Inhibition of Cholesterol Efflux by 7-Ketocholesterol: Comparison between Cells, Plasma Membrane Vesicles, and Liposomes as Cholesterol Donors†

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ABSTRACT: Cholesterol removal from lipid-loaded macrophages is an important, potentially antiatherogenic process, and we have previously shown that an oxysterol, 7-ketocholesterol (7K), can impair efflux to lipid-free apoprotein A-1 (apoA-1). This publication investigates whether incorporation of 7K into membranes could account for this impairment of cholesterol efflux. Cholesterol efflux was studied from lipoprotein-loaded THP-1 cells, from plasma membrane vesicles obtained from these cells, and from artificial, protein-free liposomes. Impairment of cholesterol efflux by 7K was observed for all cholesterol donor systems whether measured as decline in cholesterol removal rates or as the percentage mass of total cellular cholesterol exported. 7-Ketocholesterol itself was not removed by apoA-1 from any of the cholesterol donor systems. Increasing membrane cholesterol content increased the rate of cholesterol removal by apoA-1 (as seen with plasma membrane vesicles), the quantity of cholesterol removed at equilibrium (liposomes), or both (whole cells). Although the minimum inhibitory 7K concentrations varied between the cholesterol donor systems, 7K inhibited cholesterol efflux in all systems. It was concluded that 7K induces alteration in membranes which decreased the efficiency of cholesterol efflux and the quantity of removed cholesterol induced by apoA-1. As cell membrane proteins are not essential for cholesterol efflux in these systems, the impairment of such by 7K suggests that its effect on membrane lipid composition and its structure are key regulatory elements in this efflux process.

High-density lipoproteins (HDLs) (*1*, *2*) and their major protein component, apolipoprotein A-1 (apoA-1)[1] (*3*), are regarded as antiatherogenic agents. Part of this activity is probably due to their capacity to remove excess cholesterol from peripheral cells in a cholesterol transport system known as reverse cholesterol transport (RCT) (*4*). Currently, there are two major hypotheses for the initial step of RCT, the interaction of lipid-accepting, extracellular particles with lipid-loaded cells (*5*). When these particles contain substantial amounts of lipids, such as in HDL, it is believed that cholesterol can diffuse between cholesterol donor and acceptor along a concentration gradient (*6*). The aqueous diffusion model is therefore a bidirectional transport model which does not require the acceptor particle to bind or penetrate the cellular plasma membrane (*7*, *8*). Lipid-free HDL proteins, in particular apoA-1, can also remove cholesterol from lipid-enriched cells, and there is in vivo evidence for such a lipid-poor apoA-1 pool, suggesting that this mechanism might contribute significantly to RCT (*9*, *10*). In this case, direct interaction between apoA-1 and the plasma membrane to remove both phospholipid and cholesterol seems the most likely mechanism, although the nature of this interaction is not yet fully understood.

A number of receptors for HDL apolipoproteins have been suggested although most candidates such as SR-B1 and HB2 have broad ligand-binding characteristics and allow nonspecific lipid interaction (*11*). SR-B1, for example, is regarded as a low-affinity HDL-binding protein, and its influence on cholesterol removal by lipid-free apoA-1 is controversial (*12*−*14*). However, efflux to apoA-1 (but not HDL) is dependent on the membrane transporter protein ABC1 (*15*). Mutations in ABC1 are the underlying cause of Tangier disease in which cellular cholesterol efflux to apoA-1 is severely impaired (*16*). Recently, the relationship between ABC1 expression and cholesterol efflux to apoA-1 (*17*) and specific binding of apoA-1 to ABC1 (*18*) have been shown, although the mechanism by which ABC1 stimulates efflux to apoA-1 is not yet known.

The alternative to such protein−protein interaction is direct interaction between apoA-1 and the lipid components of the plasma membrane; this mechanism has been proposed in the

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[1] Abbreviations: 7K, 7-ketocholesterol; 7KAcLDL, 7K-enriched AcLDL; 7KLDL, 7K-enriched LDL; 7KE, 7K esters; ACAT, acyl-CoA:cholesterol acyltransferase; AcLDL, acetylated LDL; apoA-1, apolipoprotein A-1; CH, cholesterol; CE, cholesterol esters; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FCS, fetal calf serum; HRP, horseradish peroxidase; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MBS, MES-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; PC, L-α-phosphatidylcholine; PM, plasma membrane; PMA, phorbol 12-myristate 13-acetate; RCT, reverse cholesterol transport; SDS, sodium dodecyl sulphate; SPM, sphingomyelin; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

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microsolubilization hypothesis (*19*). This suggests that amphipathic α-helices of such apolipoproteins can be inserted into the plasma membrane, simultaneously solubilizing free cholesterol and phospholipids (*20*). Mutation of the α-helices in apoA-1 decreased cholesterol efflux, while modification to nonhelical parts of apoA-1 had little effect (*21*−*24*). Furthermore, synthetic amphipathic α-helices can also stimulate efflux from the plasma membranes (*25*), and nonmodified apoA-1 can remove cholesterol from artificial lipid membranes (*26*), lending support to the microsolubilization theory.

We have previously investigated the effect of oxysterols, oxidized forms of cholesterol, on the reverse cholesterol transport system (*27*−*29*). It was found that 7-ketocholesterol (cholest-5-en-3β-ol-7-one, 7K), the major form of oxidized cholesterol present in oxidized LDL and atherosclerotic lesions, inhibits cholesterol efflux from macrophages to apoA-1. In this paper, we investigate the hypothesis that it is the presence of 7K in the plasma membrane which directly inhibits apoA-1-induced cholesterol efflux. Due to the presence of a keto group on the seventh carbon, 7K is less efficient in condensing membranes than cholesterol (*30*, *31*). Hence one possible mechanism for the efflux-impairing effect of 7K is that this oxysterol alters membrane lipid composition or packing. Cholesterol efflux from cells, plasma membrane vesicles, and artificial membranes were compared under conditions of varying 7K content. Comparison between cholesterol efflux to apoA-1 from cells and plasma membrane-derived vesicles allowed discrimination between intracellular alterations by 7K and its effect on the plasma membrane. Furthermore, a comparison between cholesterol efflux from plasma membranes and protein-free liposomes was conducted to determine direct effects of 7K on the ability of apoA-1 to solubilize membrane cholesterol.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade, and all solvents were of HPLC grade (Mallinckrodt). If not otherwise stated, all buffer components and salts were purchased from Sigma. Bovine serum albumin (BSA, essentially fatty acid free), Dulbecco's phosphate-buffered saline (PBS), chloramphenicol, and cholesterol were purchased from Sigma. "Cell buffer" (8.0 g/L NaCl, 0.2 g/L KCl, 0.1 g/L $CaCl_2$, 0.1 g/L $MgCl_2$, 1.9 g/L HEPES, pH 7.4) was used as a phosphate-free alternative to PBS. [^{3}H]Cholesterol (48.0 Ci/mmol) was obtained from Amersham. RPMI-1640 (Trace Biosciences) was supplemented with 2 mM L-glutamine (Trace Biosciences) and penicillin/streptomycin (100 units/100 μg in 1 mL, Sigma) at the time of use. Heat-inactivated fetal calf serum (FCS) was purchased from Trace Biosciences. Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Sandoz 58-035 [3-(decyldimethylsilyl)-*N*-(2-(4-methylphenyl)-1-phenylethyl)propanamide], was a gift from Sandoz Inc. For immunoblotting, rabbit anti-human caveolin-1 and mouse anti-human flotillin-1 antibodies were purchased from Transduction Laboratories and mouse anti-human transferrin receptor antibodies from Zymed Laboratories. Secondary antibodies, anti-rabbit horseradish peroxidase (HRP) conjugates and anti-mouse HRP conjugates, were purchased from Sigma and Amersham, respectively.

Lipoprotein Preparation and Acetylation. Incorporation of 7K into LDL, lipoprotein isolation, and LDL modification have been described previously (*28*). In brief, plasma from fasted normolipidemic subjects was collected, anticoagulated with 2 mM EDTA, and incubated with a small volume of an ethanolic solution of 7K (final 7K concentration 2.5 mM, final ethanol concentration 2% v/v) for 6 h at 37 °C with gentle shaking. The 7K-enriched LDL (7KLDL) or native LDL (not incubated with 7K; density 1.021−1.055) was separately isolated by density gradient ultracentrifugation, dialyzed against deoxygenated PBS containing EDTA (1 g/L) and chloramphenicol (0.1 g/L), and filter-sterilized (0.45 μM). LDL and 7KLDL were acetylated and analyzed as described previously (*32*). EDTA was removed by gel filtration (PD10; Pharmacia) before acetylated LDL (AcLDL) and 7KAcLDL were incubated with cells. The lipoprotein preparations were stored under N_2 at 4 °C in the dark and used within 7 days.

Prior to incubation with cells, AcLDL and 7KAcLDL were enriched with [^{3}H]cholesterol. The AcLDL/7KAcLDL preparations (final total concentration 0.1 mg of LDL of protein/mL) were incubated with FCS (final concentration 1% v/v), one-tenth of the final volume of RPMI, and an ethanolic solution of [^{3}H]cholesterol (final concentration 1 μCi/mL; final ethanol concentration 0.1% v/v) for 6 h or overnight at 37 °C and then diluted with RPMI to the final concentrations (*33*).

