Effects of Enrichment of Fibroblasts with Unesterified Cholesterol on the Efflux of Cellular Lipids to Apolipoprotein A-I*

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Kristin Gillotte-Taylor‡§, Margaret Nickel¶, William J. Johnson¶§, Omar L. Francione**, Paul Holvoet‡‡, Sissel Lund-Katz‡, George H. Rothblat¶, and Michael C. Phillips§§

From the Department of Biochemistry, MCP-Hahnemann University, Philadelphia, Pennsylvania 19129.
¶The Children’s Hospital of Philadelphia, University of Pennsylvania School of Medicine, Abramson Research Center, Philadelphia, Pennsylvania 19104-4318, **Central Research Division, Pfizer Inc., Groton, Connecticut 06340, and ‡‡Center for Experimental Surgery and Anesthesiology, University of Leuven, B-3000 Leuven, Belgium

This study elucidates the factors underlying the enhancement in efflux of human fibroblast unesterified cholesterol and phospholipid (PL) by lipid-free apolipoprotein (apo) A-I that is induced by cholesterol enrichment of the cells. Doubling the unesterified cholesterol content of the plasma membrane by incubation for 24 h with low density lipoprotein and lipid/cholesterol dispersions increases the pools of PL and cholesterol available for removal by apoA-I from about 0.8–5%; the initial rates of mass release of cholesterol and PL are both increased about 6-fold. Expression of the ABCA1 membrane enzyme in cholesterol-enriched fibroblasts over 24 h increases ABCA1 mRNA about 12-fold. The presence of more ABCA1 and cholesterol in the plasma membrane results in a 2-fold increase in the level of specific binding of apoA-I to the cells with no change in binding affinity. Characterization of the species released from either control or cholesterol-enriched cells indicates that the plasma membrane domains from which lipids are removed are cholesterol-enriched with respect to the average plasma membrane composition. Cholesterol enrichment of fibroblasts also affects PL synthesis, and this leads to enhanced release of phosphatidylcholine (PC) relative to sphingomyelin (SM); the ratios of PC to SM solubilized from control and cholesterol-enriched fibroblasts are ~2/1 and 5/1, respectively. Biosynthesis of PC is critical for this preferential release of PC and the enhanced cholesterol efflux because inhibition of PC synthesis by choline depletion reduces cholesterol efflux from cholesterol-enriched cells. Overall, it is clear that enrichment of fibroblasts with unesterified cholesterol enhances efflux of cholesterol and PL to apoA-I because of three effects, 1) increased PC biosynthesis, 2) increased PC transport via ABCA1, and 3) increased cholesterol in the plasma membrane.

Plasma high density lipoprotein (HDL) levels are inversely correlated with the onset of coronary artery disease, and this protective effect is due at least in part to the role of HDL in mediating cholesterol transport from peripheral cells to sites of reutilization and degradation in the reverse cholesterol transport process (for a review, see Ref. 1). HDL and its main protein moiety, apolipoprotein (apo) A-I, drive the initial unesterified (free) cholesterol (FC) efflux from the periphery as well as the subsequent esterification of the cholesterol by lecithin-cholesterol acyltransferase (2, 3) and selective uptake of the cholesterol ester in the liver by scavenger receptor class B type 1 (SR-B1) (4). HDL comprises a very heterogeneous class of particles, particularly with respect to the particle size, density, protein content, and surface charge; the structure of the particles affects their efficiency in participating in the various steps of the reverse cholesterol transport pathway (1).

The efflux of cellular FC is initiated by one of two distinct mechanisms, depending on the degree of lipidation of the apo-A-I (5, 6). Fully lipidated apoA-I or mature HDL species have been established to mediate this process through passive diffusion. In this aspect of FC efflux, FC molecules spontaneously desorb from the plasma membrane, diffuse through the aqueous phase surrounding the cell, and incorporate into an HDL particle upon collision. This so-called aqueous diffusion mechanism has been extensively described in the literature (for a review, see Ref. 7). This simple diffusion process can be facilitated by the presence of scavenger receptor class B, type 1 (SR-B1) in the plasma membrane of the cell (8). Apo-A-I molecules that have dissociated from HDL particles and exist in a lipid-free (poor) state (9) mediate cellular phospholipid (PL) and FC efflux by an active mechanism involving interaction of apo-A-I with the cell surface (3, 10). The resulting protein-lipid complexes enlarge in size through the further incorporation of lipids and interaction with lecithin-cholesterol acyltransferase as they progress through the reverse cholesterol transport pathway (2).

The efflux of cellular lipids to incompletely lipidated apo-A-I molecules is a critical pathway for supply of PL to the HDL pool. Recent studies of Tangier disease have shown that mutations that inactivate the ATP binding cassette transporter 1 (ABCA1) are the cause of the low HDL levels in this disease (for a review, see Ref. 11). ABCA1 is critical for efflux of cellular PL and FC to apo-A-I because FC-loaded Tangier disease fibro-
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bacteria are defective in this regard (12). This behavior is in striking contrast to that of normal human fibroblasts, which exhibit significant stimulation of FC and PL efflux when FC-loaded (13–15). The above observations are consistent with a key role for ABCA1 in PL and FC efflux because expression of ABCA1 in fibroblasts is up-regulated by cholesterol loading (15, 16). Expression of ABCA1 can be regulated by ligands for the nuclear receptor LXR (17) and by cAMP (18). The appearance of the transporter at the cell surface enhances apoA-I binding and efflux of cellular PL and FC (18–20). Comparison of different cell types indicates that there is a general correlation between expression of ABCA1 and lipid efflux to apoA-I (21).

ABCA1 is likely to be involved in PL and FC transport to the cell surface because related transporters translocate PL molecules across membranes (22). However, FC loading of cells up-regulates expression of other proteins likely to modulate lipid flux within cells. Thus, caveolin mRNA levels are up-regulated by FC loading of fibroblasts, and the rate of FC efflux is proportional to the caveolin mRNA levels (23). Likewise, FC loading of macrophages and fibroblasts stimulates phosphatidylinositol (PC) biosynthesis and up-regulation of CTPT:phosphocholine cytidylyltransferase (24, 25). Active trafficking of lipid vesicles between the plasma membrane and the cell interior is critical because disruption of Golgi membranes by agents such as brefeldin (26, 27) and of calcium-dependent retroendo- cytosis (28) inhibits apoA-I-mediated FC and PL efflux. In sum, it seems clear that a continuing supply of PL and FC to the exofacial leaflet of the plasma membrane is required for efficient efflux to apoA-I.

