ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I

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Abstract **Prior studies provide data supporting the notion that ATP binding cassette transporter A1 (ABCA1) promotes lipid efflux to extracellular acceptors in a two-step process: first, ABCA1 mediates phospholipid efflux to an apolipoprotein, and second, this apolipoprotein-phospholipid complex accepts free cholesterol in an ABCA1-independent manner. In the current study using RAW264.7 cells, ABCA1-mediated free cholesterol and phospholipid efflux to apolipoprotein A-I (apoA-I) were tightly coupled to each other both temporally and after treatment with ABCA1 inhibitors. The time course and temperature dependence of ABCA1-mediated lipid efflux to apoA-I support a role for endocytosis in this process. Cyclodextrin treatment of RAW264.7 cells partially inhibited 8Br-cAMP-induced efflux of free cholesterol and phospholipid to apoA-I. ABCA1-expressing cells are more sensitive to cell damage by high-dose cyclodextrin and vanadate, leading to increased lactate dehydrogenase leakage and phospholipid release even in the absence of the acceptor apoA-I. Finally, we could not reproduce a two-step effect on lipid efflux using conditioned medium from ABCA1-expressing cells pretreated with cyclodextrin.**—Smith, J. D., W. Le Goff, M. Settle, G. Brubaker, C. Waelde, A. Horwitz, and M. N. Oda. **ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I.** ***J. Lipid Res.*** **2004.** 45: **635–644.**

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Cellular expression of ATP binding cassette transporter A1 (ABCA1) promotes the efflux of both free cholesterol (FC) and phospholipids (PLs) to extracellular acceptors such as apolipoprotein A-I (apoA-I); however, the mechanism of this efflux is not understood. Fielding et al. (1) proposed a two-step mechanism in which ABCA1 mediates PL efflux to apoA-I, which in turn can then pick up FC in an ABCA1-independent autocrine or paracrine manner. This conclusion was based on two types of experiments: *1*) PL efflux from vascular smooth muscle cells to apoA-I is less sensitive to vanadate inhibition than FC efflux, and *2*) medium containing apoA-I that is conditioned on smooth muscle cells can lead to FC efflux from vascular endothelial cells that do not express ABCA1 (1). Wang et al. (2) provided evidence for the two-step pathway by demonstrating that *1*) a 30 min pretreatment of ABCA1-expressing cells with 20 mM 2-hydroxypropyl-β-cyclodextrin reduced FC efflux to apoA-I without reducing PL efflux, and *2*) medium containing apoA-I that is conditioned on cyclodextrin-pretreated ABCA1-expressing cells could lead to FC efflux from cells that do not express ABCA1. These experiments and others from Chimini and colleagues (3, 4), who demonstrated that ABCA1 could mediate phosphatidylserine translocase activity, have led to the notion that the primary activity of ABCA1 is the assembly of PL onto acceptors and that FC efflux follows passively by a mechanism not dependent on ABCA1.

We demonstrate here that FC and PL efflux to apoA-I is concurrent in the RAW264.7 murine macrophage cell line, in which ABCA1 expression is inducible by cAMP analogs (5, 6). ABCA1-mediated lipid efflux has delayed kinetics and is abolished at room temperature, results that are consistent with the need for endocytosis and vesicular trafficking for efflux to occur. We can further explain some of the observed effects of high-dose cyclodextrin and vanadate as a result of cell damage, as ABCA1-expressing cells are sensitized to both of these treatments, which result in lactate dehydrogenase (LDH) and PL release by a mechanism independent of extracellular lipid accep-

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Abbreviations: AcLDL, acetylated LDL; DGGB, DMEM supplemented with 20 mM glucose, 2 mM glutamine, and 0.2% BSA; FC, free cholesterol; PL, phospholipid.

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tors. In the case of cyclodextrin pretreatment, the released PL is associated with membrane shedding. It certainly remains possible that various treatments, such as cyclodextrin, FC loading (7), or the expression of stearoyl-CoA desaturase (8), can alter the ratio of FC to PL efflux mediated by ABCA1; thus, ABCA1-mediated FC and PL efflux can be uncoupled under certain conditions. However, our data support the hypothesis that ABCA1 can directly and concurrently mediate the assembly of PL and FC onto apolipoprotein acceptors to generate nascent lipoproteins. In RAW264.7 and HEK293 cells, we find no evidence that conditioned medium from ABCA1-expressing cells can act as a subsequent acceptor of cellular lipids in an ABCA1-independent manner. Thus, we find no evidence to support the two-step pathway of lipid efflux.

EXPERIMENTAL PROCEDURES

Cell culture and lipid efflux assays

RAW264.7 cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum. The general protocols for lipid efflux are as follows, with specific alterations noted in the figure legends. [^{3}H]Cholesterol (Amersham) was dried down and dissolved in ethanol at 1 mCi/ml. On day 1, cells were plated in 24-well dishes with 200,000 cells per well. On day 2, the cells were cholesterol labeled and loaded by incubation overnight in serum-free DMEM supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA (DGGB) and containing 50 μg/ml acetylated LDL (AcLDL) and 0.3 μCi/ml [^{3}H]cholesterol. On day 3, the labeling medium was removed and the cells were treated for 16–24 h in DGGB in the presence or absence of 0.1–0.3 mM 8Br-cAMP (Sigma) to induce ABCA1. On day 4, the cells were chased for the specified times in DGGB in the presence or absence of apoA-I (Biodesign, Saco, ME) or methyl-β-cyclodextrin (Sigma catalog no. C-4555; estimated molecular weight = 1,320). Radioactivity in medium aliquots was determined after a 30 s spin in a microfuge to pellet debris. Cell radioactivity was determined by extraction in hexane-isopropanol (3:2) and evaporation of the solvent in a scintillation vial. The percentage FC efflux was calculated as 100 × (medium dpm)/(medium dpm + cell dpm). To measure FC and choline PL efflux, the cells were labeled as described above using [^{14}C]cholesterol (Amersham) and [methyl-^{3}H]choline (Amersham) such that the labeling medium contained 0.5 μCi/ml [^{14}C]cholesterol and 2 μCi/ml [^{3}H]choline. Radioactivity in the chase medium was determined after lipid extraction in 1 volume of methanol and 2 volumes of $CHCl_3$. The solvents were dried down in scintillation vials before determining radioactivity. All samples were counted in a Beckman LS6500 using quench and dual-label spill corrections. A 100 mM orange-colored stock of sodium decavanadate was prepared from sodium orthovanadate (Sigma) by boiling at pH 10 to produce the colorless sodium mono-orthovanadate, placing on ice, and adjusting the pH to 4.0. Cholesterol efflux from wild-type or ABCA1-green fluorescent protein (GFP) stably transfected HEK293 cells (9) was as described above with the following changes. Cells were plated onto collagen-coated 24-well dishes at a density of 200,000 cells per well. Cells were labeled by overnight incubation with 1 μCi/ml [^{3}H]cholesterol in DMEM containing 10% FBS.

