedures." The cells were incubated for up to 30 min at 37°C in MEM-HEPES containing 100 mM 2OHpCD. The data are expressed as the fraction of [3H]cholesterol remaining and were fitted using a biexponential decay equation (see "Experimental Procedures"). The values are means ± S.D. for triplicate determinations. Where not visible, error bars are within the points.

Temperature Dependence of Cyclodextrin-mediated Cholesterol Efflux from Fu5AH Cells and LUV—Arrhenius plots of the rates of cholesterol efflux obtained when Fu5AH cells were incubated for 10 min with either 2OHpCD or apo-HDL-PC at temperatures ranging from 4 to 37°C are shown in Fig. 7. The experimental activation energy for the transfer of cholesterol to apo-HDL-PC was 20 kcal/mol. In contrast, the activation energy for cholesterol efflux to 2OHpCD was 7 kcal/mol. Activation energies of 9 and 7 kcal/mol were obtained when the rates of cholesterol release were measured by incubating the cells with the 2OHpCD for 30 s and 5 min, respectively. In similar experiments using mouse L-cells as cholesterol donors, an activation energy of 8 kcal/mol was obtained. The Arrhenius plot of the data when LUVs composed of POPC/POPG/cholesterol (40:15:45) were incubated with 2OHpCD at temperatures ranging from 4 to 50°C for times up to 2 min yielded an activation energy of 2 kcal/mol for cholesterol efflux from the LUV donors.

Fig. 5. Kinetics of cholesterol efflux to 2OHpCD from Fu5AH cells that were exposed to various cyclodextrin-cholesterol complexes that changed cell cholesterol content. Fu5AH cells were labeled with [3H]cholesterol, equilibrated, and put in suspension as described under "Experimental Procedures." The cells were then incubated for up to 30 min at 37°C in MEM-HEPES alone or with 100 mM 2OHpCD solutions that contained no cholesterol (○), solutions that were saturated with cholesterol (●), or solutions in which saturated cyclodextrins were mixed with cholesterol-free cyclodextrins at a ratio of 1:1 (●; 50% saturated) or 1:0.5 (●; 66% saturated) (panel A). Efflux is expressed as the fraction of [3H]cholesterol remaining. The data were fitted to a biexponential decay equation. Also shown in the inset are the masses of cholesterol remaining in the cells after the 30-min incubation with the different treatments. The mass data are expressed as µg of cholesterol/mg of cell protein. The relationship between the percentage size of the fast pool and the mass of cholesterol in the cells after the 30-min incubation is shown in panel B. The vertical dashed line represents the cholesterol content of the same cells after a 30-min incubation with MEM-HEPES alone. All values are means ± S.D. of triplicate determinations. Where not visible, error bars are within the points.

**DISCUSSION**

Since our previous studies (10) had demonstrated the ability of cyclodextrins to promote the rapid efflux of cholesterol from cells in culture, we initiated the present investigation to further examine the release of cholesterol to cyclodextrins. In this study we obtained detailed kinetic data on cholesterol efflux and additional information that has permitted the formulation of a model that can explain the mechanism of action of cyclodextrins in promoting cholesterol efflux.
Comparison of Cholesterol Efflux from Cell Monolayers and Cells in Suspension—The efficiency of any acceptor in stimulating cell cholesterol efflux is linked, in part, to factors that affect the ability of the acceptor to readily diffuse through the unstirred water layer and cell glycocalyx and concentrate at the cell surface (4, 25). Among the factors that will influence acceptor diffusion are the size of the acceptor particles (25, 26) and the incubation conditions under which the experiments are conducted. These conditions include incubation time, monolayer versus suspension cultures, and shaking versus stationary incubations (11). Sampling time and acceptor size are important and related determinants, since smaller acceptors will diffuse to the cell surface more rapidly than larger particles (25). Thus, short incubation times will accentuate the importance of small particles as acceptors. Suspension cultures have more cell surface exposed for efflux, and diffusional barriers are reduced (11); in addition, the effects of the unstirred water layer are reduced even more when the cells in suspension are shaken. Previous studies have shown that the rate of cholesterol release from cells to acceptors that contain phospholipid (i.e. apo-HDL-PC, HDL) is faster from cells in suspension than that from cell monolayers (11), and we have reexamined these factors using the ultimate in small acceptors, 2OHpβCD, which has a molecular weight of approximately 1500. The present studies show that the rate of cholesterol efflux from cells to cyclodextrins is affected both by mixing and by maintaining the cells in suspension (Fig. 1). First, the rate of cholesterol release to cyclodextrins is enhanced from cell monolayers and cells in suspension when these are shaken. Second, the efflux of cholesterol from cells to cyclodextrins is increased if the cells are put in suspension, when compared with being maintained as monolayers. Although the effects of these two factors on the rate of cholesterol release is additive, maintenance in suspension appears to have a greater impact on efflux than does mixing.