In prior studies (6) using human skin fibroblasts containing normal levels of FC, we examined the kinetics of efflux of cellular FC and PL to lipid-free apoA-I (pre-β-HDL). It was shown that in a 10-min incubation FC and PL are released together and that a finite pool of about 1% of plasma membrane cholesterol is available. This process was termed membrane microsolubilization and defined as follows. “This process involves apolipoprotein-mediated solubilization into the extracellular medium of a small amount of plasma membrane PL and FC; the mechanism is facilitated by interaction of apolipoprotein with the plasma membrane and occurs without obvious cellular damage” (6). Subsequently, the features of the apolipoprotein required for membrane microsolubilization were investigated (29). It was concluded that the mechanism of apoA-I-mediated FC efflux involves two essential features, 1) the membrane lipids must be organized in a manner that allows insertion of α-helices among the lipids, and 2) the α-helices must exhibit sufficient binding affinity to induce the apolipoprotein-lipid association. It now seems apparent that ABCA1 activity is critical for the first feature, but the sizes and composition of the particles released by apoA-I remain to be established.

FC loading of fibroblasts can have direct effects on plasma membrane composition and structure by altering the FC/PL ratio and indirect effects via up-regulation of ABCA1 (15, 16). To better define these contributions, we have examined how FC enrichment of fibroblasts affects FC and PL efflux to apoA-I. Since FC loading of fibroblasts has opposite effects on PC and sphingomyelin (SM) biosynthesis (25, 30), the contribution of PL synthesis to ABCA1-mediated release of lipids to apoA-I was investigated. Enrichment of the cells with FC enhances efflux of FC because of increases in both the amount of FC in the plasma membrane and the expression of ABCA1; the latter effect combined with increased PC biosynthesis stimulates release of PC.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-oleoylphosphatidylcholine and bovine brain sphingo-
myelin were purchased from Avanti Polar Lipids (Birmingham, AL) (+99% grade). [1,2-H]cholesterol (43.5 Ci/mmol), [4-14C]cholesterol (51.3 mCi/mmol), and [methyl-3H]cholesterol chloride (81 Ci/mmol) were obtained from PerkinElmer Life Sciences. Minimal essential medium and phosphate-buffered saline (PBS) were purchased from BioWhit-
taker (Walkersville, MD). Bovine calf and fetal serum were supplied by Invitrogen. Methyl-β-cyclodextrin and 2-hydroxypyropyl-β-cyclo-
dextrin were generous gifts from Cerestar (Hammond, IN). All other reagents were purchased from Sigma.

Methods

Preparation of Lipoproteins and ApoA-I—HDLs and LDL were isolated from fresh plasma obtained from normolipemic donors by sequential ultracentrifugation, and apoA-I was obtained from HDL as described previously (6, 29). A previously described (29) apoA-I deletion mutant (∆190–243) in which a C-terminal segment was removed was used in selected experiments. Reconstituted HDL (rHDL) particles containing 100:1 (mol:mol) 1-palmitoyl-2-oleoylphosphatidylcholine/ apoA-I were prepared and purified using the cholate dispersion/ Bio-Bead removal technique as described in detail previously (31).

Preparation of Cell Monolayers—Normal human skin GM3468A fibroblasts (passages <30) were plated in 22-mm 12-well plates and grown to confluence in minimal essential medium/bicarbonate (MEM) supplemented with 10% fetal calf serum plus 0.5% gentamicin in a 37 °C humidified incubator (95% air, 5% CO2). Upon reaching confluence, the cells were labeled with either 10–20 μCi/ml [3H]cholesterol diluted in MEM supplemented with 10% lipoprotein-deficient serum (LPDS) or 15 μCi/ml [3H]cholesterol chloride diluted in MEM with 10% LPDS for 48–72 h (6, 29). For experiments utilizing unesterified (free) cholesterol (FC)-enriched monolayers, the radiolabeled fibroblast mono-
layers were incubated an additional 24 h with an FC-loading medium (13). This medium contained 2 μg/ml of Sandoz compound 58035 to inhibit FC esterification, 100 μg of FC/ml FC:PL dispersions (>2:1, mol:mol), 50 μg of protein/ml human LDL, 2 mg/ml fatty acid-free bovine serum albumin, and either 10–20 μCi/ml [3H]cholesterol or 20 μCi/ml [3H]cholesterol chloride. After this preparation, the monolayers (35–50 μg of cell protein/well) were washed 4× with MEM containing HEPES (50 mM) (MEM-HEPES), and 1 ml of an acceptor preparation was applied for FC or PL efflux measurements. The cellular content of ABCA1 mRNA was determined by Northern analysis as described previously (21).

MEM devoid of choline was prepared for experiments designed to understand the importance of PL synthesis in apoA-I-mediated FC efflux. In these studies all manipulations were carried out in parallel plates with media either containing or lacking choline. Control and FC-enriched fibroblasts were grown in the choline-deficient media for 3–6 days; this treatment resulted in decreases of cellular PL phosphor- us relative to protein of about one-third. This result indicated that incubation of the fibroblasts in choline-free medium inhibited FC syn-
thesis as expected (24).

In some experiments, methyl-β-cyclodextrin (CD) or FC complexes at a final CD concentration of 5 mM (CD:cholesterol (mol:mol) = 12:1) were utilized to rapidly enrich the monolayers 2–3-fold with FC (32). In one experiment 2-hydroxy-propyl-β-CD was utilized to ensure that effects were not specific for methyl-β-cyclodextrin; in this case the CD solution was prepared at 25 mM with a CD:cholesterol molar ratio of 40:1. The results with 2-hydroxypropyl-β-CD were identical to those obtained with methyl-β-cyclodextrin treatments.