ApoA-I cellular uptake assay

His-tagged apoA-I with a cysteine replacement (L218C) was prepared as previously described (10). The single cysteine residue was labeled with tetramethylrhodamine iodoacetamide (Molecular Probes), repurified by nickel column chromatography, and dialyzed against phosphate-buffered saline. Time-of-flight mass spectrometry confirmed a single rhodamine substitution per apoA-I with a labeling efficiency of ~66%. This labeled apoA-I was fully functional as an ABCA1-dependent acceptor of cellular FC (data not shown). RAW264.7 cells were plated in 35 mm dishes and treated overnight in the presence or absence of 0.3 mM 8Br-cAMP to induce ABCA1. A total of 5 μg/ml rhodamine-labeled L218C apoA-I in DGGB was added to the cells for a 1 h uptake period at 37°C or 21°C. After fixation in 10% phosphate-buffered formalin, Vectorshield with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was applied to mount a glass coverslip. Using constant settings for each fluorophore, rhodamine and DAPI epifluorescent photographs were taken using a 63× oil-immersion lens.

LDH release fluorometric assay

LDH activity was assessed in RAW264.7 cells in 0.5 ml of DGGB. At the end of the incubation period, 275 μl of conditioned medium was removed and the cells were lysed by the addition of 25 μl of 10× lysis reagent from the CytoTox-ONE assay kit (Promega). A total of 50 μl of the conditioned medium or 2 μl of the cell lysate plus 50 μl of DGGB was placed in wells of a 96-well dish and equilibrated to 22°C. Fifty microliters of the reconstituted CytoTox-ONE assay reagent was added and incubated in the dark at 22°C for 10 min. Then, 50 μl of the stop reagent was added, and fluorescence was measured in a 96-well fluorescence plate reader with excitation at 560 nm and emission at 590 nm. Total LDH activity in the medium and lysate was calculated based on the total volumes and the assay volumes, and the percentage LDH released was calculated as 100 × (medium LDH)/(medium LDH + lysate LDH).

Transmission electron microscopy

RAW264.7 cells were cholesterol loaded by overnight incubation with 50 μg/ml AcLDL in DGGB and then treated overnight in the presence or absence of 0.1 mM 8Br-cAMP in DGGB. The cells were treated for 30 min in the presence or absence of 20 mM methyl-β-cyclodextrin in DGGB, washed, and replenished with DGGB, and 0.1 mM 8Br-cAMP was readded to the cells that were initially treated. Four hours later, the medium was aspirated, and the cells were washed once gently in prewarmed phosphate-buffered saline and fixed in prewarmed 2.5% glutaraldehyde. A small section of the tissue culture dish was cut out, and adherent cells were stained with 1% osmium tetroxide and 1% uranyl acetate. After dehydration, the samples were embedded in Epon, and 85 nm sections were cut perpendicular to the tissue culture dish surface for viewing on a Philips CM-12 electron microscope. For each condition, at least six cells were photographed, and the entire experiment was repeated once with similar results.

Statistics

All graphs were made and statistical analyses were performed using Prism software (GraphPad, San Diego, CA). All data are shown as means ± SD. Comparison of two samples was performed by a two-tailed *t*-test, and for three or more samples, ANOVA was performed with either Dunnett's multiple comparison posttest (for each group versus control) or Newman-Keuls multiple comparison posttest (for all groups versus each other).

RESULTS

ABCA1 has been proposed to mediate a two-step assembly of PL and FC onto apolipoprotein acceptors (1, 2); if this is true, one would expect that PL efflux would precede FC efflux. To address this, we examined the early time course of FC and PL efflux to apoA-I from RAW264.7 cells pretreated overnight in the absence or presence of 0.3 mM 8Br-cAMP (**Fig. 1A**, B), which induces lipid efflux to apolipoprotein acceptors via a greater than 50-fold induction of ABCA1 mRNA in these cells (11). Comparing RAW264.7 cells with or without 8Br-cAMP pretreatment to induce ABCA1, there was a time lag of ~15 min before the effects of ABCA1 could be observed on the efflux of either FC or PL. This time lag is consistent with our hypothesis that endocytosis and resecretion of apoA-I may play a role in ABCA1-mediated lipid efflux. Subtracting the efflux to apoA-I from cells pretreated in the absence of 8Br-cAMP, it is clear that 8Br-cAMP-dependent PL efflux did not precede FC efflux, even at the 15 and 20 min time points, the first to show any significant ABCA1-induced lipid efflux (Fig. 1C). The 8Br-cAMP-dependent lipid efflux rate did not appear linear over the first 90 min, with the rate increasing after 60 min. In contrast, the rate of cholesterol efflux to 1 mM methyl-β-cyclodextrin was most rapid during the first 5 min (Fig. 1D). For the 5 min time point, FC efflux to methyl-β-cyclodextrin was equivalent from cells treated in the presence or absence of 8Br-cAMP, whereas there was significantly increased FC efflux from the 8Br-cAMP-treated cells expressing ABCA1 only at the 20 and 30 min time points (23% higher for the 30 min time point; $P < 0.01$). The lack of an 8Br-cAMP-mediated effect on FC efflux to methyl-β-cyclodextrin at the initial 5 min time point suggests that ABCA1 expression did not lead to an increase in the steady-state level of FC on the cell surface. There was evidence that ABCA1 could mobilize more FC in a pool that was accessible to the methyl-β-cyclodextrin only after 15 min, when vesicular trafficking could play a role.

ApoA-I uptake as well as lipid efflux to apoA-I and methyl-β-cyclodextrin were also examined at 21°C, a temperature at which receptor-mediated endocytosis for many ligands is dramatically reduced (12). We first examined fluorescently labeled apoA-I uptake by RAW264.7 cells at 37°C, and as we previously observed (9), 8Br-cAMP pretreatment led to a large increase in the uptake of apoA-I into intracellular vesicles (**Fig. 2A**, B). However, when apoA-I was added to cells at 21°C, there was evidence of an 8Br-cAMP-induced increase in cell surface binding, but not of internalization into intracellular vesicles (Fig. 2C, D). There was practically no FC efflux to apoA-I at 21°C from 8Br-cAMP-pretreated cells (**Fig. 3A**). These data are consistent with our hypothesis that endocytosis and resecretion of apoA-I may play a role in ABCA1-mediated lipid efflux. In contrast, efflux to 10 mM methyl-β-cyclodextrin was robust at 21°C, indicating that the initial removal of cholesterol from the plasma membrane by methyl-β-cyclodextrin is not dependent on endocytosis and vesicular trafficking (Fig. 3B). There was no effect of ABCA1 induction during the initial 5 min efflux of FC to 10 mM methyl-β-cyclodextrin at 21°C. At later time points, there were only small increases in FC efflux from the 8Br-cAMP-treated cells.