Culturing and Sterol Loading of THP-1. THP-1 human macrophages were used throughout this study as a model for human foam cells as previously suggested (*34*). THP-1 monocytes are routinely cultured in RPMI containing 10% (v/v) FCS at 37 °C in 5% CO_2 at densities between 0.4 and 1.2×10^6 cells/mL. For experiments, cells were plated at a density of 1.2×10^6 cells/mL (4 cm^2) in RPMI with 10% (v/v) FCS and 50 ng/mL PMA (Sigma) and matured into differentiated macrophages for 72 h before use (*33*).

Matured THP-1 cells were washed three times with FCS-free RPMI and then incubated with the lipoprotein-containing media (total lipoprotein concentration 0.1 mg of protein/mL) and 50 ng/mL PMA for 48 h. Altering the proportion of AcLDL to 7KAcLDL leads to a constant cellular cholesterol but variable 7K content. Cells were then washed again, incubated overnight in RPMI containing 1 mg/mL BSA (fatty acid free) and 50 ng/mL PMA, and washed three times in serum-free RPMI before efflux.

To inhibit sterol esterification, THP-1 cells were exposed to an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Sandoz S-58035 (5 μg/mL), continuously during lipoprotein loading, equilibration, and subsequent efflux periods. The ACAT inhibitor was prepared as an ethanolic stock solution at 10 mg/mL and therefore increased the final ethanol content of the respective solutions by 0.05% (v/v).

Cholesterol Efflux from Cells. Cholesterol efflux was measured in RPMI containing 1 mg/mL BSA, 50 ng/mL PMA, ±25 μg/mL delipidated apoA-1, and ±5 μg/mL Sandoz 58035 at 37 °C (total volume 1 mL). Lipid-free apoA-1 was prepared as described previously (*28*). Cholesterol efflux was quantified by [^{3}H]cholesterol appearing in the media. Aliquots (100 μL) of the efflux media were taken at various times and centrifuged in Eppendorf tubes at 800*g* to remove detached cells. Eighty microliters of the supernatant was transferred to scintillation vials and counted. The volumes of the extracellular media progressively declined with each sampling, and this was taken into account in the

final calculation of cholesterol efflux. At the end of the time course, the residual medium was removed, cells were washed twice in ice-cold PBS and lysed in 1 mL NaOH (0.2 M, Sigma), and 100 μL was counted. Efflux (unless otherwise stated) is expressed as a percentage of the total counts in the system. Cell death was routinely checked after lipoprotein loading and cholesterol efflux by measuring the release of lactate dehydrogenase and cell protein.

Cellular Sterol Analysis. Supernatants of the efflux media and cell lysates were extracted as described previously (*28*), and cholesterol, 7K, and their respective esters were analyzed by reverse-phase HPLC before and after saponification (*34*, *35*).

Subcellular Fractionation and Plasma Membrane Fraction. Plasma membrane fractions were isolated by subcellular fractionation as described previously (*27*, *36*). Briefly, cell homogenates prepared by shear lysis were loaded onto a 1−22% (w/v) Ficoll gradient with a 45% (w/v) Nycodenz cushion. The samples were spun in a Beckman Ti 65.2 rotor at 50 000 rpm for 90 min, and 26 fractions of 200 μL were collected from the bottom. Fractions 15−20 were identified as the plasma membrane fractions by enzymatic markers (see below) and fractionated according to the method of Roy et al. (*37*). The plasma membrane fractions were pooled, incubated for 5 min in a sonicating water bath, and adjusted to a final concentration of 45% (w/v) sucrose, using 90% (w/v) sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl). Two milliliters of the plasma membrane fraction was overlaid with 2.2 mL of 35% (w/v) sucrose, 2.0 mL of 30% (w/v) sucrose, 2.0 mL of 25% (w/v) sucrose, and 2.0 mL of 5% (w/v) sucrose (all in MBS). The sucrose gradient was spun at 40 000 rpm in a Beckman SW41 rotor for 16 h. Twelve fractions of 0.85 mL were collected from the top. Fractions were analyzed for protein (Bio-Rad assay) and sterol (HPLC). Proteins were precipitated with ice-cold TCA [final concentration 15% (w/v)], washed with ice-cold acetone, resuspended in SDS sample buffer [2% (w/v) SDS, 100 mM DTT, 50 mM Tris-HCl, pH 6.8, 0.1% (w/v) bromophenol blue, 10% glycerol; all from Sigma], boiled for 5 min, resolved by SDS−PAGE on 10% acrylamide gels, transferred to nitrocellulose (Amersham), and analyzed by immunoblotting. Immunoblots were analyzed by densitometry (Gel Doc, Bio-Rad).

Protein Determination. LDL and cell protein concentrations were determined by the bicinchoninic acid assay method using BSA as a standard (*28*). The Bio-Rad protein assay was used for Ficoll and sucrose gradient fractions, according to the manufacturer's instructions.

Plasma Membrane Vesicle Preparations and Cholesterol Efflux Therefrom. Cells (50×10^6) were differentiated and loaded with lipoproteins as described above and then washed in cell buffer. Plasma membrane "blebbing" was induced by incubating the loaded cells with 50 mM formaldehyde and 2 mM DTT (Sigma) in cell buffer at 37 °C overnight (*38*, *39*). The buffer was collected, and loose cells were removed by centrifugation at 800*g*. The supernatant was then centrifuged for 3 h at 50 000 rpm in a Ti70 rotor (Beckman). The pellet was resuspended in cell buffer and the second centrifugation repeated to ensure that all formaldehyde and DTT were removed. Finally, the plasma membrane vesicles were resuspended in cell buffer, and small vesicles were removed by concentrating the solution using 0.1 μm filters (Ultrafree-MC, Millipore). The protein concentration of membranes was determined. Typical yields were ∼100 and ∼200 μg from 50×10^6 non-loaded and lipoprotein-loaded cells, respectively.

Efflux from such plasma membrane vesicles has been described previously (*40*−*42*). The plasma membrane vesicles (20 μg of protein) were incubated in 1 mL of RPMI containing 25 μg of apoA-1. The mixture was incubated at 37 °C, and two aliquots of 100 μL were taken at various time points. To separate the apoA-1 from the plasma membrane vesicles, the mixture was centrifuged through 0.1 μm filters (Millipore) for 30 s at 2000 rpm (∼400*g*) in a benchtop centrifuge; 200 μL of RPMI was then added and the sample recentrifuged for 60 s at 2000 rpm. The eluted and retained fractions were counted separately. The filter was washed in 100 μL of 0.2% (v/v) Triton X-100, and the wash was added to the retained fraction. A separate sample containing apoA-1 without vesicles was included in each experiment to measure the proportion of apoA-1 present in the eluted and retained fractions. This spinning and washing procedure routinely achieved separation of >60% of unbound apoA-1 so that the "yield" of separation was determined by this control sample: y = (eluted apoA-1)/(total apoA-1). Cholesterol efflux was calculated for each sample as (eluted [^{3}H]cholesterol)/(total [^{3}H]cholesterol) × 1/y. At least 97% of the plasma membrane vesicles were retained on the filter as determined in control samples containing plasma membrane vesicles but no apoA-1. Total recovery of radioactivity was 90−99%.

Characterization of Plasma Membrane Vesicles. Plasma membrane vesicles were extracted, and the cholesterol and 7K content were determined by reverse-phase HPLC as described for cell lysates (*32*, *34*).

Phospholipids were extracted by the Bligh−Dyer method (0.8 volume sample in 3.0 volumes of chloroform/methanol mixture (1:2), 1.0 volume of chloroform, and 1.0 volume of H_2O) (*43*). The solvent was evaporated, the samples were resuspended in a small volume of TLC solvent (chloroform/methanol/H_2O, 65/35/4) (*44*), and a fraction of the sample was run onto silica TLC plates with pure phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine (all from Sigma) as standards. After the lipids were visualized in iodine vapor, the bands were cut out and together with the pre-TLC samples were analyzed for phosphate using inorganic phosphate as a standard (*45*). [^{3}H]Cholesterol was used to determine the yield of the Bligh−Dyer extraction, which was usually 60−65%. The recovery of phospholipids after TLC separation was 85 ± 9%.

A number of enzyme assays were used to determine the purity of the plasma membrane preparation (*46*). All assays were carried out on plasma membrane and cell samples both before and after formaldehyde/DTT treatment. The activities of marker enzymes (activity/mg of protein) in the plasma membrane preparation and cell sample post formaldehyde/DTT treatment were quantified as the ratio of activity in plasma membrane over that in whole cell. Aryl sulfatase was used as a lysosomal marker (*36*, *47*), succinate dehydrogenase as a mitochondrial marker, galactosyl transferase for Golgi apparatus, glucose-6-phosphatase for endoplasmic reticulum, and alkaline phosphatase (*46*) and (Na^+,K^+)-ATPase (*48*) as plasma membrane markers. The glucose-6-phosphatase and ATPase assay required the determination

of inorganic phosphate (*48*). Typical ratios (plasma membrane:whole cell homogenate) of the specific activities for markers were Golgi marker, 0.25 (±0.03); ER, 0.05 (±0.03); lysosomal, 0.17 (±0.01); and mitochondrial, 0.15 (±0.02); while the ratio for the PM markers was 3−5. Over a series of experiments, the contamination of the plasma membrane with internal membranes was between 6% for Golgi apparatus and 1% for ER. Eighty-five to ninety-seven percent of all plasma membrane vesicles preparations were intact, sealed, right-side-out vesicles and stable during a 5 h incubation period at 37 °C as calculated from the ratio of the specific activity of the ATPase assay in the absence and presence of 0.2% (v/v) Triton X-100 (*49*).