Efflux of Cellular Cholesterol and Phospholipid—FC and PL efflux measurements were conducted as described before (6, 29, 33). The kinetic models used to analyze the measured time courses of FC and PL efflux from fibroblasts are presented under “Appendix.” As described before for this system (33, 34), Model 1, in which all the FC is available for exchange and located in either the cellular pool or the acceptor pool, was used to analyze FC efflux to rHDL particles. The efflux time courses were fitted to Equation 1 by nonlinear regression, and the rate constants for FC flux were derived from the fitting variables using Equations 2–6. The apparent rate constant for efflux (k eff) was used to calculate the halftime (t 1/2) of efflux from the relationship t 1/2 = ln 2/k eff. Model 3, in which there are accessible and inaccessible pools of cellular FC and PL, was applied to the efflux induced by the presence of lipid-free apoA-I in the extracellular medium. Equation 16 was used to estimate the initial distribution of cellular FC between accessible and isolated pools.
Effect of Plasma Membrane Protein Modification on Cellular Cholesterol Efflux—Fibroblasts grown to confluence and labeled with [1^4C]cholesterol as described above were incubated with 0.2% w/v trypsin at 0 °C for 10 min. The trypsin was removed, and the monolayers were washed 2× with MEM-HEPES containing 0.1% (w/v) soybean trypsin inhibitor and 2× with MEM-HEPES, warming the cells to 37 °C. Cholesterol efflux to apoA-I was performed as described above. The effectiveness of the trypsin treatment in removing cell surface proteins was assessed by using cells in which the surface proteins were radioiodinated (35). Briefly, confluent cells in 35-mm dishes were washed on ice with PBS then incubated for 20 min with 1 ml of PBS containing 400 μg of glucose, 40 μg of lactoperoxidase, 20 μg of glucose oxidase, and 100 μCi of Na[^125I]. The reaction was quenched by rinsing cells 2× with PBS containing 1 mM tyrosine and 10 mM sodium metabisulfite followed by 2 additional washes with PBS. The labeled cell monolayers were then treated with trypsin as described above, and the radioactivity released into the media was compared with the total[^125I] incorporated into the monolayers. Approximately 50% of the total[^125I] was released upon trypsin treatment; this value was corrected for the release of counts measured in the absence of trypsin.

The effects of cAMP on cellular cholesterol efflux were examined because this treatment has been shown in macrophages and fibroblasts to promote expression of ABCA1 (15, 18, 20, 21). Chlorophenylthio-cAMP was incubated with radiolabeled control or FC-enriched fibroblasts for 2.5 h in MEM-HEPES at 37 °C before the initiation of cholesterol efflux measurements. For comparison, MEM-HEPES alone was incubated for 18 h before the efflux experiment.

Binding of ApoA-I to Control or FC-Enriched Human Fibroblasts—As described previously (36), radioiodinated apoA-I was incubated with the fibroblast monolayers at concentrations ranging from 0.1 to 100 μg/ml for 1 h at 4 °C to measure the total binding of apoA-I to the cell surface. The nonspecific binding was determined by measuring the same concentration dependence of binding with a 40-fold excess of unlabeled apoA-I present at each concentration. From the difference between total and nonspecific binding, the specific binding of apoA-I was calculated and fitted by nonlinear regression to the one-site binding equation, $Y = B_{\text{max}} \times X/(K_a + X)$.

Preparation of Plasma Membrane Vesicles—Cells labeled with [1^4C]cholesterol and [1^4H]choline chloride were washed 4× with PBS, and the monolayers were exposed to 50 mM formaldehyde and 2 mM dithiothreitol in PBS for 90 min at 37 °C (37, 38). The incubation buffer was removed and discarded, and the monolayers were washed with hypertonic PBS (0.44 M NaCl, pH 7.6). This treatment resulted in the release of a large number of plasma membrane vesicles (0.5–5.0-μm diameter (39)); the buffer was removed and collected for analysis. Aliquots were extensively extracted as described above to remove any free [1^4H]choline present, and radioactivity was determined by liquid scintillation counting. The composition of the plasma membrane vesicles, which is assumed to be approximately representative of the plasma membrane, was computed from the specific activities of the FC and PL in the cell at $t = 0$.

Analytical Methods—Protein concentration was determined by a modification of the Lowry assay (40). Total and FC contents of the cellular lipid extracts were quantified by gas-liquid chromatography using cholesteryl methyl ether as an internal standard. Total PL mass was measured by an enzymatic assay (Wako). The composition of the choline-containing PL appearing in the medium upon stimulation of PL efflux from the cell monolayers was determined by a one-dimensional TLC separation procedure. Samples were collected and extracted from free [1^4H]choline as described above. Further isolation of PL and separation into the various PL subclasses was achieved through two sequential developments in chloroform:methanol: ammonium hydroxide (75:25:4 (v/v/v)) (Silica Gel G). Migration distances of lysophosphatidylcholine, sphingomyelin, and phosphatidylcholine were determined by iodine staining of lanes containing purified standards of these species. Areas of the TLC plate corresponding to the migration distances of the standards were scraped from each sample lane and analyzed by liquid scintillation counting to determine the percentage of radioactivity associated with each lipid subclass.

RESULTS

Influence of Cellular Cholesterol Content on FC and PL Efflux—It is not known whether apoA-I-mediated efflux of plasma membrane FC and PL occurs by the same mechanism in fibroblasts containing control and enriched levels of FC or if increasing the cellular FC content stimulates a different process. Fig. 1 demonstrates that in the first 10 min of apoA-I exposure to fibroblasts with twice the FC content of control cells, there is a stimulation in both FC (panel A) and PL (panel B) efflux to the apolipoprotein as compared with the efflux observed under the same conditions from control fibroblasts. These results indicate that there is an essentially simultaneous solubilization of FC and PL molecules from the plasma membrane by apoA-I regardless of whether the membrane has control or enriched levels of FC. FC loading of the cells increased the masses of PL and FC released to apoA-I in 10 min by ~4 and 9 pmol/ml of medium, respectively (Fig. 1). The fractional lipid efflux measured during the 10-min time course is also increased; FC efflux increased from 0.09 to 0.29% of total cell FC, and PL efflux increased from 0.06 to 0.20% of total cell PL upon FC enrichment of the plasma membrane. This effect is specific for lipid efflux mediated by lipid-free apoA-I because enhanced FC fractional efflux is not observed from FC-enriched monolayers when lipidated rHDL discoidal complexes (1-palmitoyl-2-oleoylphosphatidylcholine-apoA-I, 100:1 (mol:mol)) are utilized as an acceptor (Fig. 2). Over the 6 h time course, fractional FC efflux was the same or greater from control cells compared with FC-enriched cells; the same results were found when PL efflux was measured (data not shown). In this case, the so-called aqueous diffusion mechanism drives the efflux of FC, and the fraction of FC released is not affected by changes in plasma membrane FC content (42).
then increasing the volume of the incubation medium at constant acceptor concentration decreases the influx by a factor proportional to the increase in medium volume. Changing the volume of medium containing 50 μg/ml apoA-I by a factor of up to 5 had no effect on the fraction of FC released in 4 h from either control or FC-loaded fibroblasts (data not shown). This indicates that under the experimental conditions used here, Model 3 with no return FC flux from the medium is appropriate, and the efflux kinetics conform to Equation 16 (“Appendix”). In this treatment, cellular FC exists in two pools, one of which is effectively isolated from apoA-I during the efflux experiment, and another, which is accessible to apoA-I. As summarized in Table I, this kinetic model was used to determine the size of the pool of cellular FC solubilized by apoA-I. A limited amount of PL and FC is accessed by apoA-I, with the pools of FC and PL removed from control and FC-enriched fibroblasts in the range of about 1–5% (Table I). Consistent with the enhanced PL and FC association with apoA-I observed in the FC-enriched cell system, the cellular lipid pool available is increased about 6-fold (Table I). By fitting FC efflux time courses to Equation 16 it was possible to estimate the rate constants for efflux from the accessible pool (k_a in Equation 16) to apoA-I. The rate constants for efflux from control and FC-enriched cells were ~0.6 and 0.2 h⁻¹, respectively; these values correspond to t½ of about 1 and 3 h. The estimated t½ values for PL efflux are about twice as large.