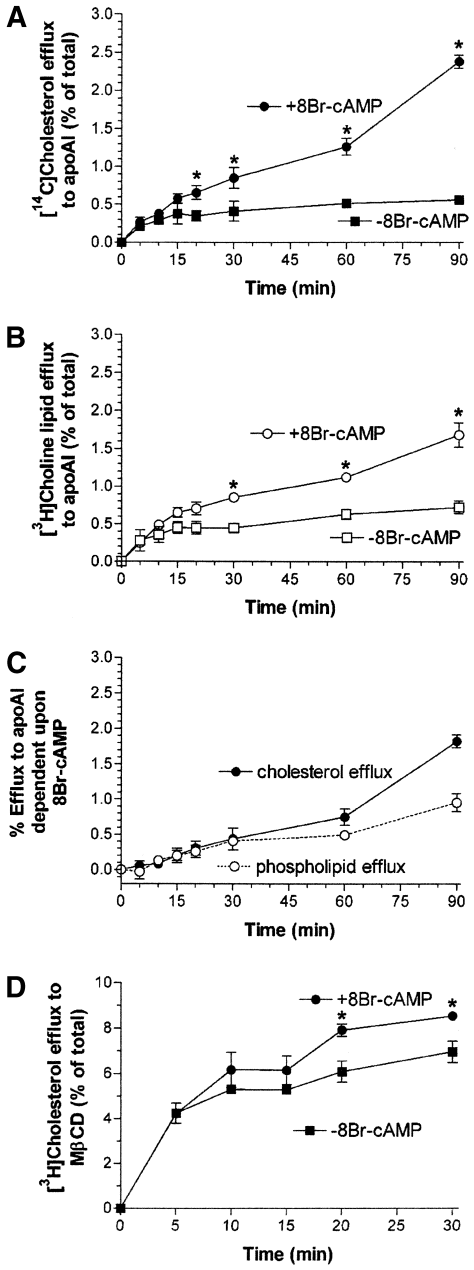

Fig. 1. Early time course of lipid efflux to apolipoprotein A-I (apoA-I) and cyclodextrin at 37°C. A–C: RAW264.7 cells were double labeled with [^{14}C]cholesterol and [^{3}H]choline, and free cholesterol (FC) and phospholipid (PL) efflux to 5 μg/ml apoA-I over 90 min was assessed in cells pretreated overnight in the presence (circles) or absence (squares) of 0.3 mM 8Br-cAMP to induce ATP binding cassette transporter A1 (ABCA1), as described in Experimental Procedures. A: FC efflux. B: Choline PL efflux. C: FC (closed circles) and PL (open circles) efflux to apoA-I from 8Br-cAMP-treated cells minus the efflux to control treated cells. D: FC efflux to 1 mM methyl-β-cyclodextrin (MβCD) from cells pretreated in the presence (circles) or absence (squares) of 0.3 mM 8Br-cAMP. * $P < 0.05$ compared with the absence of 8Br-cAMP treatment; n = 4 for A–C and n = 3 for D. Error bars are mean ± SD.

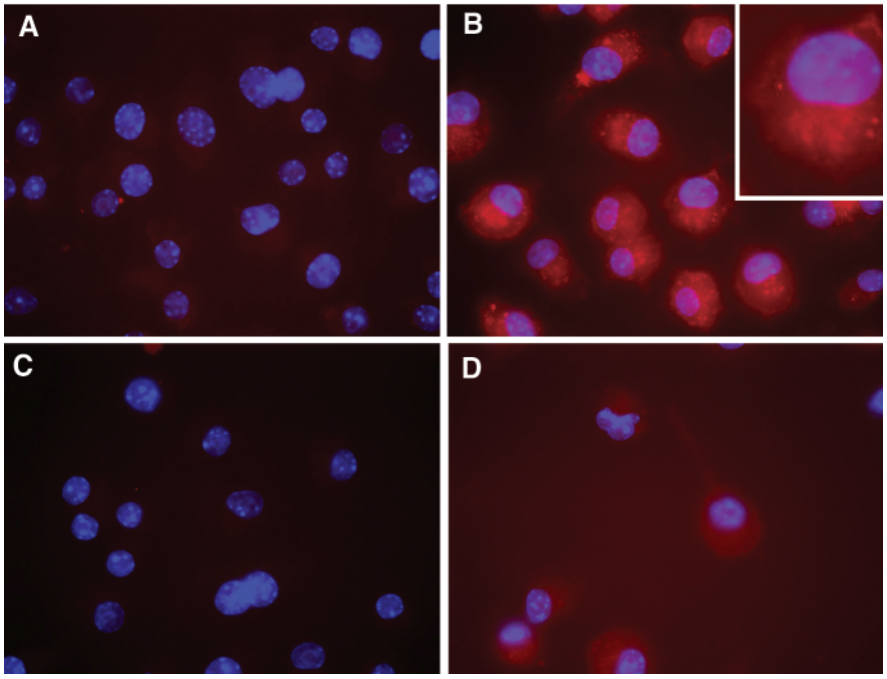

Fig. 2. Cell uptake of apoA-I at 37°C and 21°C. Rhodamine-labeled apoA-I was incubated with RAW264.7 cells for 1 h, and nuclei were stained with 4′,6-diamidino-2-phenylindole. A: Cells pretreated in the absence of 8Br-cAMP and incubated with labeled apoA-I at 37°C. B: Cells pretreated in the presence of 8Br-cAMP and incubated with labeled apoA-I at 37°C. The inset shows an enlargement of one cell demonstrating vesicular uptake of apoA-I. C: Cells pretreated in the absence of 8Br-cAMP and incubated with labeled apoA-I at 21°C. D: Cells pretreated in the presence of 8Br-cAMP and incubated with labeled apoA-I at 21°C.

We then examined the effects of glyburide and decavanadate on ABCA1-dependent FC and PL efflux to apoA-I. In contrast to the results from Fielding et al. (1), who used to demonstrate vascular smooth muscle cells that decavanadate inhibited FC efflux to a greater extent than PL efflux, we found that decavanadate decreased FC and PL efflux to apoA-I with approximately the same dose response (**Fig. 4A**), similar to what we observed for glyburide (Fig. 4B). Thus, we could not replicate in RAW264.7 cells the finding that FC and PL efflux could be uncoupled by the use of decavanadate. The data in Fig. 4A, B were calculated by subtracting FC or PL efflux in the absence of apoA-I from the efflux in the presence of apoA-I. We also noted that PL efflux from 8Br-cAMP-treated cells chased in the absence of apoA-I was significantly increased by either 1 mM decavanadate (1.33-fold) or glyburide (1.54-fold) (Fig. 4C). Because decavanadate had been used by Fielding et al. (1) to demonstrate a dissociation of FC and PL efflux, we examined the effect of decavanadate in RAW264.7 cells on LDH release as a measure of cellular integrity. We found that LDH release from 8Br-cAMP-pretreated RAW264.7 cells was increased from 1.5 ± 0.4% to 3.2 ± 0.2% of total cellular LDH content by treatment with 1 mM decavanadate (n = 6 ± SD, $P < 0.0001$). Thus, the increased PL release from decavanadate-treated ABCA1-expressing cells even in the absence of apoA-I might be attributable to cell damage and the release of cellular debris.

