# Efflux of Cholesterol from Different Cellular Pools†

M. Page Haynes, Michael C. Phillips, and George H. Rothblat*

*Department of Biochemistry, MCP−Hahnemann University, Philadelphia, Pennsylvania 19129*

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ABSTRACT: Free cholesterol is very efficiently removed from cells by 2-hydroxypropyl-$\beta$-cyclodextrins. The efflux of cholesterol occurs from two distinct kinetic pools: the half-times ($t_{1/2}$) for the two pools in CHO-K1 cells are 15 ± 5 s and 21 ± 6 min and they represent 25% ± 5% and 75% ± 5% of the readily exchangeable cell cholesterol, respectively. In this study we have determined that the fast pool and the majority of the slow kinetic pool for cholesterol efflux are apparently present in the plasma membrane. Numerous agents that inhibit intracellular cholesterol trafficking are unable to affect either the size or the $t_{1/2}$ for efflux of either kinetic pool. In contrast, treatment of the cells with *N*-ethylmaleimide (NEM), exogenous lipases such as sphingomyelinase and phospholipase C, calcium ionophore A23187, or heat resulted in the dramatic increase in the size of the fast kinetic pool of cholesterol. These changes in the kinetics of cholesterol efflux are not specific to the nature of the extracellular acceptor indicating that they are a consequence of changes in the cell plasma membrane. The above treatments disrupt the normal organization of the lipids in the plasma membrane via either hydrolysis or randomization. The phosphatidylcholine and sphingomyelin present in the plasma membrane are critical for maintaining the two kinetic pools of cholesterol; any alteration in the amount or the location of these phospholipids results in an enhancement of efflux by redistributing cholesterol into the fast kinetic pool.

The first step in reverse cholesterol transport is the efflux of free cholesterol from the plasma membrane of peripheral cells to an extracellular acceptor (*1*). This movement of cholesterol is governed by both cellular and extracellular factors. Many studies have focused on the extracellular acceptors of cellular cholesterol, specifically how modifications of these acceptors can positively enhance cholesterol efflux (see ref *1* for review). It is generally believed that reverse cholesterol transport is mediated by high-density lipoprotein (HDL).[1] With phospholipid-containing acceptors such as HDL, the rate-limiting step is the desorption of cholesterol molecules from the plasma membrane. The present studies have utilized cyclic oligosaccharides termed $\beta$-cyclodextrins (CD), which are much more efficient acceptors of cell cholesterol than HDL (*1, 2*). By use of these very efficient cholesterol acceptors it is possible to rapidly access the majority of the cellular cholesterol and begin to define cellular factors that regulate the efflux of cholesterol.

Cellular cholesterol has been shown to exist in various cellular pools both in the plasma membrane and in intracellular membranes (see ref *3* for review). Intracellular membranes are relatively cholesterol-poor when compared to the plasma membrane (*4*−*9*). Cholesterol continuously moves through the cell from intracellular organelles to the plasma membrane. The plasma membrane contains cholesterol domains in the inner and outer leaflet and laterally separated domains such as caveolae. These domains have been defined on the basis of numerous criteria such as their cholesterol content, the phospholipids and proteins associated with the cholesterol, their resistance to detergent, the physical state of the lipids in the domain, and the kinetic parameters of cholesterol efflux (*1, 2, 4, 10*−*13*). The organization of cholesterol in the plasma membrane affects the flux of cholesterol between cells and extracellular acceptors. Previous studies from this laboratory have established that the release of cellular cholesterol to very efficient CD acceptors occurs from two clearly distinct kinetic pools (*2*). The aim of this study was to further examine the cellular location of these pools of cholesterol and to begin to define the factors that influence the sizes of the pools and the rate of release of cholesterol from them. This was accomplished by treating cells with various agents known to affect intracellular lipid trafficking and membrane lipid organization and monitoring their effects on the two kinetic pools of cholesterol.