Nuclear contamination was not detectable in plasma membrane preparations. This was determined by measuring the increase in fluorescence (λ_{em} = 597 nm, λ_{ex} = 365 nm) in the presence of 0.5% (v/v) Triton X-100 in ethidium bromide (final concentration 10 μg/mL) and using whole cell lysates as positive control samples.

Liposome Preparation and Efflux from Liposomes. L-α-Phosphatidylcholine (PC, Sigma) and sphingomyelin (SPM, Sigma) were both derived from egg yolk. Egg PC contains saturated and unsaturated fatty acids (with a typical content of 34% of 16:0, 32% of 18:1, 18% of 18:2, and 11% of 18:0) while egg SPM contains only saturated fatty acids (84% of 16:0, 6% of 18:0, and 4% each of 22:0 and 24:0).

For the preparation of liposomes, PC, SPM, cholesterol/[^{3}H]cholesterol, and 7K (Steraloids Inc.) were each prepared as 10 mg/mL stock solutions in chloroform. The phospholipids and sterols were mixed in the appropriate proportions, and the solvent was removed under vacuum. The pellet was resuspended in cell buffer by extensive vortexing and then subjected to five cycles of freezing in liquid N_2 and thawing at 37 °C (*50*). The liposomes were then extruded through a pretested 0.2 μm filter under N_2 pressure to yield unilamellar liposomes with an average diameter of 220 nm (±27%) (*51*). The recovery of [^{3}H]cholesterol after the preparation was 91 ± 8%.

For fluorescence anisotropy measurements, 10 μL of 10 mM 1,6-diphenyl-1,3,6-hexatriene (DPH, Sigma) in THF was added to 1 mL of liposomes in cell buffer (0.5 mg of lipid/mL) and incubated for 90 min at 37 °C. DPH fluorescence (λ_{ex} = 365 nm, λ_{em} = 428 nm) was strong in the presence of liposomes but negligible in the absence of lipids. Fluorescence anisotropy was determined according to Lakowicz (*52*) using a Perkin-Elmer LS50B fluorometer.

For cholesterol efflux, liposomes (~1 μM total lipid in 1 mL) were incubated with 50 μg/mL apoA-1 (2 μM) in RPMI at 37 °C. At intervals, three aliquots of 100 μL were taken, and apoA-1 was separated from liposomes by centrifuging the mixture with 0.1 μm filters (Millipore) for 30 s in a benchtop centrifuge at 2000 rpm. The eluted and retained fractions were analyzed separately for [^{3}H]cholesterol and by HPLC for total cholesterol and 7K. The filters were washed in 100 μL of RPMI, and the wash was added to the retained fractions. Total recovery of radioactivity was 93 ± 5%. As a control, during each spin an apoA-1-containing sample was treated without liposomes and the protein recovery determined in eluted and retained fractions separately. Between 40% and 50% of the unbound apoA-1 was eluted by this procedure, and efflux data were calculated as described for efflux from plasma membrane vesicles. As a further control, liposomes without apoA-1 were analyzed in the same fashion. This was regarded as "nonspecific" or "background" efflux and was found to be 3 ± 1%.

Kinetic Analysis for Cholesterol Efflux from Plasma Membrane Vesicles and Liposomes. Kinetic data of cholesterol efflux from plasma membrane vesicles and liposomes were empirically fitted to the following equation for efflux, E:

$$E = E_m - E_m \exp(-kt)$$

with the time in hours. k is the rate constant in h^{-1} for cholesterol removal from the membrane to apoA-1, while E_m is the maximum efflux and is expressed as a percentage of total cholesterol in the system. The data were fitted to the equation by a least-squares fit using a Levenberg−Marquardt algorithm which gives the values for K and E_m and their respective standard errors. Statistical analysis for data presented in Tables 1−3 was carried out with unpaired, two-tailed Student t-tests assuming equal variance.

RESULTS

Sterol Loading of THP-1 Cells. Differentiated THP-1 cells, like other macrophages, endocytose AcLDL leading to intracellular cholesterol accumulation (*34*). In these experiments, model foam cells were generated by loading mature THP-1 cells with either AcLDL or a combination of 7KAcLDL and AcLDL. Supplementation of AcLDL with this specific oxysterol generates a cholesterol-loaded foam cell containing controlled amounts of 7K; this can be varied by adjusting the proportions of AcLDL and 7KAcLDL used. A detailed study of the incorporation of 7K into LDL and the subsequent uptake and metabolism of 7KAcLDL by macrophages have been previously described by our group (*28*). Cell toxicity was not significant when cells were incubated with AcLDL ± 7KAcLDL under the conditions used here. Six different loading conditions were used to vary the proportion of 7K, as shown in Table 1. Nonloaded cells had a total cholesterol content of 25−32 nmol/mg of cell protein. AcLDL loading alone increased total cholesterol at least 3-fold. The extent of esterification after AcLDL loading varied between 30% and 55%. 7K incorporation did not significantly alter free cholesterol loading of the cells nor the proportion of cholesterol esterified. However, supplementation with 7K did increase the total sterol by up to 50% (Table 1). In mouse macrophages, the extent of cholesterol esterification was higher (70−90%), and 7KAcLDL loading did not increase the total sterol content of the cells (~405 nmol/mg of cell protein) (*28*).

Incubation with an ACAT inhibitor (S-58035) during and after lipoprotein loading inhibited esterification of both cholesterol and 7K. While sterol esterification is completely inhibited by S-58035, unesterified cholesterol and 7K were increased in the presence of S-58035. For AcLDL-loaded cells, total cholesterol levels were comparable between S-58035-treated and nontreated cells. Supplementation with 7K in the presence of S-58035 did not affect total cholesterol levels (as seen with cells not treated with S-58035) while it increased the total sterol content although the latter was not as notable in S-58035-treated cells as in their untreated

Table 1: Sterol Content of THP-1 Macrophages after 48 h Incubation with AcLDL ± 7KAcLDL and ± ACAT Inhibitor (S-58035, 5 μg/mL)[a]

loading conditions (μg/mL)		free CH (nmol/mg of cell protein)	CH esters (nmol/mg of cell protein)	total CH (nmol/mg of cell protein)	7K (nmol/mg of cell protein)	7KE (nmol/mg of cell protein)	total 7K (nmol/mg of cell protein)	total sterol (nmol/mg of cell protein)
AcLDL	7KAcLDL							
(−) S-58035								
100	0	63.21 (6.64)[1,a]	28.69 (5.19)	91.90 (8.43)[b,c]	nd	nd	nd	91.90 (8.43)[6,d]
95	5	57.49 (5.04)	40.55 (6.85)	98.04 (8.50)	3.42 (0.42)	0.189 (0.06)	3.61 (0.42)	101.65 (8.51)
90	10	46.72 (3.28)	69.37 (15.61)	116.09 (5.14)	6.74 (0.74)	8.97 (1.82)	15.71 (1.96)	131.79 (5.50)
85	15	55.74 (5.03)	45.61 (10.31)	101.34 (11.47)	9.10 (1.39)	8.53 (3.19)	17.63 (3.48)	118.97 (11.98)
80	20	62.23 (5.47)	61.54 (15.57)	123.77 (13.71)	12.55 (1.17)	14.52 (1.72)	27.07 (2.08)	150.84 (13.87)
75	25	52.76 (6.54)[2,a]	63.02 (7.63)	115.78 (10.05)[3,b]	12.53 (1.48)[4]	18.07 (4.32)	30.60 (4.56)[5]	146.38 (11.03)[6,7]
(+) S58035								
100	0	80.64 (7.09)[1,e]	<1	80.64 (7.09)[c,f]	nd	nd	nd	80.64 (7.09)[8,d]
95	5	79.79 (9.89)	<1	79.79 (9.89)	4.26 (0.40)	nd	4.26 (0.40)	84.05 (9.90)
90	10	95.13 (8.59)	<1	95.13 (8.59)	9.53 (1.45)	<0.01	9.53 (1.45)	104.66 (8.71)
85	15	93.86 (6.60)	<1	93.86 (6.60)	14.37 (1.69)	<0.01	14.37 (1.69)	108.23 (6.81)
80	20	90.88 (9.54)	<1	90.88 (9.54)	17.63 (1.95)	<0.02	17.63 (1.95)	108.51 (9.74)
75	25	88.92 (7.80)[2,e]	<1	88.92 (7.80)[3,f]	22.28 (2.74)[4]	<0.02	22.28 (2.74)[5]	111.20 (8.27)[7,8]

[a] 1.2×10^6 cells were incubated with a total of 0.1 mg/mL AcLDL, in which the ratio of AcLDL to 7KAcLDL was varied as indicated. For loading conditions and sterol analysis see Materials and Methods. CH gives values for unesterified cholesterol, and 7K gives values for unesterified 7K. Nonloaded cells have 20.31 (±2.11) nmol/mg of unesterified cholesterol and 10.64 (±1.50) nmol/mg esterified cholesterol and no detectable 7K. Treating nonloaded cells with S-58035 under the same conditions as lipoprotein-loaded cells leads to 25.15 (±3.81) nmol/mg unesterified cholesterol and no detectable cholesterol esters or 7K. All sterols above are given in nmol/mg of cell protein and are the means of triplicate cell cultures. Standard deviations are given in parentheses. nd stands for not detectable. Superscript pairs of 1−8 indicate a statistically significant difference of $p < 0.05$, and superscript pairs of a−d indicate that $p > 0.05$ as tested with unpaired two-tailed *t*-tests assuming equal variance.

counterparts (Table 1). Total cellular 7K expressed as a percentage of total sterol is similar for cells loaded with and without ACAT inhibitor S-58035 (in both cases around 20%).