The concentration dependencies of FC and PL efflux from control and FC-loaded fibroblasts to apoA-I were also investigated. Comparison of the k_a values for FC and PL efflux from control monolayers indicates that these values are not significantly different, as determined by Student’s t test; the same phenomenon is observed with FC-enriched monolayers (Table

### Table I

| Cell condition | FC efflux | Cell pool available | PL efflux | Cell pool available |
|---------------|-----------|---------------------|-----------|---------------------|
|               | K_m | μg/ml | % | K_m | μg/ml | % |
| Control       | 0.4 ± 0.2 | 0.8 ± 0.1 | 0.8 ± 0.3 | 0.7 ± 0.2 |
| FC-enriched   | 0.7 ± 0.2 | 4.9 ± 0.8 | 1.2 ± 0.4 | 5.1 ± 0.9 |

FC contents of control and FC-enriched cells were 19 ± 2 and 40 ± 5 μg/mg cell protein, respectively.

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**FIG. 2.** Influence of human fibroblast FC levels on rHDL-mediated FC efflux. Human fibroblasts were radiolabeled with [3H]cholesterol and FC-enriched using LDL/FC/PL dispersions as described under “Methods.” 1-Palmitoyl-2-oleoylphosphatidylcholine:apoA-I (100:1 mol:mol) rHDL discoidal complexes diluted to 50 μg of apoA-I/ml were incubated with control or FC-enriched monolayers for 0–6 h. At each time point an aliquot of the media was removed and filtered, and radioactivity was determined by liquid scintillation counting. The fractional release was determined from the total [3H]FC associated with the monolayers at t = 0. The symbols represent the average (±1 S.D.) fraction of FC released from control (△) or FC-enriched (■) monolayers; all values were determined in triplicate and have been corrected for any release of [3H]FC to MEM-HEPES alone. Curves were generated by fitting the data to a mono-exponential rate equation (cf. Equation 1 under “Appendix”), and the inset focuses on time points up to 0.5 h.


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I). This is consistent with coordinate release of FC and PL molecules from cell monolayers containing either normal or high levels of cholesterol. Furthermore, the concentration dependencies of apoA-I-mediated efflux of FC (as reflected in the $K_m$ values, Table I) and PL are similar regardless of the cholesterol content of the plasma membrane. This is consistent with lipid solubilization from either control or FC-enriched fibroblast membranes occurring by the same mechanism.

Because the $t_{1/2}$ for FC efflux from unloaded cells is about 1 h, a single 4-h incubation of cells with 50 µg/ml lipid-free apoA-I would be expected to deplete the pool to about 6% of its initial size since 4 half-times would have elapsed. However, the amount of FC released in two subsequent 4-h exposures of control or FC-loaded fibroblasts to apoA-I was not reduced to 6% but was reduced only by a factor of about two (data not shown). Similar effects were observed when FC-loaded cells were handled in this fashion. These results indicate that the FC pool accessible to apoA-I can be repleted, presumably by the action of ABCA1; this repletion is sufficiently slow so that the inaccessible FC pool assumed in Model 3 (“Appendix”) is effectively isolated from apoA-I during an efflux experiment.

The fibroblast monolayers were routinely enriched with FC through incubation for 24 h with a FC-loading medium consisting of LDL and FC/PL dispersions. During this incubation period, the cell cholesterol content doubled, and the level of ABCA1 mRNA in the cells increased by a factor of about 12 (Fig. 3), consistent with prior reports for fibroblasts (15, 16). As shown in Fig. 4, there was a concomitant 4-fold increase in the rate of FC efflux. The FC loading of the cells is critical because there is no enrichment or enhanced FC efflux when cells are incubated with LDL and FC-free PL dispersions (data not shown). To examine the effects of FC enrichment of the plasma membrane in the absence of induced protein expression, a rapid loading system involving CD-FC complexes was utilized (32); in this case, molecular FC is incorporated directly into the plasma membrane. A 2-h incubation of these complexes with the control fibroblast monolayers resulted in a 2.5–3-fold increase in cellular FC content that is similar to the 2–2.5-fold enrichment obtained with the LDL-FC/PL dispersion loading mixture. When apoA-I-mediated FC and PL efflux was measured from monolayers enriched with FC by CD-FC complexes, the release was similar to that observed from cells with control levels of cholesterol (Fig. 4). This is in contrast to the 3–4-fold increase in membrane microsolvilization induced by the 24-h LDL-FC/PL dispersion loading. In an attempt to examine the influence of the duration of the FC-loading period, the CD-containing medium was removed after the 2-h enrichment period, and the FC-enriched fibroblasts were maintained on MEM for 22 h before the incubation with apoA-I. Unfortunately, this method consistently resulted in cell toxicity, presumably due to the combination of the large increase in FC content and the presence of an ACAT inhibitor (43). For this reason, the CD- and LDL-based methods of enriching the cells with FC could not be compared at the 24-h time point. Nevertheless, the modification of the loading procedure through the use of CD-FC complexes clearly demonstrates that the structural alteration of the plasma membrane induced by FC enrichment alone does not stimulate enhanced apoA-I-mediated lipid release. Expression of ABCA1 by the cells is required to induce the enhanced efflux of FC and PL to apoA-I.