Increasing doses of methyl-β-cyclodextrin were assayed for their effects on FC and PL efflux during a 30 min chase period in cells pretreated with or without 8Br-cAMP. FC efflux to 1 mM methyl-β-cyclodextrin was increased compared with that in the absence of methyl-β-cyclodextrin and was 15% higher in cells pretreated with 8Br-cAMP, a result comparable to that observed in Fig. 1D (**Fig. 5A**). 8Br-cAMP treatment boosted FC efflux to 15 mM and 20 mM methyl-β-cyclodextrin, 18% and 48% ($P < 0.05$), respectively. In contrast, there was no appreciable PL efflux to 1 mM methyl-β-cyclodextrin (Fig. 5B). Fifteen and 20 mM methyl-β-cyclodextrin chases led to significant PL efflux, and this efflux was also boosted by 8Br-cAMP pretreatment, such that there was a 3-fold increase in PL efflux to 20 mM methyl-β-cyclodextrin ($P = 0.005$). The same cells were then evaluated for FC and PL efflux to apoA-I during a subsequent 4 h chase (Fig. 5C, D). 8Br-cAMP-induced FC efflux to apoA-I was reduced in a dose-dependent manner by methyl-β-cyclodextrin pretreatment (overall ANOVA, $P < 0.0001$). In the absence of 8Br-cAMP induction of ABCA1, the basal FC efflux was also reduced in a dose-dependent manner by methyl-β-cyclodextrin pretreatment, decreasing from 0.78% (control) to 0.28% after pretreatment with 20 mM methyl-β-cyclodextrin (Fig. 5C). 8Br-cAMP-induced PL efflux to apoA-I was also reduced by the methyl-β-cyclodextrin pretreatment (overall ANOVA, $P = 0.004$); however, this reduction was maximal at 15 mM methyl-β-cyclodextrin, and 20 mM methyl-β-cyclodextrin yielded a smaller reduction in PL efflux (Fig. 5D). In this experiment, 8Br-cAMP mediated 11.6- and 4.2-fold inductions of FC and PL efflux, respectively,

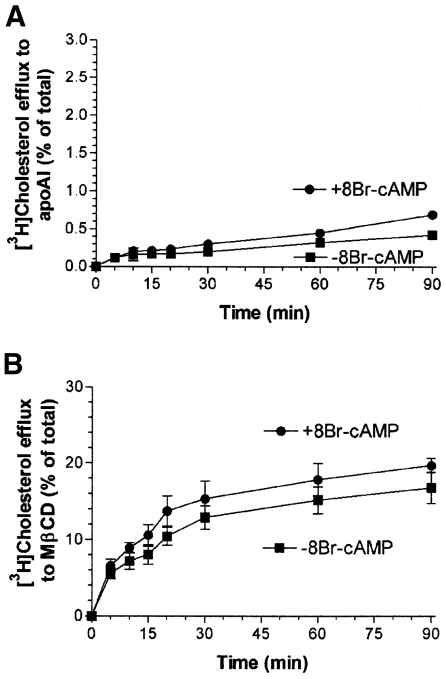

Fig. 3. Early time course of FC efflux to apoA-I and cyclodextrin at 21°C. A: FC efflux to 3 μg/ml apoA-I from cells pretreated in the presence (circles) or absence (squares) of 0.1 mM 8Br-cAMP to induce ABCA1 (n = 3). B: FC efflux to 10 mM methyl-β-cyclodextrin (MβCD) from cells pretreated in the presence (circles) or absence (squares) of 0.1 mM 8Br-cAMP (n = 3). Error bars are mean ± SD.

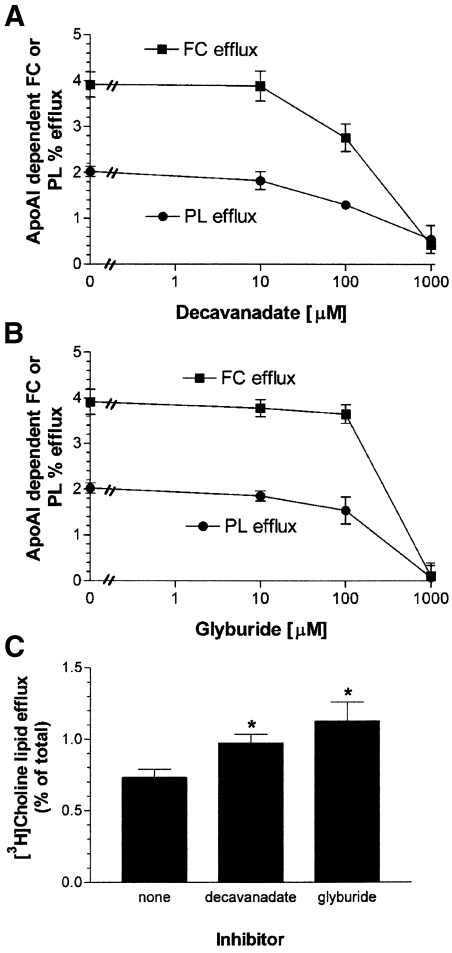

Fig. 4. Effects of decavanadate and glyburide on FC and PL efflux. A and B: FC (squares) and PL (circles) efflux over 4 h to 5 μg/ml apoA-I minus efflux in the absence of apoA-I from RAW264.7 cells pretreated with 0.3 mM 8Br-cAMP in the presence of increasing concentrations of decavanadate (A) or glyburide (B). C: Effect of 1 mM decavanadate or glyburide on PL efflux for 4 h in the absence of apoA-I from RAW264.7 cells pretreated with 0.3 mM 8Br-cAMP. * $P <$ 0.001 versus control by ANOVA (n = 3). Error bars are mean ± SD.

in the absence of methyl-β-cyclodextrin pretreatment. We then calculated the 8Br-cAMP-mediated fold induction of lipid efflux at each dose of methyl-β-cyclodextrin and plotted the percentage inhibition of FC and PL efflux compared with the control value (Fig. 5E). This plot demonstrates that 1 mM methyl-β-cyclodextrin inhibited FC and PL efflux to an equal extent, but at high doses, FC efflux was inhibited to a greater extent than was PL efflux. The inhibition of ABCA1-dependent efflux of PL was markedly reversed by the 20 mM methyl-β-cyclodextrin pretreatment, whereas the inhibition of efflux to FC was only moderately reversed at this dose (Fig. 5E).