## EXPERIMENTAL PROCEDURES

*Materials.* Tissue culture flasks and plates were products of Corning (Corning, NY) or Falcon (Lincoln, NJ). Eagle's minimal essential medium (EMEM), Dulbecco's minimal essential medium (DMEM), Hams F12 medium, and phosphate-buffered saline (PBS) were purchased from Bio-Whittaker. [1,2-$^3$H]Cholesterol (51 Ci/mmol), [2,8-$^3$H]-adenine (28.8 Ci/mmol) and [*methyl*-$^3$H]choline chloride (60 Ci/mmol) were obtained from Dupont NEN (Boston, MA). 2-Hydroxypropyl-$\beta$-cyclodextrin (2OHp$\beta$CD) was a generous gift from Cerestar USA, Inc. (Hammond, IN). Heat-

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* Corresponding author: The Children's Hospital of Philadelphia, Abramson Building, Rm. 302B, 3516 Civic Center Blvd., Philadelphia, PA 19104-4318. Tel. 215-590-0585; Fax 215-590-0583; E-mail rothblat@email.chop.edu.

[1] Abbreviations: 2OHp$\beta$CD, 2-hydroxypropyl-$\beta$-cyclodextrin; CHO-K1, Chinese hamster ovary cells; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; PC-PLC, phosphatidylcholine-specific phospholipase C; NEM, *N*-ethylmaleimide.

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inactivated fetal bovine serum (FBS), gentamicin, cholesterol, trypsin, sphingomyelinase (from *Staphylococcus aureus*), phosphatidylcholine-specific phospholipase C (from *Bacillus cereus*, type IV), *N*-ethylmaleimide, bafilomycin $A_1$ (from *Streptomyces griseus*), cytochalasin D, sodium azide, iodoacetic acid (sodium salt), iodoacetamide, 2-deoxyglucose, potassium cyanide, sodium fluoride, brefeldin A, dithiothreitol, calcium ionophore A23187, and the bioluminescent somatic cell assay kit were all purchased from Sigma (St. Louis, MO). All organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Pierce (Rockford, IL). U18666A was a gift from the Upjohn Corp. (Kalamazoo, MI). The acyl-coenzyme A:cholesterol acyl transferase (ACAT) inhibitor CP-113,818 was a generous gift from Pfizer Pharmaceuticals (Groton, CT).

*Cell Culture.* Chinese hamster ovary cells (CHO-K1) were cultured in Hams F12 medium containing 7.5% FBS. All other cells (GM3468, FU5AH, W138VA13) were cultured in EMEM containing 10% FBS. All medium were buffered with sodium bicarbonate, supplemented with 50 $\mu$g of gentamicin/mL, and incubated at 37 °C in a humidified incubator with 5% $CO_2$.

*Radiolabeling of Cell Monolayers.* For labeling of cellular free cholesterol pools, cells were plated in either 12-well tissue culture plates or 100 mm Petri dishes and labeled with 1−2 $\mu$Ci/mL [$^3$H]cholesterol (for efflux to 2OHp$\beta$CD acceptors) or 6 $\mu$Ci/mL [$^3$H]cholesterol (for efflux to $HDL_3$ acceptors) or 1 $\mu$Ci/mL [$^{14}$C]cholesterol (for dual labeling experiments) and 3−5% FBS for 18−24 h. The radioactive cholesterol was dissolved in ethanol and all medium containing radioactive lipids had less than 0.1% final ethanol concentration. After radiolabeling, the cells were equilibrated with EMEM buffered with 50 mM HEPES (EMEM/HEPES) for 2 h. The Pfizer ACAT inhibitor CP-113,818 (2 $\mu$g/mL, <0.1% dimethyl sulfoxide final concentration) was included in both the labeling and equilibration medium. Cells were labeled to equilibrium so that all cholesterol pools contained [$^3$H]cholesterol. For analysis of cell toxicity, cells were plated in either 12-well tissue culture plates or 100 mm Petri dishes and labeled with 1−2 $\mu$Ci/mL [$^3$H]adenine and 7.5% FBS for 2−3 h; the cells were then washed 3 times with EMEM/HEPES and equilibrated in EMEM/HEPES for 10 min. For the incorporation of [*methyl*-$^3$H]choline chloride into cellular phospholipids, monolayers of cells were incubated with 3 $\mu$Ci/mL of [*methyl*-$^3$H]choline chloride in Hams F12 medium supplemented with 3% FBS and 50 $\mu$g/mL gentamicin for 18 h; the cells were then washed three times and equilibrated with EMEM/HEPES for 2 h.