PC biosynthesis in fibroblasts is also up-regulated in response to FC loading (25). To investigate the importance of PL synthesis with regard to the enhanced apoA-I-mediated efflux observed from FC-enriched fibroblasts, we depleted the choline content of the cells and examined the effect on FC efflux. Control and FC-enriched fibroblasts were cultured and tested in either MEM or choline-depleted MEM. Choline depletion did not affect the increased FC/protein content of the enriched monolayers but decreased the PL/protein ratio by ~30% in both the control and FC-enriched cells. The increase in ABCA1 expression induced by FC loading was unaffected by growing the cells in choline-free medium (data not shown). The decrease in cellular PL content reduced apoA-I-mediated FC efflux at 4 h from both control and FC-enriched fibroblasts (Fig. 5). It is apparent that inhibition of PC synthesis in the FC-enriched cells significantly reduced the degree to which apoA-I-mediated FC efflux was enhanced by FC loading. This apparent requirement of PL synthesis for enhanced removal of FC and PL by apoA-I is consistent with the observations with cells loaded rapidly with FC from CD (Fig. 4). It should be noted that choline depletion had no effect on FC efflux to rHDL particles present at 50 µg/ml apoA-I (data not shown).
mechanism in either cell condition because the cells (27). The process of apoA-I binding occurs by a similar are consistent with those previously reported for fibroblast this experiment. The monolayers were radiolabeled with [3H]choles-

terol, and those indicated as FC-enriched were incubated with LDL-
FC/PL dispersions as described under “Methods.” ApoA-I diluted to 50
µg/ml was incubated with the control or FC-enriched monolayers for 4
h. An aliquot of the media was then removed, filtered, and extracted, and radioactivity was determined by liquid scintillation counting. The bars represent the average (±1 S.D.) percent FC efflux from cells maintained in the presence (open bars) or absence (cross-hatched bars) of choline. The efflux values represent the means from at least three experiments, each conducted in triplicate, and have been corrected for any release of [3H]FC to MEM-HEPES alone. A t test showed that choline depletion significantly (p < 0.05) reduced efflux from control and FC-loaded cells.

**Interaction of ApoA-I with the Plasma Membrane**—The enhanced apoA-I-mediated FC and PL efflux observed from fibroblasts with increased FC content may be due to greater interaction of apoA-I with the plasma membrane. To address this question, the binding of apoA-I to control and FC-enriched fibroblasts was compared. It is apparent from Fig. 6 that increasing the FC content of the monolayer results in a significant increase in apoA-I binding; the B_max values determined for control and FC-enriched monolayers are 0.3 ± 0.1 and 0.7 ± 0.1 µg/mg cell protein, respectively. These binding characteristics are consistent with those previously reported for fibroblast cells (27). The process of apoA-I binding occurs by a similar mechanism in either cell condition because the K_d or apoA-I concentration resulting in half the maximal binding is the same (21 ± 6 and 19 ± 4 µg of apoA-I/ml for control and FC-enriched monolayers, respectively). These results indicate that enriching the cells with FC does not alter the nature of the apoA-I binding sites but roughly doubles their number. This effect was unaltered by growing the fibroblasts on choline-free medium (data not shown).

To better define the relationship between the binding of apoA-I to the plasma membrane and the efflux of cellular FC and PL, the concentration dependencies of the processes were compared. Fig. 7 compares the binding and FC efflux data for the FC-enriched cell system; an identical relationship was observed with the control fibroblast monolayers. The dependence of these processes on the concentration of apoA-I is quite different because the K_d of specific binding is 19 ± 4 µg of apoA-I/µl (−0.7 µM apoA-I), and the K_m of FC efflux is 0.7 ± 0.2 µg of apoA-I/µl (−0.025 µM apoA-I). These results indicate that maximal binding of apoA-I is not required to achieve the maximal rate of removal of FC by apoA-I.

Previously, we have shown that removal of a C-terminal segment reduces the ability of the apoA-I molecule to penetrate into a monolayer of PC molecules (29). This reduction correlates with lower FC and PL efflux from fibroblasts to this type of variant apoA-I than to wild-type apoA-I. To determine whether or not this effect is sensitive to the FC content of fibroblasts, we compared the abilities of apoA-I (Δ190–243) and recombinant wild-type apoA-I to induce FC efflux from control and FC-loaded cells. ApoA-I (Δ190–243) was excluded from penetrating into an egg PC monolayer spread at the air-water interface (29) at a surface pressure of 29–30 dyn/cm, which is about a 10% lower value than the value for recombinant wild-type apoA-I (32–33 dyn/cm). With control fibroblasts, the wild-type and C-terminal deletion apoA-I molecules removed 1.3–1.5 and 1.2% (average of triplicate wells in two separate experiments), respectively, of cellular FC when incubated with the cells at 20 µg/ml for 4 h. In FC-loaded fibroblasts, the equivalent FC efflux values for the wild-type and C-terminal
FC enrichment on this process (Fig. 3). Treatment with cAMP utilizing control monolayers, the composition of lipids released from FC-enriched cells by apoA-I is different from that of membrane vesicles generated from the cells; the average FC/PL mol ratios are 2.5 and 1.7 for the lipids removed by apoA-I and the plasma membrane vesicles, respectively. These results suggest that, similar to the determinations performed with control cells, apoA-I accesses regions that are more FC-enriched than the plasma membrane in general. In addition, the PL released to apoA-I from the FC-loaded fibroblasts is more enriched in PC relative to SM (PC/SM = 4.7 ± 0.4) as compared with the situation with control cells (cf. Ref. 16). The PL released from FC-loaded cells is selectively enriched in PC relative to the average plasma membrane PL composition (Table II). The fact that alterations in the plasma membrane FC/PL ratio change the FC/PL ratio of the solubilized lipids indicates that there is not a unique FC/PL stoichiometry involved in the lipid removal process. Indeed, it is possible to dissociate the release of cellular PL and FC to apoA-I (16, 45).

**DISCUSSION**

The phenomenon of lipid-free apoA-I-mediated cellular FC efflux and the key role for ABCA1 are established (reviewed in Ref. 11), but the molecular mechanism has not been defined. Previously, we have presented evidence that apoA-I simultaneously (at least in times as short as 3 min) removes human fibroblast plasma membrane FC and PC and that the domains of apoA-I exhibiting high lipid affinity are critical (6, 29). The effects of cholesterol enrichment on the kinetics of lipid release to apoA-I and the roles of PL biosynthesis and apoA-I interaction with the cell surface have been investigated in the current work.