Cholesterol Efflux from Cells. We have previously described cholesterol efflux from cholesterol- and 7K-loaded mouse macrophages (*28*). ApoA-1 at 25 μg/mL was used routinely in this study, preliminary studies confirming that higher apoA-1 concentrations did not further increase cholesterol efflux (data not shown) as previously shown for mouse macrophages (*32*). Figure 1 shows cholesterol efflux from THP-1 cells whose sterol contents are described in Table 1. Efflux is expressed as a percentage of the total sterol (CH, CH esters, 7K, 7K esters) in each cell culture and is averaged over triplicate cultures.

Although the rate of cholesterol efflux in Figure 1a was nonlinear for the first 3 h, a significant dose-dependent decline in cholesterol efflux was observed with increasing 7K supplementation. The inhibitory effect of 7K on cholesterol efflux was also found over a longer time frame (24 h, Figure 2a). Basal cholesterol efflux from AcLDL-loaded cells in the absence of apoA-1 was 1.5−3% after 3 h and 3−6% after 24 h. Additionally, 7K efflux from 7KAcLDL-loaded cells to apoA-1 (1.5 ± 0.61% over 3 h and nondetectable to BSA) was less, both in proportional and in mass terms, than cholesterol efflux. These observations are consistent with previous data for mouse macrophages (*28*) and human monocyte-derived macrophages (*32*) and suggest that selective enrichment with 7K is a strong and consistent inhibitor of cholesterol efflux from macrophage foam cells (*28*).

To simplify interpretation of the effect of 7K on cholesterol efflux, the cholesterol efflux after a 3 h incubation with apoA-1 is plotted against the 7K content of the cell culture (Figure 3). The inhibitory effect of 7K can be clearly seen for cells which have not been treated with an ACAT inhibitor. For these cells, cholesterol efflux decreases linearly and significantly with increasing 7K incorporation so that even at low 7K concentrations (3−5% total cell sterol) cholesterol efflux is markedly decreased (by 30%).

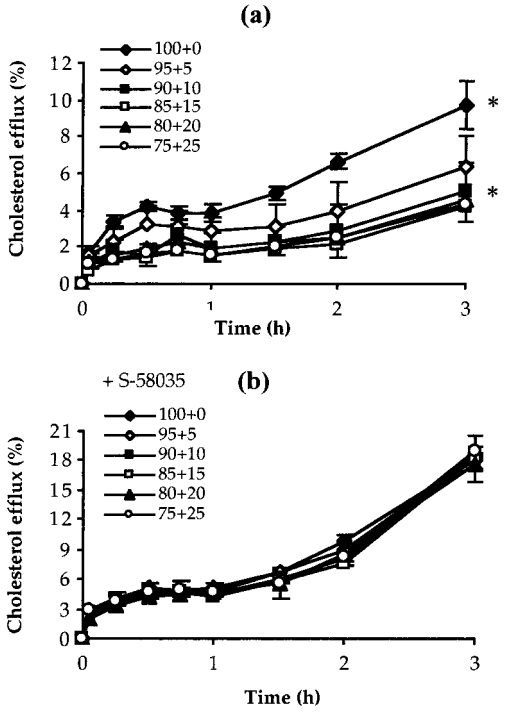

FIGURE 1: Kinetics of cholesterol efflux to apoA-1 (25 μg/mL) from sterol-loaded THP-1 cells (a) or from cells additionally treated with S-58035 (5 μg/mL) (b). Total LDL concentration was 100 μg/mL; proportions of AcLDL and 7KAcLDL in the loading media are given in the legends, with AcLDL being the first number. For experimental details see Materials and Methods. Efflux is given as a percentage of the total [^{3}H]cholesterol released. Data are means and standard deviations of triplicate measurements from one experiment representative of three. Asterisks indicate a significant difference between the loading conditions of $p < 0.05$.

Incubation of THP-1 cells with an ACAT inhibitor throughout sterol loading, equilibration, and efflux periods efficiently blocked synthesis of cholesteryl and 7K esters (see Table 1). Cholesterol efflux from cells loaded with AcLDL only was higher (~18% vs ~10%, Figure 3) compared to efflux from cells loaded in the absence of an

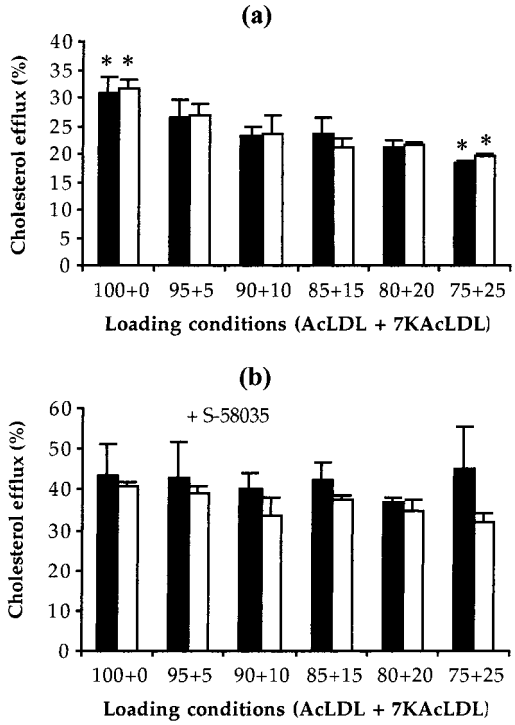

FIGURE 2: Cholesterol efflux to apoA-1 (25 ng/mL) after 24 h from cells untreated (a) and treated with S-58035 (5 μg/mL) (b). Conditions are described in Figure 1. The efflux was measured by release of either [^{3}H]cholesterol (open bars) or measurement of cholesterol mass (solid bars), and both are expressed as the percentage of total [^{3}H]cholesterol or cholesterol mass in the cultures, respectively. The data are means and standard deviations of triplicate cultures. Asterisks indicate a significant difference between the loading conditions of $p < 0.05$.

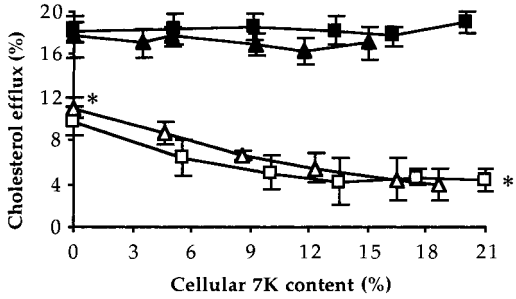

FIGURE 3: Correlation between cellular cholesterol efflux after 3 h incubation with apoA-1 (25 μg/mL) and the 7K content in the cell culture from two experiments (experiment 1, □; experiment 2, △) with S-58035 (5 μg/mL, solid symbols) and without (closed symbols) as described for Figure 1. 7K content is calculated as the percentage of free 7K and esterified 7K with respect to the total cellular sterol content. Data are means and standard deviations of triplicate cultures. Asterisks indicate a significant difference of $p < 0.05$.

ACAT inhibitor. In addition, 7K had no significant effect on cholesterol efflux from cells treated with the ACAT inhibitor (Figures 1b and 3). Increased cholesterol efflux to extracellular acceptors from cells treated with an ACAT inhibitor has been reported previously (*53*−*55*), and it has been suggested that ester hydrolysis is a rate-limiting step in cholesterol export and that this is bypassed when ester formation is blocked (*56*).

Sterol Loading of Plasma Membrane Vesicles and Their Characterization. Cholesterol efflux from cells may be regulated at several sites distal from the interaction of apoA-1 with plasma membrane. S-58035, for example, increases the pool of plasma membrane cholesterol (see Table 3), which may also contribute to the increased cholesterol efflux and subsequently decreased capacity of 7K to inhibit cholesterol efflux. To examine the importance of direct effects of 7K on the capacity of plasma membranes to deliver cholesterol to apoA-1 for efflux, plasma membrane vesicles were prepared from THP-1 foam cells by inducing blebbing with formaldehyde and DTT (*38*, *39*). This method has the advantage that it readily forms sealed and right-side-out vesicles. Since distribution of lipids and proteins is asymmetric between the two faces of the plasma membrane, the orientation of the plasma membrane leaflets may be important for apoA-1-membrane interaction and cholesterol efflux, and so this method was preferred over more generic plasma membrane isolation methods.