*Preparation of Cyclodextrin Solutions and Cyclodextrin−Cholesterol Complexes.* Uncomplexed (cholesterol-free) 50 mM 2OHp$\beta$CD solutions were readily solubilized in EMEM/HEPES. CD:cholesterol solutions were prepared as previously described (*14*). Briefly, the appropriate amount of cholesterol from a stock solution prepared in chloroform−methanol (1:1) was dried onto the walls of a glass container. The appropriate mole ratio for 100% saturated solution for this lot of 2OHp$\beta$CD (G8120) was empirically determined to be 40:1 CD:cholesterol as previously described (*14*) An appropriate volume of 50 mM 2OHp$\beta$CD solution prepared in EMEM/HEPES was then added. The sample was sonicated briefly in a bath sonicator and incubated overnight in a shaking water bath. Immediately before use, the cholesterol-saturated 2OHp$\beta$CD solution was filtered through a 0.45 $\mu$m filter (Millipore) to remove any uncomplexed cholesterol. This solution was considered to be 100% saturated with cholesterol. The saturated 2OHp$\beta$CD solution was then diluted with 50 mM 2OHp$\beta$CD free of any exogenous cholesterol to yield a 50% saturated cholesterol:CD solution. Pfizer ACAT inhibitor was added at a concentration of 2 $\mu$g/mL to the 50% saturated 50 mM cholesterol:2OHp$\beta$CD solution.

*Efflux of Labeled Cholesterol from Cells in Suspension.* Cell monolayers were plated in 100 mm Petri dishes and radiolabeled with [$^3$H]cholesterol to equilibrium as described above. The cell monolayers were washed 3 times with PBS and incubated at 37 °C with 2 mL of trypsin (900 BAEE units/mL) for 3−5 min. Then, 10 mL of EMEM/HEPES was added. The suspended cells were harvested by brief centrifugation (1000 rpm for 5 min), reuspended in 50-fold excess of EMEM/HEPES, and washed three times with a 50-fold excess of EMEM/HEPES, to remove any residual trypsin. Cells were then resuspended in EMEM/HEPES to an approximate cell density of $4 \times 10^6$ cells/mL (cell concentrate). Three milliliters of acceptor medium was added to a 7 mL glass scintillation vial in a shaking water bath at the indicated temperature. At the beginning of the efflux phase, 600 $\mu$L of the cell concentrate was added to the efflux medium. Aliquots of the cells and the acceptor medium were removed at different time points and filtered through a 0.45 $\mu$m Multiscreen (96 screen) filtration plate (Millipore Corp.) to remove the cells. Aliquots of the filtrate were then taken for analysis by liquid scintillation counting of the radioactivity released into the acceptor medium. Aliquots of the whole cell suspension (cells and acceptor medium) were counted by liquid scintillation to determine the total amount of radioactivity in each assay. For experiments in which various agents were tested for their effect on the efflux of radiolabeled cholesterol, these agents were incubated with the suspension cell concentrate for the indicated time periods prior to the addition of the cells to the efflux medium. The acceptor medium was either 50 mM 2OHp$\beta$CD solution that was 50% saturated with cholesterol (prepared as described previously) or human $HDL_3$. This percent of saturation of the 2OHp$\beta$CD solution has been previously determined to promote rapid exchange of cholesterol without altering the amount of cholesterol mass present in the cell (*2, 14*). Thus, in the efflux experiments only the exchange of labeled cholesterol with no net depletion of cell cholesterol mass was monitored. Human $HDL_3$ (1.125 g/mL < $d$ < 1.21 g/mL) was separated from plasma by ultracentrifugation (*15*) and was dialyzed against 0.01 M Tris/0.15 M sodium chloride buffer before use. The $HDL_3$ acceptor medium was used at a concentration of 1.3 mg of protein/mL as determined by a modified Lowry method (*16*).

The extent of cholesterol efflux was expressed as the fraction of cholesterol remaining in the cells as a function of time. The large excess of acceptors present ensured that the efflux of radiolabeled cholesterol was effectively unidirectional. The time course of release of labeled cholesterol was fit by nonlinear regression to a biexponential decay equation describing simultaneous release of cholesterol from two independent pools. The equation utilized was $Y = Ae^{-k_1 t} + Be^{-k_2 t}$, where $Y$ is the fraction of radiolabeled cholesterol

remaining in the cell at time *t*, *A* is the size of the cellular cholesterol pool 1, and *B* is the size of the cellular cholesterol pool 2. $k_1$ and $k_2$ are the apparent rate constants for the release of cholesterol from pools 1 and 2, respectively. The apparent half-times of cholesterol efflux were calculated as $t_{1/2} = \ln 2/k$ (*2, 17*). In this analysis any movement of cholesterol between pools 1 and 2 was not considered since for this process $k \ll k_1$; $t_{1/2}$ for efflux of cholesterol from the fast pool was ≈15 s, whereas $t_{1/2}$ for regeneration of the fast pool from the slow pool was ≈20 min (*2*).