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![Fig. 6. Kinetics of the transfer of [3H]cholesterol from the slow pool of Fu5AH and L-cells to the fast pool. Fu5AH cells or L-cells were labeled with [3H]cholesterol, equilibrated, and put in suspension as described under “Experimental Procedures.” One set (●, dotted line) of the cells in suspension was then incubated at 37 °C in MEM-HEPES containing 100 mM 2OHpβCD that was 50% saturated with cholesterol (panel A, Fu5AH; panel C, L-cells). To deplete the cells of the fast pool of [3H]cholesterol, the other 3 sets of cells in suspension were incubated for 2.5 min at 37 °C in MEM-HEPES containing 100 mM 2OHpβCD that was 50% saturated with cholesterol. The cells were then quickly pelleted by centrifugation for 2 min at 4000 rpm. After a chase period to allow for restoration of the fast pool, the cells were resuspended in 1 ml of MEM-HEPES and were held in MEM without cyclodextrin prior to reexposure to 100 mM 2OHpβCD. The times for the chase of Fu5AH cells were 0 min (●), 5 min (○), 35 min (△), and 30 min (□). Efflux data are expressed as the fraction of [3H]cholesterol remaining in cells and are fitted to a biexponential decay equation. Shown in panels B and D are the percentage size of the fast pool plotted against the incubation time of the chase for Fu5AH and L-cells, respectively. All values are the mean ± S.D. of triplicate determinations. Where not visible, error bars are within the points.](image)

![Fig. 7. Arrhenius plot of the rate constants versus temperature for the efflux of [3H]cholesterol from Fu5AH cells and liquid-crystalline POPC/POPG/Chol (40:15:45) LUV donors. Fu5AH cells were labeled with [3H]cholesterol, equilibrated, and put in suspension as described under “Experimental Procedures.” The Fu5AH cells were incubated for 10 min at temperatures ranging from 4 to 37 °C in MEM-HEPES containing either 100 mM 2OHpβCD (●) or 1 mg of apo-HDL-PC phospholipid/ml (○). LUVs were prepared and labeled with [3H]cholesterol as described under “Experimental Procedures.” They were then incubated for times up to 2 min at temperatures ranging from 4 to 50 °C in Tris buffer containing 100 mM 2OHpβCD (●). At the end of the incubation, the LUVs were separated from cyclodextrins as described under “Experimental Procedures.” All values are the means of triplicate determinations.](image)
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When saturating concentrations of cyclodextrins are used as acceptors, the efflux time course is biexponential, indicating at least two kinetic pools for cholesterol release from Fu5AH cells, mouse L-cells, and human skin fibroblasts. The fast pool is released with half-times ranging from 19 to 23 s, and the slow pool has half-times ranging from 15 to 35 min (Fig. 3). The location and interrelationship between these pools remain to be resolved. Because of its very fast efflux it is most likely that the fast pool of cholesterol is located in the plasma membrane. Three locations can be suggested for the slow pool: 1) intracellular cholesterol, 2) cholesterol present in the cytoplasmic monolayer of the plasma membrane bilayer, and 3) a separate lateral lipid domain in the plasma membrane. A number of investigations have indicated that the majority (>90%) of the free cholesterol in cells is located in the plasma membrane (27, 28), so it is unlikely that the slow pool is comprised of intracellular cholesterol since the estimated sizes of this pool range from approximately 50 to 80% of the cellular free cholesterol. Another reason to believe that the fast and slow pools are both located in the plasma membrane is the observation that two kinetic pools exist for cholesterol release from LUV to cyclodextrins (Fig. 4). In this vesicle system all the cholesterol is located in membrane, so both kinetic pools must reside in the LUV bilayer.