We hypothesized that the apparent recovery of 8Br-cAMP-mediated PL efflux at high doses of methyl-β-cyclodextrin might be an artifact of ABCA1-expressing cells being more susceptible to cell damage by this treatment, resulting in the release of PL-rich cell debris. We tested the effect of a 30 min 15 mM methyl-β-cyclodextrin or control pretreatment on PL efflux during a subsequent 4 h chase period in the absence of the apoA-I acceptor and found that this methyl-β-cyclodextrin treatment increased PL efflux only in cells pretreated with 8Br-cAMP to induce ABCA1 (**Fig. 6A**; $P < 0.01$ compared with all other treatments by ANOVA posttest). Cell integrity was measured in a similar experiment by assaying LDH release. RAW264.7 cells were treated overnight in the presence or absence of 8Br-cAMP to induce ABCA1 and then for 30 min in the presence or absence of methyl-β-cyclodextrin to determine if these treatments led to the subsequent release of LDH during the next 24 h (Fig. 6B). Pretreatment with 8Br-cAMP alone had no effect on LDH release. The 30 min treatment with 20 mM methyl-β-cyclodextrin led to a 1.6-fold increase in LDH release versus untreated cells, but this difference was not significant. However, the combined 8Br-cAMP pretreatment and the 20 mM methyl-β-cyclodextrin treatment led to a 3.0-fold increase in LDH release compared with that in untreated cells, which was significantly different from all other conditions ($P < 0.001$ by ANOVA posttest). This assay provides direct evidence that the 8Br-cAMP-pretreated cells were significantly more susceptible to cellular damage caused by methyl-β-cyclodextrin treatment. We also examined these cells by transmission electron microscopy at 4 h after methyl-β-cyclodextrin treatment. Compared with un-

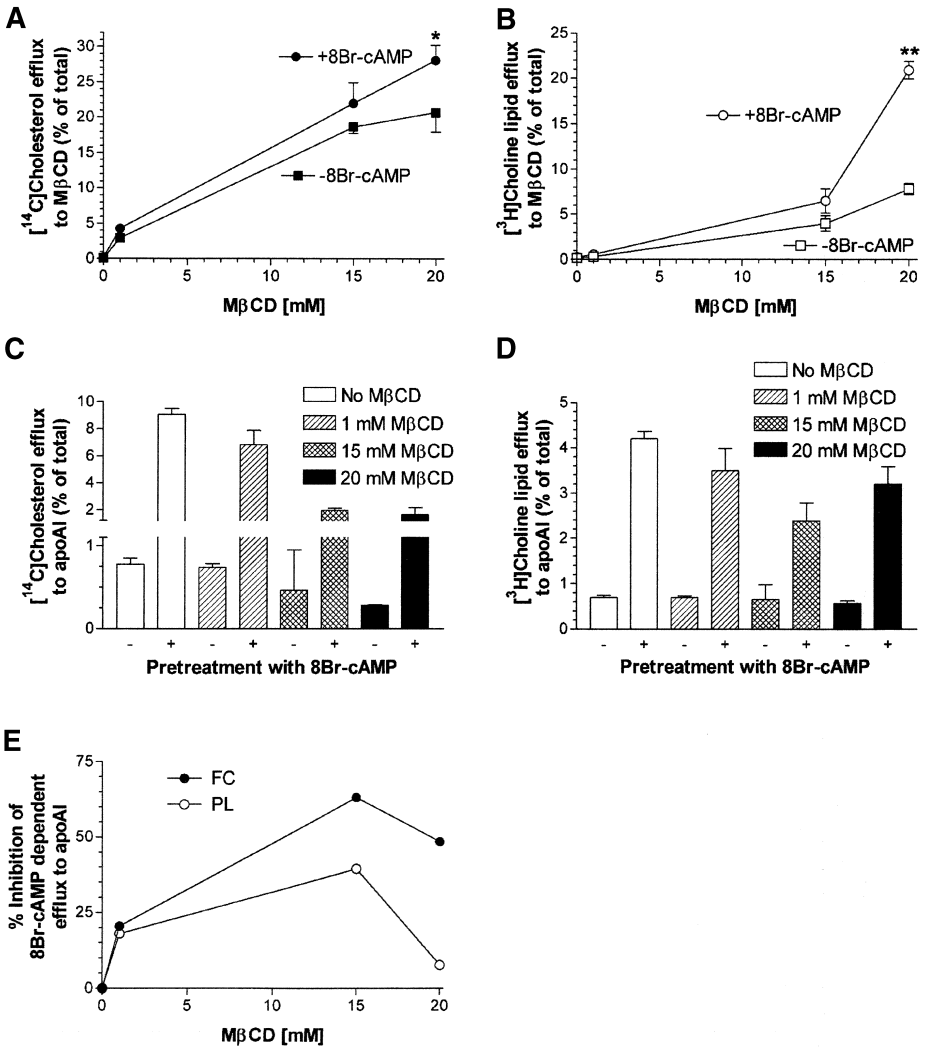

Fig. 5. Effect of increasing doses of methyl-β-cyclodextrin (MβCD) on FC and PL efflux to methyl-β-cyclodextrin or to apoA-I. A and B: FC and PL efflux, respectively, from RAW264.7 cells with or without 0.3 mM 8Br-cAMP pretreatment to varying doses of methyl-β-cyclodextrin during a 30 min chase period. C and D: FC and PL efflux, respectively, from the same cells after methyl-β-cyclodextrin treatment to 5 μg/ml apoA-I for 4 h. E: Percentage inhibition of FC (closed circles) and PL (open circles) efflux to apoA-I by increasing doses of methyl-β-cyclodextrin, calculated from the data in C and D by comparing the 8Br-cAMP-mediated fold effect on efflux to apoA-I at each dose of methyl-β-cyclodextrin compared with cells treated without methyl-β-cyclodextrin. For all panels, the cholesterol-loaded and double-labeled cells were treated on day 3 overnight in the presence (circles) or absence (squares) of 0.3 mM 8Br-cAMP, as described in Experimental Procedures. On day 4, the cells were chased for a 30 min period to assess efflux to varying doses of methyl-β-cyclodextrin. The medium was replaced and the cells were then chased for an additional 4 h with apoA-I (n = 3; * $P < 0.05$, ** $P = 0.005$ compared with cells treated in the absence of 8Br-cAMP). Error bars are mean ± SD.

treated cells, neither 0.1 mM 8Br-cAMP nor 20 mM methyl-β-cyclodextrin treatment alone had a noticeable effect on the cholesterol-loaded RAW264.7 cells (**Fig. 7A–C**). However, the cells treated with both 8Br-cAMP and methyl-β-cyclodextrin had evidence of extracellular debris that consisted of membranes and organelles (Fig. 7D, E). These experiments could not distinguish whether the LDH and organelle release occurred from dying cells or from cells that had transient interruptions of membrane integrity and subsequently survived.