The sterol content of the plasma membrane vesicles was determined with reverse-phase HPLC (Table 2), and enzyme assays were used to assess the purity of the plasma membrane preparation (see Materials and Methods). Table 2 shows the analysis of one experiment representative of three independent experiments. No esterified cholesterol or 7K could be detected in plasma membrane vesicles, as expected. Autoxidation of cholesterol during the plasma membrane isolation process was negligible since 7K levels in plasma membrane preparation from cells without 7KAcLDL loading were either not detectable (Table 2) or less than 0.2% of the total sterol levels (Table 3). As seen with whole cell lysates, incorporation of 7K did not significantly alter the cholesterol levels of plasma membrane vesicles obtained from the 7K-enriched cells. Therefore, with increasing 7K content, sterol levels in the plasma membrane vesicles were elevated. However, the proportion of 7K (as a percentage of total sterol) in the plasma membrane vesicles was greater than that in the cells from which the plasma membrane vesicles were derived. For example, in cells loaded with 75 μg/mL AcLDL and 25 μg/mL 7KAcLDL, the 7K content is 20−22% of the total cell sterol (Table 1). A similar percentage was also found in cells after formaldehyde/DTT treatment (19−22%) and in the whole plasma membrane fraction after subcellular fractionation (21−23%; Table 3). However, in the corresponding plasma membrane vesicles prepared from these cells, the 7K content was much higher, 40−55% of the total sterol (cholesterol + 7K; Table 2). Kilsdonk et al. have previously found a similar unequal distribution of 25-hydroxycholesterol between whole cell lysates and plasma membrane vesicles (*41*).

The above observations suggest that the plasma membrane vesicles generated during formaldehyde/DTT treatment contained areas of membrane relatively enriched in 7K. Previous studies (*42*, *57*) used similar preparations of plasma membrane vesicles as representative of whole plasma membranes. To determine whether the plasma membrane vesicles actually represent specific sections of the plasma membrane, whole plasma membranes were prepared, disrupted, and separated according to their buoyancy (data not shown). Commonly, lighter fractions are associated with caveolae or lipid rafts while nonraft regions of the plasma membrane partition into higher densities (*58*). When cells loaded with 75 μg/mL AcLDL and 25 μg/mL 7KAcLDL were fractionated, the lighter fractions were enriched in 7K with 30−40% of the sterol in raft fractions being 7K (or 25−31% when cells were additionally treated with S-58035).

Table 2: Sterol Content and [^{3}H]Cholesterol Efflux Characteristics of Plasma Membrane Vesicles[a]

plasma membrane vesicles cellular loading conditions (μg/mL)		cholesterol (nmol/mg)	7K (nmol/mg)	total sterol (nmol/mg)	7K/sterol (%)	rate constant (h^{-1})	max efflux E_m (%)	correlation coefficient R
AcLDL	7KAcLDL							
(−) S-58035								
100	0	56.42 (2.54)[1,a]	nd	56.42 (2.54)[5,6]	0	1.16 (0.16)	20.18 (0.98)	0.981
95	5	53.13 (1.84)	13.38 (1.02)	66.51 (2.10)	20.12	0.92 (0.21)	16.76 (1.49)	0.955
90	10	49.56 (2.68)	30.67 (1.45)	80.23 (3.05)	38.23	0.82 (0.19)	16.62 (1.61)	0.970
85	15	49.79 (1.52)	40.29 (2.12)	90.08 (2.61)	44.72	0.35 (0.11)	16.74 (3.34)	0.977
80	20	53.22 (1.24)	55.39 (2.78)	108.61 (3.02)	50.99	0.29 (0.43)	7.91 (4.59)	0.643
75	25	56.53 (1.57)[2,a]	70.37 (3.11)[4]	126.90 (3.45)[5,7]	55.45			<0.05
(+) S-58035								
100	0	106.65 (5.11)[1,3]	nd	106.65 (5.11)[6,b]	0	2.18 (0.43)	20.12 (1.04)	0.963
95	5	106.49 (7.59)	4.06 (0.45)	110.49 (7.60)	3.67	3.96 (0.82)	18.94 (0.72)	0.967
90	10	86.52 (6.89)	7.36 (0.81)	93.88 (6.94)	7.84	3.73 (1.14)	21.81 (1.27)	0.922
85	15	65.80 (5.06)	8.95 (1.11)	74.75 (5.18)	11.97	4.86 (1.23)	18.85 (0.76)	0.963
80	20	68.28 (4.38)	13.36 (1.15)	81.64 (4.53)	16.36	2.46 (0.42)	16.46 (0.70)	0.976
75	25	80.10 (5.68)[2,3]	15.86 (1.21)[4]	95.96 (5.81)[7,b]	16.52	2.76 (0.52)	22.11 (0.96)	0.967

[a] The plasma membrane vesicles were obtained after cells were loaded with lipoproteins in the absence or presence of S-58035 (5 μg/mL) as indicated. All values for sterol are in nmol/mg of PM vesicle protein and refer to unesterified sterols. Steryl esters were not detected in any of the plasma membrane preparations. The sterol analysis data are the means of triplicate measurements from a single PM vesicle preparation, and the standard deviation is given in parentheses. Cholesterol efflux from PM vesicles with the above sterol loading is shown in Figure 4b,c. The experimental efflux E was fitted to $E = E_m - E_m \exp(-kt)$; the rate constant k is given in h^{-1} and the maximum efflux E_m in % of plasma membrane cholesterol. R is the correlation coefficient for the fitting, and the standard error of the fitted values is given in parentheses. Superscript indices of 1−7 indicate a statistically significant difference of $p < 0.05$, and superscript indexes a and b indicate that $p > 0.05$ as tested with unpaired two-tailed t-tests assuming equal variance.

Table 3: Sterol Content of Plasma Membranes Isolated from Subcellular Fractionation[a]

PM fraction after subcellular fractionation, cellular loading (μg/mL)		cholesterol (nmol/mg)	7K (nmol/mg)	total sterol (nmol/mg)	7K/sterol (%)
AcLDL	7KAcLDL				
(−) S-58035					
100	0	87.03 (3.4)[1,a]	0.15 (0.05)	87.18 (3.4)[5,6]	<0.2
75	25	81.45 (3.5)[2,a]	22.09 (1.9)[4]	103.54 (3.9)[5,7]	21.4
(+) S-58035					
100	0	117.51 (8.2)[1,3]	0.20 (0.04)	117.71 (8.2)[6,8]	<0.2
75	25	141.16 (7.1)[2,3]	43.19 (2.3)[4]	184.35 (7.4)[7,8]	23.3

[a] THP-1 cells were sterol loaded with and without 5 μg/mL S-58035, and subcellular fractionation was performed as described in Materials and Methods. Sterol content was analyzed by HPLC and is given as nmol of sterol/mg of plasma membrane protein and is an average of three measurements. Superscript indexes of 1−8 indicate a statistically significant difference of $p < 0.05$ while superscript a indicates that $p > 0.05$ as tested with unpaired two-tailed t-tests assuming equal variance.

These lighter fractions contain the raft proteins flotillin and, to a lesser extent, caveolin but no transferrin receptor (*59*) and may be associated with caveolin-poor rafts (for lipid rafts ± caveolin, see refs *60* and *61*). Nonraft fractions, on the other hand, contain no caveolin-1 but have transferrin receptor and display a 7K content of 20−22% as seen with whole PM preparations.

Plasma membrane vesicles contained caveolin and some flotillin but only low amounts of transferrin receptor (data not shown). Compared with whole plasma membranes, we concluded that plasma membrane vesicles are relatively enriched in caveolin and flotillin (or deprived of transferrin receptor). Some variation in protein expression between the plasma membrane vesicles from cells loaded differently was observed and cannot be readily explained (but suggests that formaldehyde/DTT-induced blebbing is dependent on the cellular sterol loading). The relatively high 7K concentration in the plasma membrane vesicles (Table 2) and the raft-like plasma membrane fractions (data not shown) led to the hypothesis that 7K can accumulate in lipid-raft-like membrane domains (as also reported in ref *62*) and that plasma membrane vesicles are enriched in such domains.

Plasma membrane vesicles from cells loaded with AcLDL in the presence of the ACAT inhibitor S-58035 contained twice as much free cholesterol as vesicles from cells loaded with AcLDL alone (Table 2). A similar observation was made with whole plasma membrane preparations obtained by subcellular fractionation where membranes from ACAT-inhibited cells contained 1.4−1.8-fold more cholesterol than those from cells with functional ACAT (Table 3). In addition, the total sterol content of the plasma membrane vesicles did not increase with 7K incorporation in the presence of an ACAT inhibitor (Table 2; consistent with sterol loading of whole cells in the presence of S-58035, Table 1). Furthermore, the proportion of 7K in plasma membrane vesicles from S-58035-exposed cells matched that of the parent cells. 7K accumulation within lipid-raft domains (after fractionation of whole plasma membranes) is not as marked in S-58035-treated cells compared to their nontreated counterparts. In summary, S-58035 treatment may not alter the overall cellular sterol levels (see Table 1), but it does alter cholesterol and 7K distribution by increasing plasma membrane sterol levels (Table 2) and altering 7K distribution within the plasma membrane. Thus, plasma membrane vesicles derived from S-58035-treated cells should be treated as a different subset of vesicles.