**Effects of FC Enrichment on the Rates of FC and PL Efflux**

When efflux of FC is mediated by passive diffusion, in which mass action kinetics apply, the mass of FC released per unit time is influenced both by the rate constant of FC efflux (k_e) and the FC content of the plasma membrane ([FC]_PM) because the flux = k_e[FC]_PM (5, 7). In the aqueous diffusion mechanism

| Cell condition          | FC/PL | % of choline-containing PL |
|-------------------------|-------|---------------------------|
|                         | mol/mol |                         |
| ApoA-I-induced efflux   |       |                           |
| Control                 | 1.4 ± 0.3 | 62 ± 9 33 ± 9 5 ± 1 |
| FC-enriched             | 2.5 ± 0.2 | 80 ± 3 17 ± 2 3 ± 0.3 |
| Membrane vesicles       |       |                           |
| Control                 | 0.9 ± 0.1 | 62 ± 8 34 ± 7 4 ± 0.2 |
| FC-enriched             | 1.7 ± 0.1 | 67 ± 6 30 ± 6 3 ± 1 |

| TABLE II |
|----------|
| Compositional analysis of plasma membrane lipids released to apoA-I |

| Cell condition | FC/PL | % of choline-containing PL |
|----------------|-------|---------------------------|
| ApoA-I-induced efflux |       |                           |
| Control | 1.4 ± 0.3 | 62 ± 9 33 ± 9 5 ± 1 |
| FC-enriched | 2.5 ± 0.2 | 80 ± 3 17 ± 2 3 ± 0.3 |
| Membrane vesicles |       |                           |
| Control | 0.9 ± 0.1 | 62 ± 8 34 ± 7 4 ± 0.2 |
| FC-enriched | 1.7 ± 0.1 | 67 ± 6 30 ± 6 3 ± 1 |

*The average mol ratios were significantly increased upon enrichment (p < 0.006 and 0.0006 for lipids removed by apoA-I and membrane vesicles, respectively) as determined by the t test. Comparison of the FC/PL mol ratios for the lipids removed by apoA-I and membrane vesicles indicates a marginally significant difference (p = 0.05) between the control cell values and a significant difference (p < 0.003) between the FC-enriched cell values.*

*Percent of choline-containing PL subspecies present in the extracellular medium was determined by TLC analysis as described under "Methods."

*After a 10 min incubation with [14C]FC- and [3H]PL-dual-labeled fibroblasts, apoA-I-containing medium was removed for analysis (n = 6). All measurements were corrected for FC and PL efflux to media in the absence of apoA-I.*

*Plasma membrane vesicles were prepared from dual-labeled control or FC-enriched monolayers through treatment with dithiothreitol/formaldehyde as described under "Methods."

deletion apoA-I molecules were 2.6–2.8 and 1.9–2.2%, respectively. Comparison of these data reveals that removal of residues 190–243 from the apoA-I molecule reduces the rate of efflux of cellular FC by 20 ± 5% from both control and FC-loaded fibroblasts. These results indicate that the 10% reduction in the ability of apoA-I to penetrate into a monolayer of PC molecules induced by removal of C-terminal residues has the same effect on FC efflux with control and FC-loaded fibroblasts. This is consistent with the molecular mechanism of lipid acquisition by apoA-I from the fibroblasts being unaltered by changes in their FC content.

Prior work with other cell types indicates that apoA-I may interact with a plasma membrane protein such as ABCA1 to mediate the release of cellular FC and PL (18–20, 36). The involvement of a fibroblast cell surface factor sensitive to proteolytic treatment was investigated through treatment of the cells with trypsin. Relative FC efflux from treated cells as compared with untreated cells was 0.96 ± 0.08 and 0.8 ± 0.2 for control and FC-enriched cells, respectively. Therefore trypsinization and removal of about half of the cell surface proteins did not affect the apoA-I-mediated FC efflux.

It has been shown that elevated levels of cyclic AMP can increase expression of ABCA1 and apoA-I-mediated cholesterol efflux (15, 18, 21). In light of this, the significance of a cAMP-dependent component in the solubilization of the fibroblast plasma membrane was investigated. Consistent with a prior report (21), pretreatment of the control fibroblast monolayers with chlorophenylthio-cAMP for 18 h led to only a marginal increase in the pool of FC available for release to apoA-I (data not shown). This increase is small compared with the effects of FC enrichment on this process (Fig. 3). Treatment with cAMP did not further increase FC efflux from FC-enriched fibroblasts. In fact, there was a decrease in FC efflux, probably due to some cell toxicity effects.

The nature of the lipid regions of the plasma membrane involved in apoA-I-mediated efflux was examined through determining the composition of lipids released, and these data are summarized in Table II. In this study, only the choline-containing PL constituents of the plasma membrane have been monitored; phosphatidylethanolamine is an additional contributor to apoA-I-mediated efflux of PL from fibroblasts but represents <10% of the lipids released (13, 44). The FC/PL mol ratio of the lipids removed by apoA-I was increased with FC-enriched cells as compared with control cells. The control cellular efflux values were compared with plasma membrane vesicles generated from the normal fibroblasts. The average FC/PL mol ratio of the particles released from the cells by apoA-I is about 50% greater than that of the plasma membrane vesicles (Table II). If the lipids released by apoA-I reflect the composition of the region of the plasma membrane where solubilization occurs, then these results suggest that apoA-I interacts with regions of the plasma membrane that are more enriched in FC than the plasma membrane in general. In contrast, the PL subclasses associated with apoA-I in the extracellular medium or present in the plasma membrane vesicles are the same; the PC/SM ratios are 1.9 ± 0.2 and 1.8 ± 0.1 for efflux medium and plasma membrane vesicles, respectively. Therefore, the lipids in control cells solubilized by apoA-I are representative of the average plasma membrane PL composition. Similar to the results utilizing control monolayers, the composition of lipids released from FC-enriched cells by apoA-I is different from that of membrane vesicles generated from the cells; the average FC/PL mol ratios are 2.5 and 1.7 for the lipids removed by apoA-I and the plasma membrane vesicles, respectively. These results suggest that, similar to the determinations performed with control cells, apoA-I accesses regions that are more FC-enriched than the plasma membrane in general. In addition, the PL released to apoA-I from the FC-loaded fibroblasts is more enriched in PC relative to SM (PC/SM = 4.7 ± 0.4) as compared with the situation with control cells (cf. Ref. 16). The PL released from FC-loaded cells is selectively enriched in PC relative to the average plasma membrane PL composition (Table II). The fact that alterations in the plasma membrane FC/PL ratio change the FC/PL ratio of the solubilized lipids indicates that there is not a unique FC/PL stoichiometry involved in the lipid removal process. Indeed, it is possible to dissociate the release of cellular PL and FC to apoA-I (16, 45).