Conditioned medium experiments were performed to determine whether ABCA1 could mediate the assembly of apoA-I particles that could act as acceptors on cells lacking ABCA1 expression. We first performed this experiment with ABCA1-inducible RAW264.7 cells, in which medium was conditioned on the unlabeled A plates and tested for efflux ability on the [^{3}H]cholesterol-labeled B plates. **Fig. 8A**, lane 1, shows the result of a positive control experiment in which apoA-I was conditioned on an untreated A plate and placed onto a B plate that had been pretreated with 8Br-cAMP to induce ABCA1, resulting in robust FC efflux. In the test conditions, the A plates were

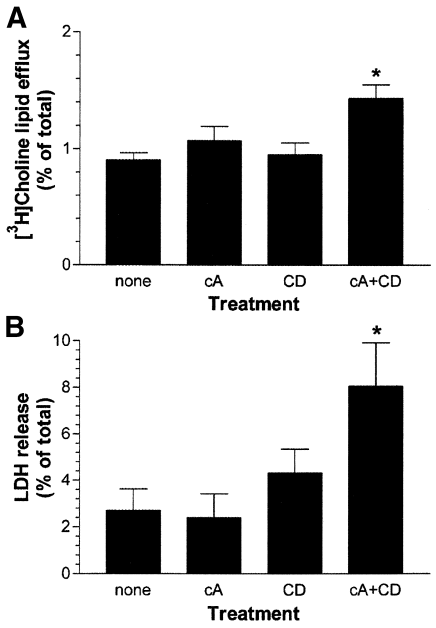

Fig. 6. Effect of methyl-β-cyclodextrin (CD) on PL efflux and lactate dehydrogenase (LDH) release in the absence of an acceptor. A: PL efflux over 4 h without apoA-I acceptor. Cholesterol-loaded cells were labeled with [^{3}H]choline and treated overnight in the presence or absence of 0.1 mM 8Br-cAMP (cA), as described in Experimental Procedures. On day 4, the cells were incubated for a 30 min pretreatment period with or without 15 mM methyl-β-cyclodextrin just before the 4 h efflux period (n = 3; * $P < 0.01$ versus all other treatments by ANOVA). B: Cells were pretreated in the presence or absence of 0.3 mM 8Br-cAMP and then exposed to 20 mM methyl-β-cyclodextrin or control, as described in Experimental Procedures. Cell damage was assessed by the release into the medium of LDH, as assayed as the percentage of total LDH activity in the cells plus medium (n = 6; * $P < 0.001$ versus all other treatments by ANOVA). Error bars are mean ± SD.

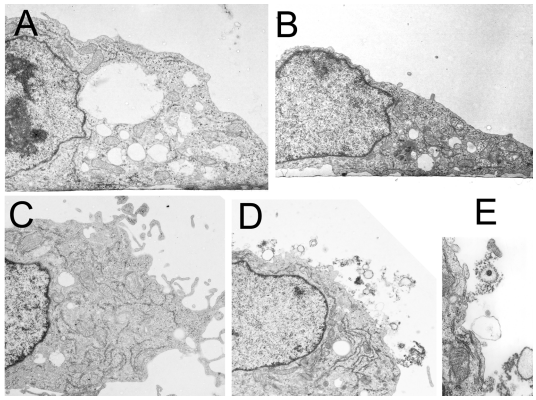

Fig. 7. Transmission electron micrographs of cell debris at 4 h after cells were pretreated sequentially with or without 8Br-cAMP and/or 20 mM methyl-β-cyclodextrin. A: Cell without pretreatment. B: Cell with 0.1 mM 8Br-cAMP pretreatment alone. C: Cell with methyl-β-cyclodextrin pretreatment alone. D and E: Cells pretreated with 8Br-cAMP and methyl-β-cyclodextrin. Magnifications are 15,000× (A–D) and 25,000× (E). These results are representative of two independent experiments in which six randomly chosen cells were photographed for each condition.

subjected to varying treatments and efflux was measured on the B plates that were not induced and thus lacked ABCA1 expression. No combination of ABCA1 induction by overnight 8Br-cAMP pretreatment, a 30 min 20 mM methyl-β-cyclodextrin pretreatment, or inclusion of apoA-I in the medium being conditioned on the A plates led to significant FC efflux on the B plate (Fig. 8A, lanes 2–9). We then performed a similar experiment using wild-type and ABCA1-GFP stably transfected HEK293 cells (Fig. 8B) (9). As a positive control, apoA-I-containing medium that was were conditioned on wild-type cells on an A plate led to robust FC efflux from ABCA1-GFP-expressing cells on the B plate (Fig. 8B, lane 1). However, no significant FC efflux was observed from the B plates when incubated with apoA-I-containing medium conditioned on A plates of wild-type or ABCA1-GFP cells, with or without 20 min pretreatment with 15 mM methyl-β-cyclodextrin (Fig. 8B, lanes 2–5). Thus, we were not able to detect ABCA1-independent cholesterol acceptor activity of the apoA-I-conditioned medium from ABCA1-expressing RAW264.7 or HEK293 cells, even when the cells were pretreated with methyl-β-cyclodextrin.

DISCUSSION

There is much debate about the mechanism of ABCA1-mediated lipid efflux to apolipoprotein acceptors. For example, protein cross-linking data directly support the notion that apolipoprotein ligands bind directly to ABCA1 (5, 13, 14), whereas other studies support the notion that ABCA1 alters the membrane PL and FC composition, allowing apoA-I to bind and absorb membrane lipids (3, 4, 15). It seems logical that ABCA1-mediated lipid efflux may occur at the plasma membrane; however, we previously proposed and provided evidence to support the hypothesis that ABCA1-mediated lipid efflux to apolipoproteins involves endocytosis of the apolipoprotein and subsequent resecretion of the nascent lipoprotein particle (9, 16). Finally, it has been proposed that ABCA1 may function in a two-step manner, with efflux of PL to the apolipoprotein acceptor occurring first, followed by the efflux of FC via a mechanism that can be ABCA1-independent (1, 2). The current series of experiments is discussed in light of some of these controversies.