The remaining two locations for the slow pool, the cytoplasmic monolayer of the plasma membrane bilayer or a lateral domain, cannot be resolved with the data currently available. If the slow pool represents cholesterol in the inner monolayer of the membrane bilayer, then the half-time for efflux associated with this pool would represent the half-time for transbilayer movement (flip-flop) between inner and outer monolayers of the plasma membrane. Numerous studies measuring cholesterol flip-flop have provided very diverse estimates of flip-flop rates, ranging from a few seconds to many hours (for a discussion see Refs. 14 and 29). The half-time for efflux from the slow pool to cyclodextrins ranges from approximately 20 to 30 min (Fig. 3), and this value is consistent with some of the estimates for flip-flop (29, 30). Equally plausible is a model in which the fast and slow pools of cholesterol reflect different lateral lipid domains within the membrane. There is considerable evidence demonstrating the presence of domains that contain different amounts of cholesterol in both the plasma membrane of cells and model membranes. The packing of the cholesterol molecules within these domains is influenced by a number of factors including phospholipid composition (31, 32), type of sterol (33), and sterol:phospholipid ratio (34, 35) (for a review see Ref. 24).

Regardless of the location of the slow domain, it is evident that cholesterol within this pool can exchange with the fast pool with approximate first-order kinetics and half-times between 20 and 30 min (Fig. 6). If the slow pool is inner monolayer cholesterol, then the rate of replenishment of the fast pool would represent flip-flop, and cholesterol from the slow pool would have to enter the fast pool prior to efflux. Alternatively, if the fast and slow pools were cholesterol in separate lateral domains, efflux could occur from both domains or could proceed only through the fast pool, with exchange occurring between slow and fast kinetic pools (for a discussion of these relationships see Ref. 36). It is interesting to note that the half-time for replenishment of the fast pool is similar to the half-time for efflux from the slow pool, a relationship that would be consistent with a model in which the cholesterol in the slow pool has to be transferred to the fast pool before it becomes available for efflux to cyclodextrin.

We cannot yet establish the relationships between the two kinetic pools of cell cholesterol observed when cells are incubated with cyclodextrin to kinetic pools studied in other experimental systems. Studies by Woodford et al. (37) following the exchange of dehydroergosterol between membrane preparations prepared from L-cells exhibited three kinetic pools, two of which had half-times of 24 min. (4%) and 150 min (30%), while the third represented 66% of the membrane cholesterol and was essentially nonexchangeable. In a study following the kinetics of the release of labeled cholesterol from three different cell types exposed to apoprotein-phospholipid complexes, efflux kinetics were generally best fit by a two-compartment model, with a fast pool having half-times of 1.5-3 h and a slow pool with half-times ranging between 15 and 20 h. The apparent size of these pools differed among cell types, and estimates of both the sizes and half-times were influenced by the concentration and composition of the acceptor particles (22, 38). It was concluded from these investigations that differences in the overall rate of release of cholesterol, which differs among cell types (39), were a reflection of the relative distribution of membrane cholesterol in fast and slow kinetic pools (22). Although the size and half-times of the pools observed with cyclodextrin acceptors differ from similar parameters determined when cells are exposed to phospholipid-containing acceptors, it has become evident that the kinetics of efflux are influenced by the nature and composition of both the cholesterol donors and the cholesterol acceptors (25, 40).