**DISCUSSION**

The phenomenon of lipid-free apoA-I-mediated cellular FC efflux and the key role for ABCA1 are established (reviewed in Ref. 11), but the molecular mechanism has not been defined. Previously, we have presented evidence that apoA-I simultaneously (at least in times as short as 3 min) removes human fibroblast plasma membrane FC and PL and that the domains of apoA-I exhibiting high lipid affinity are critical (6, 29). The effects of cholesterol enrichment on the kinetics of lipid release to apoA-I and the roles of PL biosynthesis and apoA-I interaction with the cell surface have been investigated in the current work.
describing FC efflux to lipided apoA-I species, doubling of the cellular FC content does not influence the ability of FC molecules to desorb from the plasma membrane, because the $k_d$ is unaffected (Fig. 2) (42). However, the flux of FC from the cell is doubled simply because the pool of available FC, [FC]PM, is twice as large. In the case of efflux to lipid-free apoA-I there is an ~6-fold increase in the fractional pool size on FC enrichment of the fibroblasts (Table I); this occurs because there are more ABCA1 molecules in the FC-enriched cells (Fig. 3) (15, 16). The size of this apoA-I-accessible pool in mass units is 12-fold higher than in control cells because the loading doubles the total cell FC content. This enrichment leads to a 3-fold decrease in the rate constant for FC efflux from this accessible pool from 0.6 to 0.2 h$^{-1}$. Thus, the change in flux of cholesterol mass to apoA-I on FC loading of the cells is expected to be about a 4-fold increase. The measured difference in initial efflux of FC mass between enriched and control cells of about 6-fold (Fig. 1) is in reasonable agreement with this calculation. Similar considerations apply to the efflux of PL mass to apoA-I, except that the size of the apoA-I-accessible pool in mass units is increased only ~7-fold on FC loading (Table I) because the PL content of control and FC-enriched cells is similar. This difference in the mass size of the accessible pools explains why FC loading of the cells causes a greater mass of FC than PL to be released in 10 min (Fig. 1).

The increase in the initial fractional efflux of cell FC from 0.09 to 0.29%/10 min (when normalized to total cell FC) is due to the 6-fold increase in the fractional size of the accessible pool (Table I). It is noteworthy that the tendency of FC (and PL) molecules to escape from the accessible pool is reduced by a factor of ~3 in FC-loaded cells. This implies that the activity of individual ABCA1 molecules in transporting lipid molecules is reduced by FC enrichment of the plasma membrane.

Nature of the Interaction between ApoA-I and Human Fibroblast Cells—The structural reorganization of the plasma membrane that occurs after cholesterol loading and enhanced expression of proteins such as ABCA1 and caveolin (cf. the Introduction) may directly influence the interaction between apoA-I and the plasma membrane, thereby enhancing the rates of FC and PL release. The strength of the interaction of apoA-I with the cell surface is apparently the same for control and FC-enriched fibroblasts because the $K_d$ values for specific binding are similar (Fig. 6). Rather, the enhanced binding is due to an increase in $B_{max}$, the number of interaction sites on the plasma membrane. It is interesting that only a subset of these interactions are productive in solubilizing plasma membrane FC and PL molecules because binding to a level of about 20% $B_{max}$ is sufficient for maximal efflux (Fig. 7). This indicates that not all of the interactions lead to removal of PL and FC. Overall, the enhanced FC and PL mass flux to lipid-free apoA-I that occurs with FC-enriched fibroblast cells as compared with control cells seems to be due to an increased number of the sites for solubilization (which enlarges the accessible pool of PL and FC) as well as an altered molecular organization in these domains that favors the lipid removal process. It is likely that ABCA1 forms these sites with its translocase/"vacuum cleaner" activity (46), creating a pool of PL and FC molecules in a physical state that allows ready access of apoA-I. The number of PL molecules removed from the plasma membrane by the interaction of an apoA-I molecule is not known at this time. Release of PL molecules to apoA-I is required for formation of HDL particles, but the FC/PL stoichiometry of these particles is not unique (Table II), which suggests that their FC content reflects the availability of FC in the plasma membrane.

ApoA-I/PL binding clearly has to occur to create HDL particles, but this interaction may not be the initial event in the removal of membrane lipids. ApoA-I binds to the cell surface close to ABCA1 because the two proteins can be cross-linked (18, 19), but it has not been established yet that there is an apolipoprotein binding site on ABCA1. The pump activity of the transporter could induce local rearrangement of PL molecules in the membrane so that apoA-I binds directly to the PL (47). It is interesting that the $K_d (0.7 \mu M)$ of binding of apoA-I to the fibroblast plasma membrane is similar to that reported for binding of apoA-I to PC/cholesterol vesicles (48); this suggests that the binding isotherms in Fig. 6 reflect apoA-I/plasma membrane lipid interactions. The molecular basis for the ~30-fold difference in $K_m$ and $K_d$ values (Fig. 7) is not clear at this time, but perhaps a high affinity, low capacity apoA-I/ABCA1 interaction is involved in solubilization of FC and PL. If this is the case, it is interesting that the ABCA1 is not sensitive to inactivation by trypsin, because the removal of fibroblast plasma membrane lipids by apoA-I is resistant to trypsin treatment (cf. "Results").

Composition of the Cellular Lipids Solubilized by ApoA-I—PL synthesis plays a key role in ABCA1-mediated lipid efflux to apoA-I because inhibition of PC synthesis by choline depletion reduces the extent to which FC efflux from FC-enriched fibroblasts is enhanced (Fig. 5). The compositional data in Table II further suggest that newly synthesized PL are an important source of lipid for acquisition by apoA-I. Thus, there is a selective enhancement of PC (relative to SM) release to apoA-I in FC-enriched fibroblasts. In contrast, the PC/SM ratio in the PL released from control cells by apoA-I is the same as the ratio for the average plasma membrane (Table II). Because FC loading of fibroblasts increases the synthesis of PC (25) but decreases the synthesis of SM (30), it follows that synthesis enlarges the pool of PC that is available for ABCA1-mediated transport to apoA-I. This is consistent with the reported role of ABCA1 in exporting cellular lipid from the Golgi membrane to the plasma membrane (49).