We previously presented several types of data to support the hypothesis that endocytosis may be required for ABCA1-mediated lipid efflux (9, 16): *1*) ABCA1 can mediate the binding, uptake, and resecretion of apoA-I and apoE; *2*) apoA-I uptake by ABCA1-expressing cells occurs in coated pits; *3*) inhibitors of endocytosis reduce ABCA1-mediated lipid efflux; *4*) extracellular Ca^{2+} is required for apoA-I uptake and lipid efflux at 37°C but not for ABCA1-induced apoA-I binding at 4°C; and *5*) apoA-I taken up by ABCA1-expressing cells is colocalized with ABCA1 in intracellular vesicles. In addition, the laboratories of Yokoyama (17) and Tall (18) independently reported that intracellular pools of cholesterol are the preferred source for ABCA1-mediated FC efflux to apoA-I. Neufeld et al.

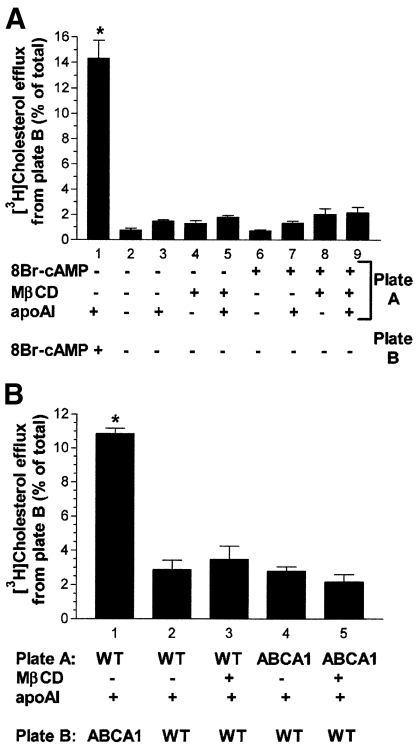

Fig. 8. FC efflux to conditioned medium. A: Using RAW264.7 cells, medium was conditioned by cells on the A plates that were pretreated, as displayed, in the presence or absence of 0.1 mM 8Br-cAMP for 16 h and then treated for 30 min in the presence or absence of 20 mM methyl-β-cyclodextrin (MβCD). The A plate cells were washed twice, and conditioned medium was prepared by incubation for 24 h with DMEM supplemented with 20 mM glucose, 2 mM glutamine, and 0.2% BSA (DGGB) in the presence or absence of 5 μg/ml apoA-I and 8Br-cAMP as indicated. FC efflux into this conditioned medium was assayed for 4 h on the cells on the B plates that had been cholesterol loaded and labeled, and in lane 1 previously treated overnight with 0.1 mM 8Br-cAMP to serve as a positive control (n = 3; * $P < 0.001$ versus all other conditions by ANOVA). B: Wild-type (WT) or ABCA1-green fluorescent protein (GFP) stably transfected (ABCA1) HEK293 cells were used on the A plates, which were pretreated for 20 min in the presence or absence of 15 mM methyl-β-cyclodextrin as indicated. The plates were washed twice, and conditioned medium was prepared by incubation for 24 h with DGGB containing 5 μg/ml apoA-I. FC efflux into this conditioned medium was assayed for 4 h on the cells on the B plates of stably transfected ABCA1-GFP cells (lane 1) or wild-type HEK293 cells (lanes 2–5) (n = 4; * $P < 0.001$ versus all other conditions by ANOVA). Error bars are mean ± SD.

(19) demonstrated bidirectional transport of ABCA1 between the plasma membrane and intracellular vesicles, evidence that is consistent with the endocytosis hypothesis. Our current data add further support to the hypothesis of the endocytic mechanism of ABCA1-mediated lipid efflux. We found a 15 min time lag before any ABCA1-mediated FC or PL efflux could be observed (Fig. 1), consistent with the previously observed time that is required for one round of transferrin endocytosis and resecretion (20). We also found that lipid efflux to apoA-I through this pathway was abolished at room temperature (21°C), which simultaneously interfered with the cellular uptake of apoA-I (Figs. 2 and 3). In contrast, FC efflux to methyl-β-cyclodextrin was both rapid, fastest in the initial 5 min (Fig. 1), and robust at room temperature (21°C) (Fig. 3), implying that the direct stripping of plasma membrane FC can occur at a temperature that does not support endocytosis and ABCA1-mediated lipid efflux. We speculate that endocytosis may be required in the ABCA1 lipid efflux pathway to allow the nascent lipoprotein to dissociate from ABCA1 and cell membranes, analogous to the acidification-induced release of iron from transferrin during its endocytic recycling. Gillotte-Taylor et al. (7) examined the initial time course of FC and PL efflux from FC-loaded and control fibroblasts to 50 μg/ml apoA-I (10-fold higher than the highest level used in our study) and observed an increased efflux from the FC-loaded cells by the first time point at 3 min. Although FC loading of these cells was shown to increase ABCA1 mRNA levels by ~12-fold, it is not clear what other effects the FC loading had.

There are several lines of evidence that ABCA1 may act primarily as a PL transporter, with FC following in an ABCA1-dependent manner, thus resulting in a two-step mechanism for FC efflux. Fielding et al. (1) reported that FC efflux from endothelial cells to apoA-I is more sensitive to vanadate inhibition. They also performed conditioned medium experiments showing robust FC efflux from endothelial cells that do not make ABCA1 when incubated with apoA-I that had been conditioned on ABCA1-expressing smooth muscle cells. Wang et al. (2) used high-dose cyclodextrin treatment (20 mM) and found that ABCA1-transfected HEK293 cells subsequently released PL but not FC to apoA-I. Medium conditioned in this way then led to effective FC efflux in HEK293 cells lacking ABCA1 expression, also supporting the two-step hypothesis (2).

During our early time course study, we observed concurrent FC and PL efflux from ABCA1-expressing cells after the 15 min lag (Fig. 1). This finding could be used to support the notion that ABCA1 can mediate the transfer of both of these lipids to apoA-I; however, these data cannot rule out the possibility that ABCA1 mediates the efflux of PL only, which is followed by a rapid (seconds rather than minutes) association of FC in an ABCA1-dependent or independent manner. Also, in contrast to the findings of Fielding et al. (1) in endothelial cells, we found a parallel reduction of ABCA1-mediated FC and PL efflux from RAW264.7 cells by the inhibitors glyburide and decavanadate (Fig. 4). These inhibitors also increased PL efflux from ABCA1-induced cells in the absence of apoA-I acceptor. This nonspecific efflux of PL could be attributable to cell damage, as we observed an increase in PL release and LDH release from decavanadate-treated, ABCA1-expressing RAW264.7 cells, even in the absence of the apoA-I acceptor. If a similar phenomenon occurred in smooth muscle cells, it might account in part for the observations of Fielding et al. (1) that decavanadate inhibited FC efflux to a greater extent than PL efflux.