*Depletion and Replenishment of the Fast Efflux Pool of Cholesterol.* Cells were placed in suspension as described previously. The cell concentrate was briefly exposed to the 50 mM 2OHp$\beta$CD efflux solution that was 50% saturated with cholesterol for 2 min at 37 °C. The cells were quickly pelleted by centrifugation at 4000 rpm for 2 min in a microfuge. The CD solution was removed leaving cells depleted of the fast pool of cholesterol. The cells depleted of the fast pool of cholesterol were then resuspended in EMEM/HEPES and either immediately reexposed to fresh 2OHp$\beta$CD efflux medium or allowed to incubate in EMEM/HEPES for various time periods at 37 °C to allow for regeneration of the fast pool of cholesterol. At the end of each time period the cells were then reexposed to fresh 2OHp$\beta$CD efflux medium to assess the amount of fast pool regeneration. For the experiments in which various agents were tested for their effect on the regeneration of the fast pool of cholesterol, these agents were added during the regeneration period in EMEM/HEPES. Pools sizes and kinetic constants were determined as previously described.

*Analysis of Cellular Toxicity and Cellular ATP Levels.* Leakage of [$^3$H]adenine was utilized as a measure of cell membrane integrity and of cellular toxicity (*18, 19*). Cell monolayers were radiolabeled with [$^3$H]adenine as described previously. The cells were placed in suspension as described previously (*20*) and incubated with various agents. Aliquots of the cells and the medium were removed at different time points and filtered through a 0.45 $\mu$m Multiscreen filtration plate (Millipore Corp.) to remove the cells. Aliquots of the filtrate were then taken for analysis of the radioactivity released into the medium by liquid scintillation counting. Aliquots of the whole cell suspension were counted by liquid scintillation to determine the total amount of radioactivity in each assay. The amount of [$^3$H]adenine released was expressed as the percentage of [$^3$H]adenine released after incubation with a particular agent relative to the percentage of [$^3$H]adenine released from untreated control cells.

The bioluminescent somatic cell assay kit (Sigma, St. Louis, MO) measured the amount of cellular ATP present after incubation with various agents. Cells grown to confluence in 100 mm Petri dishes were placed in suspension as previously described. Cells were aliquoted to 1.5 mL tubes at a cell density of $4 \times 10^6$ cells/mL and incubated with various agents for 40 min at 37 °C. The cells were then pelleted by centrifugation (14000*g* for 1 min at 4 °C) and resuspended in 1 mL of sterile Milli-Q water. Assay reagents were prepared according to the manufacturer's specifications. Aliquots of the ATP assay mix were added to the reaction vials and allowed to stand for 3 min at room temperature. Cellular ATP was released from the cells by the addition of the somatic cell ATP releasing agent. The lysed cell sample was then added to the ATP assay mix and the light emitted was immediately measured in a luminometer. The ATP assay mix was also added to the appropriate amount of an ATP standard. The light emitted from the assay mix was directly proportional to the amount of ATP present in the cell suspension. The actual amount of ATP present in each sample was determined from a standard curve.

*Preparation of Plasma Membrane Vesicles.* Cell monolayers were radiolabeled with [$^3$H]cholesterol and plasma membrane vesicles were prepared as previously described with the exception of the final step (*21*). Briefly, the cell monolayers were washed three times with PBS and then exposed to a 50 mM formaldehyde 2 mM dithiothreitol PBS solution, pH 7.6, for 90 min at 37 °C. After this incubation period the buffer containing some plasma membrane vesicles was collected. The cell monolayers were then exposed to a hypertonic PBS solution (0.44 M NaCl, pH 7.6) for 2−3 min to remove the remaining plasma membrane vesicles. The hypertonic buffer was collected and pooled with the previous buffer. The plasma membrane vesicles were collected by centrifugation (3500*g* for 30 min) and resuspended in 1 mL of PBS.