Characterization of the lipids released to apoA-I (Table II) also provides information on the nature of the plasma membrane domains accessed. Incubation of apoA-I with cells containing either control or enriched levels of FC yields extracellular particles that are, on average, FC-enriched with respect to vesicles generated from the plasma membrane. Furthermore, the FC-enriched cells give rise to extracellular particles that are comprised of more FC than those solubilized from control cells. These results suggest that the regions of the plasma membrane solubilized by apoA-I are FC-rich; caveolae (50) and glycosphingolipid-cholesterol rafts are FC-enriched regions of the fibroblast membrane (51) and could be involved in the apoA-I-mediated lipid removal process (2, 52). Consistent with this idea, caveolin-1 has been shown to be involved in cholesterol enrichment of the HDL particles created by interaction of apoA-I with THP-1 cells (53). However, it has been reported recently that ABCA1 is not located in membrane rafts (54). More work is required to establish the regions of the plasma membrane involved in apoA-I-mediated lipid efflux and the structures of the lipid particles released into the extracellular medium.

The present results with human fibroblasts support a mechanism for apoA-I-mediated lipid efflux in which apoA-I associates with FC-enriched plasma membrane domains containing ABCA1 to access PL and FC molecules. FC enrichment of the plasma membrane increases the solubilization of PL and FC through ABCA1-mediated restructuring of the membrane components to create an increased number of lipid removal sites as well as an increased FC content within them. It is also likely that ABCA1 is involved in transporting PC to these sites, where it can be preferentially removed by apoA-I. The location
of these sites within the plasma membrane and the details of the mechanism(s) by which apoA-I molecules induce release of lipid molecules remain to be elucidated.

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APPENDIX

Efflux data conforming to the general relationship,

\[ \frac{FC}{FC_0} = \left( H_1 \times e^{-kt} \right) + H_2 \]  

(Eq. 1)

where FC is the tracer FC in cells, FC_0 is the initial total tracer FC in cells, H_1, H_2, and g_1 are constants defining efflux kinetics derived from curve fitting of efflux data, e is the base of the natural logarithms, and t is incubation time, are consistent with at least two distinct compartmental models for cholesterol trafficking, 1) a single cellular pool of FC completely available for efflux interchanging with an extracellular carrier whose FC is completely available for influx (Model 1 below, where FC_m is tracer FC in medium, F_e is FC efflux, and F_i is FC influx) and 2) two cellular pools, one available for efflux and the other isolated, with the FC released from cells available for return into the accessible cellular pool (Model 2 below, where FC_a is FC in isolated cellular pools, X is the blockage of interchange between FC_a (FC in efflux-accessible cellular pool) and FC_c, and F_ea is FC efflux from accessible cellular pools).

\[ \frac{F_e}{F_i} = \frac{FC_m}{FC} \]  

(Model 1)

\[ \frac{FC_a}{FC} = X \Rightarrow \frac{FC_m}{FC} \]  

(Model 2)

In Model 1, as described previously (34, 55), a steady-state distribution of FC between cells and medium is achieved at long incubation times after sufficient FC has been released to equalize F_e and F_i. The constants H_1, H_2, and g_1 are composite terms composed of the rate constants for efflux and influx (k_e, k_i) in the following.

\[ H_1 = \frac{k_e}{k_i + k_e} \]  

(Eq. 2)

\[ H_2 = \frac{k_i}{k_i + k_e} \]  

(Eq. 3)

\[ g_1 = k_i + k_e \]  

(Eq. 4)

Thus, using kinetic data, k_e and k_i can be calculated as follows.

\[ k_e = H_1 \times g_1 \]  

(Eq. 5)

\[ k_i = H_2 \times g_1 \]  

(Eq. 6)

F_e and F_i, at any given time then are k_e \times FC_c and k_i \times FC_m, respectively. With regard to considerations in this paper, it is important to note that k_i (expressed in units of reciprocal time) is dependent on extensive variables of the incubation system showing direct proportionality to cell number or mass and inverse proportionality to medium volume. The constant k_e is not subject to the same dependencies, although it can vary between cell types and with the type and concentration of acceptor. These considerations suggest that if there is significant influx of cell-derived FC from a given acceptor, then increasing the volume of the incubation medium while keeping acceptor concentration constant would reduce k_i and, thus, slow the increase in F_i of cell-derived FC during the approach to steady state. This would lead to greater cumulative release of cellular FC both at steady state and at intermediate times beyond the initial-rate phase.

In Model 2, a steady-state distribution of FC between cells and medium is also approached at long incubation times, but in this case the final distribution is governed by both the existence of an isolated cellular FC pool and the amount of FC that needs to be released in order for F_e to equal F_i from the accessible cellular pool. To predict the efflux kinetics for Model 2 in terms of rate constants and the relative sizes of the accessible and isolated cellular pools, one can first consider the kinetics of interchange between FC_a and FC_m,

\[ \frac{FC_a}{FC_m} = \left( H_{1a} \times e^{-kt} \right) + H_{2a} \]  

(Eq. 7)

where H_{1a}, H_{2a}, and \( k_{ea} \) are terms analogous to H_1, H_2, and \( k_e \) for efflux kinetics of the accessible cellular pool, and FC_a is initial FC in the efflux-accessible cellular pool. If P_a and P_i are the initial fractions of cellular FC in the accessible and isolated pools, respectively, and FC_c = FC_a + FC_m, then,

\[ \frac{FC_a}{P_a \times FC_m} = \left( H_{1a} \times e^{-kt} \right) + H_{2a} \]  

(Eq. 8)

\[ \frac{FC}{FC_m} = \left[ P_a \times H_{1a} \times e^{-kt} \right] + \left( P_i \times H_{2a} \right) \]  

(Eq. 9)

\[ FC/FC_m = \left[ P_a \times H_{1a} \times e^{-kt} \right] + \left( P_i \times H_{2a} \right) + P_i \]  

(Eq. 10)

This derivation thus shows that for Model 2, the efflux kinetics should conform to the general relationship given in Equation 1 with

\[ H_1 = P_i \times H_{1a} \]  

(Eq. 11)

\[ H_2 = P_i \times H_{2a} + P_i \]  

(Eq. 12)

\[ g_1 = g_{1a} \]  

(Eq. 13)

To calculate the efflux rate constant from the accessible pool \( (k_{ea}) \),

\[ F_{ea} = FC_m \Rightarrow FC_a \Rightarrow FC_m \]  

(Model 3)

In this simplification, \( H_{2a} = 0, H_{1a} = 1, \) and \( g_{1a} = k_{ea} \) (see Equations 2–4 above). Thus, the efflux kinetics are predicted to conform to the following.

\[ \frac{FC_a}{FC_m} = \left[ P_a \times H_{1a} \times e^{-kt} \right] + P_i \]  

(Eq. 16)

In this case, the rate constant for efflux from the accessible pool and the initial distribution of cellular FC between accessible and isolated pools can be derived directly from the fitting of the efflux kinetic data.

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