Cholesterol Efflux from Plasma Membrane Vesicles. Prior to vesicle formation, all cells were labeled with [^{3}H]-cholesterol, either incorporated into added lipoproteins or added directly to the media for nonloaded cells; thus plasma membrane vesicles contained radiolabeled cholesterol. Cholesterol efflux was induced by the addition of apoA-1. Since

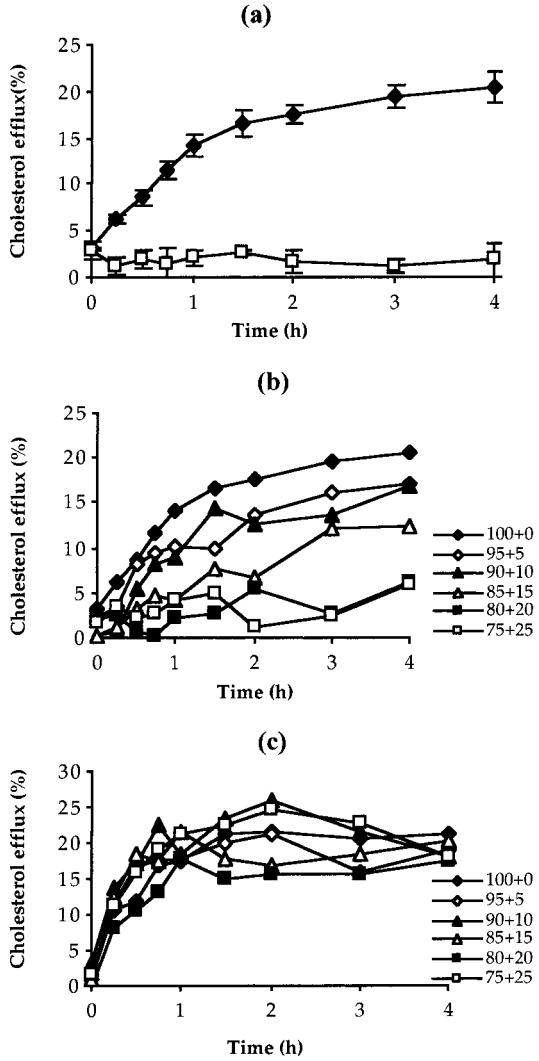

FIGURE 4: [^{3}H]Cholesterol efflux from plasma membrane vesicles. (a) PM vesicles (20 μg) from AcLDL-loaded cells were incubated with apoA-1 (25 μg/mL, ◆) and BSA (1 mg/mL, □). (b) Cells were loaded with a combination of AcLDL and 7KAcLDL as indicated in the legend, and the PM vesicles from these cells were effluxed to apoA-1. (c) Same as (b) but cells were also treated with S-58035 (5 μg/mL). The symbols in (a) are an average of triplicate measurements, with the error bars indicating the standard deviation. In (b) and (c) the data are an average of duplicates.

the plasma membrane vesicles formed relatively large structures with diameters of ~1−5 μm as determined by phase-contrast microscopy, the apoA-1-associated effluxed cholesterol was separated from the plasma membrane vesicles by 0.1 μm filters as described in Materials and Methods. Figure 4a shows efflux from plasma membrane vesicles prepared from AcLDL-loaded cells. There was a rapid initial basal efflux to 1 mg/mL BSA of approximately 4% which did not increase over the subsequent 4 h. Efflux to apoA-1 (25 μg/mL) was much more extensive, approaching saturation after ~1.5 h. The maximum efflux was ~20%, and increasing the apoA-1/vesicle ratio did not further increase the cholesterol efflux.

Effluxes from vesicles prepared from cells enriched with increasing amounts of 7K ± S-58035 are shown in panels b and c of Figure 4, respectively. A dose-dependent inhibition in cholesterol efflux was observed from plasma membrane vesicles containing increasing amounts of 7K (Figure 4b). At the highest loading of 7K, cholesterol efflux was reduced

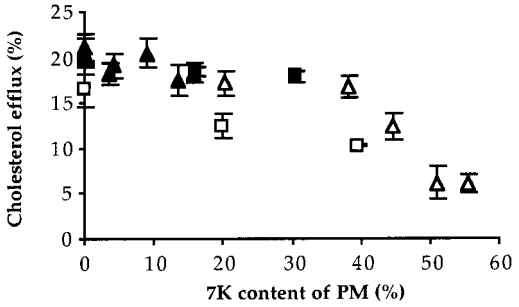

FIGURE 5: Cholesterol efflux from plasma membrane vesicles plotted against the 7K content of plasma membrane vesicles for two experiments (experiment 1, △; experiment 2, □). Solid and open symbols represent efflux from plasma membrane vesicles obtained from cells treated with and without 5 μg/mL S-58035, respectively. 7K content is calculated as the percentage of free 7K and esterified 7K over total cellular sterol content. The symbols and the error bars represent the average of four measurements and the standard deviation.

significantly to near basal rates. However, when cells treated with an ACAT inhibitor were used for plasma membrane preparation, cholesterol efflux from these plasma membrane vesicles appears to be independent of the 7K loading. In this respect, cholesterol efflux from plasma membrane vesicles followed the same response pattern to 7K supplementation as cholesterol efflux from whole cells loaded ± S-58035 (compare Figure 4a to Figure 1a and Figure 4b to Figure 1b).

The basis of the discrepancy between cholesterol efflux from plasma membrane vesicles prepared from cells ± ACAT inhibitor becomes clearer when the cholesterol efflux from plasma membrane after 4 h incubation is plotted against the 7K content of the plasma membrane vesicles (Figure 5). The plot suggests that cholesterol removal from plasma membrane vesicles is minimally affected by 7K if the 7K content is less than 30% of the total sterol. Plasma membrane vesicles from cells loaded with S-58035 contain less than 20% 7K and so show no 7K-dependent inhibition of cholesterol efflux. However, if the 7K content is between 40% and 55%, the cholesterol efflux is markedly decreased. The differences in 7K content in and subsequent cholesterol efflux from plasma membrane vesicles from cells treated with and without S-58035 may also explain the different cholesterol efflux seen from these cells. Projecting the 7K content of the plasma membrane vesicles as the *x*-axis for cellular efflux onto Figure 3 results in a similar dose-dependent inhibition of cholesterol efflux by 7K as seen in Figure 5.

The trends in Figure 5 are also evident in the rate constants (Table 2) which have been calculated from Figure 4a,b as described in Materials and Methods. The rate constants are within the same order of magnitude as the rate constants determined for cellular efflux. As with cells, rate constants from plasma membrane vesicles from S-58035-treated cells are greater than their uninhibited counterparts. In the case of the plasma membrane vesicles, the rate constants are nearly double and can be correlated to the sterol content of the plasma membrane vesicle (56 nmol/mg and $k = 1.16$ for plasma membrane vesicles from non-ACAT-inhibited cells vs 106 nmol/mg and $k = 2.18$ for plasma membrane vesicles from ACAT-treated cells).

Modeling Liposomes onto Plasma Membrane Vesicles. Plasma membrane vesicles were replaced by liposomes to

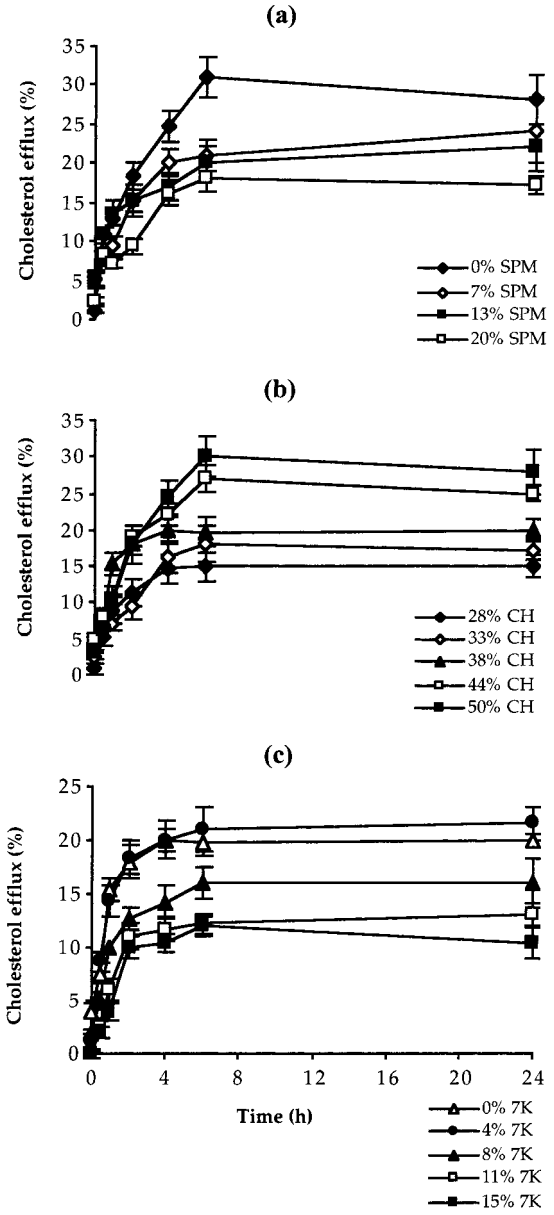

FIGURE 6: [^{3}H]Cholesterol efflux from liposomes to apoA-1. (a) All liposomes had a cholesterol:phospholipid ratio of 1:2, but the ratio of SPM to PC varied. (b) Liposomes had a constant SPM:PC ratio of 30:70, but the cholesterol:PL ratio varied. (c) Liposomes had a SPM:PC ratio of 30:70 and a sterol (cholesterol + 7K):PC ratio of 1:1.6 with 0−40% of the cholesterol replaced by 7K. The molar lipid ratios of the liposomes are also given in Table 4. The symbols and the error bars represent the average of triplicate measurements and the standard deviation, respectively.

investigate whether the inhibitory effect of 7K seen in Figure 4 could be reproduced with an artificial membrane containing no plasma membrane proteins. Since the liposomes should resemble the vesicles in their lipid composition as closely as possible, the phospholipids and their subclasses in the plasma membrane vesicles were first analyzed.