Any proposal for the role played by the kinetic pools of cell cholesterol in the metabolism of cholesterol would have to be closely linked to the determination of the cellular location of these pools. As discussed above, it is possible that the slow pool represents cholesterol in the cytoplasmic monolayer of the plasma membrane bilayer, and the half-time for efflux from this pool is a reflection of flip-flop. However, there is strong evidence to suggest that slow and fast kinetic pools of cholesterol in both biological and artificial membranes reflect lateral domains of membrane lipid (24, 41). In addition to kinetic studies tracing the movement of labeled cholesterol (22, 36) or fluorescent sterol analogs (24, 41), membrane lipid phases have been identified microscopically (33) and by differential sensitivity to cholesterol oxidase (42-44). Studies by Le Grimmel and colleagues (42–44) of renal epithelial cells have demonstrated two pools of cholesterol that are oxidized at different rates whose relative sizes are a function of the cholesterol content of the membranes. Similarly, we have observed in the present study that the distribution of cholesterol between the fast and slow pools changes as cholesterol content is varied (Fig. 5). Interestingly, a cellular pool of cholesterol that has recently been implicated in the rapid flux of cholesterol between cells and pre-β-HDL, is thought to be cholesterol located in caveolae (45), and it is this pool of cholesterol that appears to be particularly sensitive to oxidation by cholesterol oxidase (46). It is tempting to speculate that there are relationships between the fast kinetic pool of cholesterol revealed by treatment with cyclodextrin, the cholesterol oxidase-sensitive pool in membranes (43, 44), and the pool of cell cholesterol associated with caveolae (45, 47, 48). Studies are currently underway to test these relationships.

Mechanism of Cholesterol Efflux from Cells to Cyclodextrins and Acceptors That Contain Phospholipid—As discussed above, at low concentrations of cyclodextrin, efflux from cells to the cyclodextrins is dependent on the concentration of acceptor, and at high concentrations of cyclodextrin, the rate of cholesterol release becomes saturated (Fig. 2). A similar hyperbolic dependence on acceptor concentration has been described for the rate of cholesterol efflux from cells to acceptors that contain phospholipid (2, 4, 49). It has been proposed that the efflux of cell cholesterol to phospholipid-containing acceptors such as...
HDL, apoprotein-phospholipid complexes, or phospholipid vesicles requires the desorption of cholesterol from the plasma membrane into the aqueous phase followed by the incorporation of the desorbed cholesterol molecule into the acceptor part (for a discussion see Refs. 2 and 4). At low acceptor concentrations the rate of cholesterol efflux depends on the number of collisions between the acceptors and the desorbed cholesterol molecules present in the aqueous phase (2, 25, 50). At high concentrations of acceptor, the number of collisions is maximized, and the desorption of the free cholesterol molecule out of the plasma membrane into the aqueous phase becomes rate-limiting (2, 25, 50). The half-time of cholesterol efflux from various cells to acceptors that contain phospholipid varies considerably among cell types and ranges from 1 to 24 h (2, 39). These differences in the rates of cholesterol release from various cell types have been attributed to differences in the rates of desorption of cholesterol molecules from the plasma membrane (51). Our recent studies with cyclodextrins have shown that the maximal rates of cholesterol release to cyclodextrins are much greater than can be obtained with phospholipid-containing acceptors, and the difference in the rates of cholesterol efflux between cell types is largely eliminated when cyclodextrins are used as acceptors (10). To gain more insight as to what is different in the mechanism of cholesterol efflux to cyclodextrins and apo-HDL-PC, we have measured the temperature dependence of cholesterol efflux from cells to the two acceptors (Fig. 7). The activation energy for efflux from cells to cyclodextrins is 7 kcal/mol, and the activation energy for efflux from LUV to cyclodextrins is even lower (2 kcal/mol). In contrast, the activation energy for transfer from cells to apo-HDL-PC is 20 kcal/mol. This latter value is similar to those previously obtained for cholesterol exchange between HDL and LDL (18 kcal/mol) (52) and between SUV and SUV (16 kcal/mol) (53), demonstrating that the temperature dependence of cholesterol efflux or exchange to various acceptors that contain phospholipid is similar and that it is much higher than that to cyclodextrins.

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