*Efflux of [$^3$H]Cholesterol from Plasma Membrane Vesicles.* Three milliliters of 2OHp$\beta$CD efflux medium 50% saturated with cholesterol was added to a 7 mL glass scintillation vial in a shaking water bath at the indicated temperature. At the beginning of the efflux phase, 500 $\mu$L of the plasma membrane vesicle preparation was added to the efflux medium. Aliquots of the plasma membrane vesicle suspension and the acceptor medium were removed at different time points and filtered through a 0.22 $\mu$m Multiscreen filtration plate (Millipore Corp.) to remove the plasma membrane vesicles. Aliquots of the filtrate were then taken for analysis of the radioactivity released into the acceptor medium by liquid scintillation counting. The percent of radioactivity leakage through the filter was less than 6%. Pools sizes and kinetic constants were determined as previously described.

## RESULTS

*Kinetic Pools of Cellular Cholesterol.* The time course of cholesterol efflux from CHO-K1 cells in suspension was biexponential (Figure 1). This clearly indicates two kinetic pools of cellular cholesterol (fast and slow) when 2OHp$\beta$CD was used as an acceptor. In these experiments, radiolabeled cholesterol was released to a 50 mM 2OHp$\beta$CD solution that was 50% saturated with cholesterol so that we were able to monitor the release of radiolabeled cholesterol without changing the net cholesterol mass of the cells (data not shown). In these experiments, the majority of the labeled cholesterol was readily removed in the assay time frame, although 5−10% of the total labeled cell cholesterol was found in a very slowly exchangeable pool. The kinetic pool sizes and half-times were calculated on the basis of the readily exchangeable cholesterol present in the cell. In general, the half-times of efflux ($t_{1/2}$) for the fast and slow pools in CHO-K1 cells are 15 ± 5 s and 21 ± 6 min, and they represent 25% ± 5% and 75% ± 5% of cell cholesterol, respectively (Figure 1). In previous studies and this study, the size of the fast pool of cholesterol has been shown to vary among cell lines, while the $t_{1/2}$ for release of cholesterol from the fast pool does not vary (*2*). For example, the size of the fast pool of cholesterol in both normal human skin

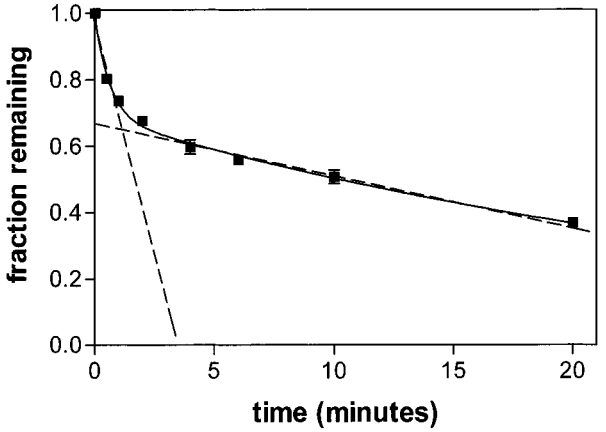


FIGURE 1: Time course of the efflux of [$^3$H]cholesterol from CHO-K1 cells in suspension. Monolayers of CHO-K1 cells were radiolabeled, equilibrated, and placed in suspension as described in Experimental Procedures. At 37 °C cells in suspension were added to 50 mM 2OHp$\beta$CD efflux medium that was 50% saturated with cholesterol. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means ± SD for triplicate determinations. The efflux values at each time point were fit to a biexponential decay equation as described in Experimental Procedures.

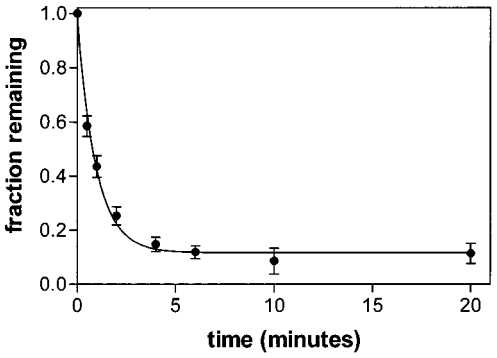


FIGURE 2: Efflux of [$^3$H]cholesterol from isolated plasma membrane vesicles. Plasma membrane vesicles were labeled and isolated from CHO-K1 cells as described in Experimental Procedures. The isolated plasma membrane vesicles were then added to 50 mM 2OHp$\beta$CD efflux medium that was 50% saturated with cholesterol and time points were taken at 37 °C. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means ± SD for triplicate determinations.

fibroblasts (GM3468) and CHO-K1 cells is approximately 25%, with a $t_{1/2}$ of about 15s. The sizes of the fast pool of cholesterol in FU5AH rat hepatoma cells and W138VA13 transformed human skin fibroblasts were somewhat larger (40%) but still had a $t_{1/2}$ of 15 s (data not shown).