An average of 111 ± 23 nmol of phospholipids/mg of plasma membrane protein was found in plasma membrane vesicles prepared from AcLDL- or 7KAcLDL-loaded cells (−ACAT inhibitor) which correlates to a cholesterol:phospholipid ratio of 1:1.15 (range 1:1.02 to 1:1.27). Sixty-five to seventy-five percent of the phospholipids were PC, 15−30% SPM, and 5−10% LPC. No significant variation in phospholipid subclasses or cholesterol:phospholipid ratio

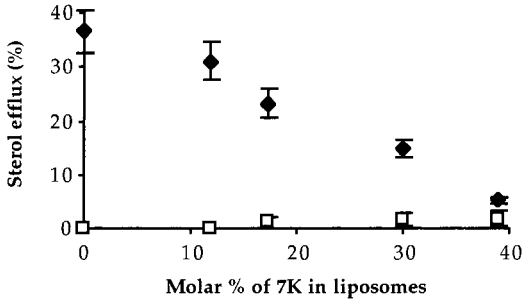

FIGURE 7: Mass of cholesterol (◆) and 7K (□) effluxed from the liposomes in Figure 6c after a 5 h incubation with apoA-1. Triplicates were pooled so that the total amount of sterol for each data point was 112 nmol. The sterol content of the media and the liposomes extracted was analyzed by HPLC as described in Materials and Methods. The percentage of 7K of the 112 nmol of sterol in the liposomes before efflux is plotted on the *x*-axis. Data represent the average and standard deviation of triplicate measurements.

between plasma membrane vesicles from ACAT- versus non-ACAT-treated cells was found. Therefore, the phospholipid composition for liposomes was chosen to be 0−30% SPM and 100−70% PC, and the cholesterol:PL ratio was adjusted between 1:1.25 and 1:1. To incorporate 7K, cholesterol was either replaced by 7K (0−40%) or 7K was added to the liposome composition (0−40% of the cholesterol in the liposomes) to maintain a constant cholesterol:phospholipid ratio.

The fatty acyl composition of macrophage phospholipids is unaffected by supplementation with AcLDL (*63*). The main saturated fatty acid associated with PC is palmitic acid (32% of 16:0) while the majority of unsaturated fatty acid is oleic acid (19% of 18:1) and linoleic acid (18% of 18:2) (*64*). SPM from egg as well as from macrophages contained primarily saturated fatty acids of which palmitic acid is the main fatty acid. Thus the egg phospholipids used here for the liposome preparations have similar fatty acyl composition to macrophage phospholipids; in particular, the ratio of unsaturated to saturated fatty acid is comparable. However, a detailed analysis of the effect of fatty acyl composition on cholesterol efflux remains to be undertaken

Efflux from Liposomes. Similar to cholesterol efflux from plasma membrane vesicles, the cholesterol efflux from unilamellar liposomes (∼220 nm diameter) induced by an excess of lipid-free apoA-1 was measured by the release of tracer amounts of [^{3}H]cholesterol into the media. Figure 6 shows the effect on cholesterol efflux to apoA-1 of liposomes of varying lipid contents. The kinetics of cholesterol efflux (0−5 h) was similar for all liposomes and was within the time frame seen for plasma membrane vesicles. Maximum efflux having been reached by ∼6 h, Table 4 gives the rate constants for cholesterol efflux from the liposomes in Figure 6 and, since the same fitting curve has been used, can be compared to the data from plasma membrane vesicles (Table 2).

Figure 6a shows the effect of changing the ratio of PC:SPM while maintaining a constant cholesterol:PL ratio (1:2). The maximum cholesterol efflux decreases with increasing amount of SPM in the liposomes, suggesting that the strong affinity between cholesterol and SPM affects the amount of cholesterol available for removal by apoA-1. However, apoA-1 efficiency, as determined by the rate constant, is unaltered by changing the SPM:PC ratio.

Table 4: Rate Constants k (in h^{-1}), Maximum Efflux E_m (in %) of Cholesterol in Liposomes, and Correlation Coefficient R for Cholesterol Efflux from Liposomes[a]

liposome composition	Figure	rate constant (h^{-1})	max efflux E_m (%)	correlation coefficient R	fluorescence anisotropy r
33% CH, 66% PC	6a, A	0.60 (0.11)	28.81 (1.71)	0.979	0.255 (0.007)
33% CH, 60% PC, 7% SPM	6a, B	0.57 (0.15)	22.80 (1.99)	0.942	0.267 (0.005)
33% CH, 53% PC, 13% SPM	6a, C	0.65 (0.19)	20.58 (1.74)	0.971	0.278 (0.008)
33% CH, 47% PC, 20% SPM	6a, D	0.67 (0.17)	17.57 (1.80)	0.935	0.286 (0.003)
28% CH, 50% PC, 22% SPM	6b, A	0.83 (0.08)	14.98 (0.39)	0.994	0.275 (0.006)
33% CH, 47% PC, 20% SPM	6b, B	0.67 (0.17)	17.57 (1.81)	0.952	0.286 (0.003)
38% CH, 43% PC, 19% SPM	6b, C	1.19 (0.27)	20.04 (1.19)	0.967	0.298 (0.005)
44% CH, 40% PC, 16% SPM	6b, D	0.61 (0.14)	25.83 (1.91)	0.961	0.304 (0.005)
50% CH, 35% PC, 15% SPM	6b, E	0.46 (0.07)	29.56 (1.55)	0.981	0.327 (0.003)
38% CH, 43% PC, 19% SPM	6c, A	1.19 (0.27)	20.04 (1.19)	0.967	0.298 (0.005)
34% CH, 4% 7K, 43% PC, 19% SPM	6c, B	1.10 (0.08)	20.974 (0.42)	0.996	0.295 (0.004)
30% CH, 8% 7K, 43% PC, 19% SPM	6c, C	0.84 (0.13)	15.65 (0.87)	0.983	0.291 (0.007)
27% CH, 11% 7K, 43% PC, 19% SPM	6c, D	0.67 (0.11)	12.95 (0.64)	0.987	0.288 (0.003)
23% CH, 15% 7K, 43% PC, 19% SPM	6c, E	0.62 (0.16)	11.46 (0.89)	0.971	0.282 (0.002)

[a] The data were obtained by fitting the data presented in Figure 6a−c to the function efflux $= E_m - E_m \exp(-kt)$ with time t in hours. The standard error for the fit is given in parentheses. Fluorescence anisotropy values were obtained as described in Materials and Methods, and standard errors are given in parentheses.

In Figure 6b, the SPM:PC ratio was kept constant (30:70) while the cholesterol:PL ratio was varied. Increasing cholesterol content increased absolute and proportional cholesterol efflux. However, the rate constants decreased with increasing amount of cholesterol in the liposomes: the value for the rate constants halved from 0.83 to 0.46 as the maximal cholesterol efflux doubled from 15% to 30%. This correlation was also seen if the liposome:apoA-1 ratio was varied so that all liposome/apoA-1 mixtures in Figure 6b had either the same amount of cholesterol in the reaction vessel (k decreased from 1.08 to 0.28 while E_m increased from 12.42% to 38.38%) or phospholipid (k decreased from 0.47 to 0.44 and E_m increased from 16.28% to 35.76%). The decrease in efficiency of cholesterol removal differed from the situation with plasma membrane vesicles as the cholesterol donors, where the rate constant increased with cholesterol content in the plasma membrane vesicles (from ACAT-inhibited cells).

We determined the fluorescence anisotropy of the lipsomes as described in Materials and Methods. Increasing the SPM or cholesterol content increased the anisotropy (decreased fluidity) as both cholesterol and SPM increase the packing order of membranes. Hence there is no linear correlation between membrane fluidity and cholesterol efflux, highlighting that efflux depends on a number of parameters of which the cholesterol concentration is only one.

In Figure 6c, the liposomes have a SPM:PC ratio of 30:70 (as in Figure 6b) and a cholesterol + 7K:PL ratio of 1:1.6, and the effects of increasing 7K content are examined, with up to 40% of the cholesterol substituted by 7K. Increasing the amount of 7K in the liposomes reduced cholesterol efflux in terms of absolute amount of effluxed cholesterol, percentage of cholesterol over liposomal sterol (cholesterol + 7K), and percentage of cholesterol over liposomal cholesterol. Additionally, 7K efflux was minimal under all conditions (<5% of the 7K in the system) and not significantly higher than the background efflux of 3 ± 1% (see Figure 7). As the 7K content of liposomes increased from 0 to 40% of the sterol content (substitution of cholesterol by 7K), the cholesterol efflux and the rate constant approximately halved. Interestingly, substitution of 30−40% cholesterol by 7K decreased the fluorescence anisotropy significantly ($p < 0.05$) while lower concentrations of 7K had no effect on the membrane fluidity.

Addition of 7K to the sterol content of the liposomes had a similar effect (with a constant cholesterol:PL ratio); rate constants decreased from 0.58 to 0.20 and the maximum cholesterol efflux decreased from 21% to 13%. In addition, cholesterol efflux decreased almost linearly with increasing 7K incorporation (Figure 7) so that cholesterol impairment was already detectable at low 7K concentrations (cholesterol:7K ratio 5:1).

The decrease in cholesterol efflux and removal efficiency by 7K was therefore observed for liposomes as well as plasma membrane vesicles, although the level of 7K required to cause efflux impairment was dependent on the cholesterol donor. However, the values for the rate constants for cholesterol efflux from these cholesterol donors were comparable, suggesting that the mechanism of inhibition by 7K is similar for both systems. In addition, 7K was not removed by lipid-free apoA-1 from any of the three cholesterol donors.