We did not observe any significant level of FC efflux when apoA-I-containing conditioned medium from ABCA1-expressing RAW264.7 cells or ABCA1-GFP-expressing HEK293

cells, regardless of methyl-β-cyclodextrin pretreatment, were incubated with cells that did not express ABCA1 (Fig. 8). Thus, the nascent HDL assembled from apoA-I and cell lipids via ABCA1 did not have the capacity to accept more FC in an ABCA1-independent manner from either RAW264.7 or HEK293 cells. Our conditioned medium findings do not agree with those of Wang et al. (2), who reported that a 20 mM cyclodextrin pretreatment of the ABCA1-transfected HEK293 cells before conditioning of apoA-I-containing medium led to the generation of PL-rich and FC-poor conditioned medium that could accept FC in an ABCA1-independent manner. Recently, Wang et al. (21) reported that they could not reproduce their original conditioned medium findings and speculated that the previously reported effects of the conditioned medium were attributable to carryover of residual cyclodextrin from the treated cells. Vaughan and Oram (15) performed a similar conditioned medium experiment using transfected BHK cells treated with a high dose of cyclodextrin and found no cholesterol-accepting activity of the apoA-I-containing conditioned medium when tested on BHK cells that do not express ABCA1, in agreement with the results in the current study and the recent results reported by Wang et al. (21). The discovery that the related ABC transporter ABCA7 promotes robust PL efflux to apoA-I, without much FC efflux, also seems to cast doubt on the two-step model of PL and FC efflux mediated by ABCA1 (21). Thus, ABCA1 apparently mediates the simultaneous assembly of both FC and PL onto apolipoprotein acceptors. However, we see no reason why ABCA1-derived nascent HDL could not have additional capacity to accept FC via SRB1 (22), and this activity may explain the results from Fielding et al. (1), who found that apoA-I-containing smooth muscle-conditioned medium evoked FC efflux from endothelial cells.

We performed additional experiments to examine more closely the lipid efflux effects of methyl-β-cyclodextrin, which is widely believed to specifically remove FC from cells. In fact, we found that a 30 min treatment with 15 or 20 mM methyl-β-cyclodextrin led to significant release of both FC and PL, whereas the 1 mM methyl-β-cyclodextrin treatment was specific for FC release (Fig. 5A, B). The 20 mM methyl-β-cyclodextrin treatment removed more FC and particularly PL from ABCA1-expressing cells versus cells without ABCA1 expression. We suspected that this dramatic increase in PL efflux from ABCA1-expressing cells might be attributable to cell damage, and we confirmed this by observing ABCA1- and cyclodextrin-dependent increases in the release of the cytoplasmic protein LDH (Fig. 6) and membranous cell debris (Fig. 7). Thus, our data support the hypothesis that ABCA1 induction alters RAW264.7 cells so that they are more sensitive to the adverse effects of high-dose methyl-β-cyclodextrin treatment; thus, caution should be used in the interpretation of experiments that rely on high doses of cyclodextrin.

The methyl-β-cyclodextrin experiments are also relevant to the nature of ABCA1-mediated changes in the plasma membrane. Chimini's group (3, 4) initially reported, and we (9) have confirmed, that ABCA1-expressing cells have increased phosphatidylserine (PS) on their surface, as measured by annexin V binding, although we (9) demonstrated that increased cell surface PS alone could not account for the ABCA1-mediated effects on cellular binding of apoA-I and lipid efflux to apoA-I. On the other hand, Vaughan and Oram (15) have demonstrated that overexpression of ABCA1 in transfected cells, to a level ~10-fold greater than that observed in cAMP-treated murine macrophage cell lines, is associated with an increase in the FC content of a lipid pool accessible to treatment with cholesterol oxidase and a lipid pool that serves as a substrate for ACAT. These observations are consistent with the conclusion that ABCA1 expression alters either the cellular distribution of cholesterol (i.e., increased FC in the plasma membrane) or its flux through cellular pools (15). Furthermore, these cholesterol pools are depleted by ABCA1-mediated lipid efflux to apoA-I (15). The cholesterol oxidase result implies that ABCA1 increases plasma membrane FC, but it is difficult to reconcile this observation with previous results that demonstrate that ABCA1 preferentially promotes the efflux of intracellular rather than plasma membrane FC (17, 18). In the current study, we found that induction of endogenous ABCA1 in RAW264.7 cells had no effect on the initial 5 min efflux of FC to 1 mM methyl-β-cyclodextrin (Fig. 1). We believe that the initial FC efflux to a low dose of cyclodextrin is a good indicator of plasma membrane FC content; thus, we think that ABCA1-mediated lipid efflux to apoA-I is not dependent on an increase in plasma membrane FC. At later time points, we observed increased FC efflux to cyclodextrin, which could be attributable to increased mobilization of cellular FC via increased vesicular trafficking. The work of Zha and colleagues (23, 24) has validated this notion by determining that ABCA1 alters both endocytic and secretory vesicular trafficking.

Several groups (5, 13, 14) have shown that apoA-I can be specifically cross-linked to ABCA1, supporting the notion of a receptor-ligand interaction between ABCA1 and apoA-I. However, if this is a ligand-receptor interaction, it has low specificity, as ABCA1 can use a variety of exchangeable amphipathic apolipoproteins as acceptors as well as specific synthetic amphipathic peptides (25, 26). In addition, Remaley et al. (25) demonstrated that synthetic amphipathic peptides made from D-isomer amino acids also appear to act as ABCA1-mediated lipid acceptors. This finding is difficult, but not impossible, to reconcile with a receptor-ligand interaction; perhaps the pattern presented by an amphipathic α-helix is sufficient for receptor binding. The current study may offer an alternative explanation for the observed lipid-acceptor activities of synthetic amphipathic peptides. The L and D synthetic peptides that promoted cholesterol efflux in an ABCA1-dependent manner also have significant detergent-like activity and promote robust PL and modest FC release even in cells lacking ABCA1 expression (25). We speculate that cells expressing ABCA1 bear an altered plasma membrane composition, which may sensitize them to the detergent-like activity of the L and D synthetic peptides. The effects observed with high doses of cyclodextrin in ABCA1-express-

ing cells may represent an analogous mechanism whereby high levels of PL are released into the medium along with the release of LDH and cell debris.

We demonstrate here that ABCA1-mediated efflux of FC and PL from RAW264.7 cells to apoA-I is concurrent and that the time course and temperature dependence of FC and PL efflux are consistent with a role for endocytosis in this process. Methyl-β-cyclodextrin, which can absorb FC from cells, has been used as a tool to study cholesterol pools as well as the effects of depleting cells of FC. Our results show that methyl-β-cyclodextrin-mediated lipid efflux from cells differs greatly in its time course and temperature dependence from ABCA1-mediated lipid efflux from apoA-I. Furthermore, we found that high doses of methyl-β-cyclodextrin also lead to PL efflux, and when given to ABCA1-expressing cells, methyl-β-cyclodextrin could lead to increased cell damage. Thus, studies relying on high-dose cyclodextrin treatment should be examined for these potential adverse effects.

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