DISCUSSION

The microsolubilization hypothesis describes the interaction of lipid-poor apoA-1 with membranes to abstract phospholipids and cholesterol. The membrane lipid−protein interaction therefore may be affected by manipulation of the lipid composition or structure of the cholesterol donor. In this study, we investigated whether 7K, an oxidized form of cholesterol, affected membrane lipid domains so that its inhibitory effect on cholesterol efflux could be explained by effects integral to apoA-1-mediated plasma membrane microsolubilization. In this context, the initial rate of efflux to apoA-1 was of particular interest, before apoA-1 is modified by the lipid uptake. The cholesterol efflux (expressed as the percentage of the total cholesterol in the system) and the kinetics of the efflux (expressed in rate constants) were investigated for lipoprotein-loaded cells, plasma membrane vesicles, and liposomes with varying cholesterol and 7K concentrations in all donor systems.

Cholesterol efflux from cholesterol-loaded THP-1 cells (in the absence of an ACAT inhibitor, Figures 1a and 2a) does not exceed more than 30−40% of the total cell cholesterol.

The efflux is reduced significantly to 17−20% (a reduction by a factor of 0.42−0.53) when 20% of the total cellular sterol is 7K. Early time points during efflux from cells to apoA-I suggest nonlinear behavior in the first 90 min (see Figure 1). This early apparent plateau in the efflux is evident to a lesser degree in some previous studies investigating apoA-I-mediated cholesterol efflux from THP-1 cells (*32*). In addition, nonlinear efflux has been observed from J774 cells to apoA-1 (*65*) and apolipoprotein-containing particles (*66*), and such behavior was attributed to two parallel efflux pathways.

To explain this phenomenon, a mathematical model was developed which indicates that cholesterol efflux derives from two parallel pathways removing cholesterol from a plasma membrane pool (M_1) and intracellular cholesterol stores (comprising cholesterol esters, intracellular free cholesterol, and plasma membrane cholesterol) (*67*). The initial efflux is consistent with cholesterol removal from the plasma membrane pool M_1. In the presence of 7K, the abundance of exportable cholesterol in this pool is diminished in addition to the rate constant describing the mobilization of intracellular cholesterol. It should be pointed out that cholesterol is unlikely to be removed without the extraction of phospholipids from the donor system and the effect of 7K on phospholipid efflux to apoprotein A-1 remains to be investigated.

The cellular efflux data suggest that 7K inhibits the removal of cholesterol and possibly phospholipids from the plasma membrane rather than affecting intracellular cholesterol distribution (*67*). Hence cellular cholesterol efflux was compared to efflux from plasma membrane vesicles obtained by formaldehyde/DTT-induced membrane blebbing. We found that these plasma membrane vesicles were enriched in 7K (50−55% 7K in plasma membrane vesicles compared to 20% 7K in the entire plasma membrane as determined by subcellular fractionation) when the parent cell contained 7K. As suggested by the original paper describing this method of plasma membrane vesicle formation, these membranes may be enriched in lipid-raft-like (cholesterol- and SPM-rich) domains (*38*).

In plasma membrane vesicles the maximal cholesterol efflux of ∼20% decreased to ∼5% (by 0.25), and the rate constant fell from 1.16 h^{-1} to 0.29 h^{-1} (by 0.25) with a 7K content of 50−55% (Figure 4b and Table 2). Although the inhibitory concentrations of 7K are obviously different in whole cells (Figure 3) and plasma membrane vesicles (Figure 7), the reduction in cholesterol efflux suggests that 7K can alter the plasma membrane (as suggested by mathematical modeling) so that apoA-1 binding, lipid solubilization, and/or lipid/apoA-1 release from the membrane is impaired. This may be due either to direct physical effects of 7K on membrane structure or to indirect effects on accessory protein function. 7K could selectively alter the expression, accessibility, or folding of plasma membrane proteins and subsequently affect the apoA-1 interaction with the plasma membrane. The importance of the membrane protein ABCA1 in apoA-1-mediated cholesterol efflux is well established (*17*, *18*). The expression of ABCA-1 is regulated by cell cholesterol loading through LXR-dependent transcriptional control. While 7K has at best only a very weak stimulatory effect on ABCA1 expression (*68*), it may affect ABCA-1 membrane distribution or function. This was not measured directly in the present study.

To discriminate the direct effects of 7K content on cholesterol efflux, cholesterol removal from protein-free liposomes was investigated (Figure 7). The maximal cholesterol efflux seen in liposomes depends on the relative concentration of phospholipid subclasses and varies between 20% and 30% of the total cholesterol (Table 4). With up to 40% of the liposomal sterols being 7K the cholesterol efflux is reduced to 10−12% (by 0.57), and the rate constant changes from ∼1.2 h^{-1} to 0.62 h^{-1} (by 0.52). The cholesterol efflux decreases almost linearly with increasing 7K content (Figure 7), highlighting the sensitivity of apoA-1-induced cholesterol removal to 7K. Since no proteins have been incorporated into the liposomes, the cholesterol efflux to lipid-free apoA-1 is obviously not dependent on receptor proteins, but their relevance in cellular systems cannot be discounted. The data presented here provide strong evidence for the hypothesis that apoA-1 can directly interact with membrane lipids inducing cholesterol removal (microsolubilization theory). Furthermore, such an interaction is impaired by 7K, underlining the sensitivity of the apoA-1−membrane interaction.

As apoA-1 is able to remove cholesterol from protein-free liposomes, the question arises as to which membrane parameter(s) is (are) critical in governing cholesterol removal. The effect of membrane curvature and fatty acyl composition on cholesterol efflux remains to be investigated. These and other factors influence the membrane lipid packing and fluidity. To date, the role of membrane structure/fluidity on cholesterol homeostasis is unclear. For example, tighter packing of cholesterol/phospholipid monolayers can be expected to reduce membrane fluidity (*69*) and reduce cholesterol efflux mediated by aqueous diffusion (*70*). However, incorporation of cholesterol into PC vesicles increased the apoA-1 binding capacity with lower affinity, which may be optimal for its reversible association and cholesterol efflux (*71*). In the liposomes used here, membrane fluidity decreased with increasing SPM and cholesterol incorporation but increased when 30−40% of the cholesterol is substituted by 7K. The latter observation is consistent with data showing that the condensing effect of 7K is lower than that of cholesterol (*30*, *31*). There is no direct relationship between membrane fluidity and cholesterol efflux as increasing SPM content decreased fluidity (increased anisotropy *r*) and cholesterol efflux while increasing the cholesterol content also decreased fluidity but increased efflux (Table 4). However, the effects of cholesterol and 7K on membrane fluidity and cholesterol efflux are consistent. Substitution of cholesterol by 7K (or lowering cholesterol levels) resulted in higher fluidity and impaired efflux. The latter observation may suggest that a sterol-induced increase in membrane order (decreased fluidity) may be beneficial for apoA-1-induced cholesterol removal.

Upregulation of membrane cholesterol in cellular systems can be achieved by the addition of an ACAT inhibitor (S-58035) during lipoprotein loading. S-58035 treatment significantly increased the cellular unesterified cholesterol content which resulted in an up to 2-fold increase in plasma membrane sterol content. It also increased the free cholesterol content in plasma membrane vesicles obtained from cells loaded under these conditions. Cholesterol efflux from these

cells was increased (~42% vs 32% above) as were the rate constants ($k_1 = 1.318\ h^{-1}$ and $k_4 = 11.69\ h^{-1}$). The increase in unesterified cholesterol content did not affect the maximal cholesterol removal from plasma membrane vesicles, but the rate constant doubled to 2.18 h^{-1} (cf. 1.16 h^{-1} above). In artificial liposomes the rate constant did not increase with increasing cholesterol loading although the maximum cholesterol removed did. This observation suggests that increasing cholesterol content increases the rate of cholesterol removal and hence the efficiency of the efflux. The fact that the rate constant for cholesterol efflux in our studies increased with cholesterol enrichment of cell membranes but not liposomes may be attributable to either differences in lipid composition between model membranes and cell membranes or to the additional effects on 7K on the expression and/or activity of essential membrane proteins that are present in cells but not model membranes.

7K incorporation into cells or plasma membrane vesicles with already high membrane cholesterol levels (stimulated by S-58035) did not alter cholesterol efflux although the 7K content can be up to 20% and 30% for cells (Figures 1b and 2b) and plasma membrane vesicles (Figure 4c), respectively. It can be suggested that the high cholesterol level, in particular high plasma membrane cholesterol levels, "neutralizes" the effect of 7K; a high cholesterol concentration may condense the membrane above a threshold beyond which 7K is ineffective. However, other factors such as ABCA1 expression are known to regulate cholesterol efflux from cells to apoA-I, and its expression increases in response to cholesterol loading. Thus the effect of cholesterol distribution manipulations, S58035, and the role of 7K in the expression of ABCA1 remain to be investigated.

In conclusion, we found that 7K can modify lipid membranes in such a way that apoA-1-induced cholesterol efflux is less efficient and less cholesterol is removed. This inhibitory effect of 7K depends on the 7K concentration as well as cholesterol concentration in cellular or artificial membranes. It is also noteworthy that removal of 7K itself by apoA-1 is not significant from any of the investigated cholesterol donors.

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