It was shown in previous studies (*2*) that the cholesterol present in the slow pool of FU5AH rat hepatoma and L-cells can replenish the fast pool of cholesterol. To demonstrate this with CHO-K1 cells, a suspension of cells was briefly incubated with a 50 mM 2OHp$\beta$CD solution (50% saturated with cholesterol) to deplete the fast pool of radiolabeled cholesterol as described in Experimental Procedures. Initially, in CHO-K1 cells 30% of the [$^3$H]cholesterol was found in the fast pool. A portion of the fast pool of cholesterol was depleted from these cells by brief 2-min exposure of the cells to the efflux medium, resulting in only 15% of the [$^3$H]-cholesterol remaining in the fast pool. When the fast pool-depleted cells were allowed to incubate in tissue culture medium prior to reexposure to the efflux medium, it was found that the fast pool of cholesterol was completely regenerated with a $t_{1/2}$ of 25 min (data not shown). Similar $t_{1/2}$ values were previously reported with FU5AH and mouse L-cells (*2*).

*Influence of Modulators of Intracellular Lipid Trafficking.* Various agents known to affect cellular metabolism and cellular cholesterol trafficking in particular were assayed for their effects on the efflux of cholesterol from the fast and slow pools, as well as the movement of cholesterol between these kinetic pools. The effects of these agents on the sizes of the two kinetic pools of cholesterol and the $t_{1/2}$ of cholesterol release were determined by placing CHO-K1 cells in suspension in the presence of these agents for 40 min prior to measuring the time course of cholesterol efflux to the 2OHp$\beta$CD solution. Compounds such as the hydrophobic amine U18666A (15 $\mu$g/mL), progesterone (40 $\mu$M), brefeldin A (4 $\mu$M) and cytochalasin D (5 $\mu$g/mL), which have previously been reported to inhibit the intracellular movement of cholesterol (*4*, *22*−*31*), did not alter either the size or the $t_{1/2}$ of the fast and slow pools of cholesterol. Energy poisons and ATPase inhibitors such as sodium azide (3−10 mM), potassium cyanide (2 mM), sodium fluoride (3−10 mM), and bafilomycin $A_1$ (3−10 nM) were also unable to change the size or the $t_{1/2}$ of either the fast or slow pool. These inhibitors of various aspects of cholesterol trafficking also did not affect the movement of cholesterol from the slow pool into the fast pool. After depletion of the cholesterol present in the fast pool, the $t_{1/2}$ of replenishment of the fast pool remained 25 min even in the presence of U18666A, progesterone, brefeldin A, cytochalasin D, sodium azide, potassium cyanide, sodium fluoride, and bafilomycin $A_1$ at the previously indicated concentrations (data not shown).

*Efflux of Cholesterol from Isolated Plasma Membrane Vesicles.* Given the possibility that the slow efflux pool of cellular cholesterol might represent cholesterol present in internal membranes, we monitored the efflux of cholesterol from plasma membrane vesicles. Plasma membrane vesicles were prepared as described in Experimental Procedures. The isolated plasma membrane vesicles were added to the 50 mM 2OHp$\beta$CD solution and the release of [$^3$H]cholesterol to the acceptor medium was monitored. The majority (84%) of the cholesterol present in the plasma membrane vesicles was found in a fast pool with a $t_{1/2}$ of 38 s (Figure 2). The remaining 16% of the cholesterol was released with a $t_{1/2}$ of 36 min. This method has been shown to generate plasma membrane vesicles that are right-side-out and that apparently maintain some phospholipid asymmetry with respect to the inner and outer leaflets of the plasma membrane, although any asymmetry linked to either an energy-requiring process or cytoskeletal structure is presumably lost (*21*, *32*−*34*). It is not known if these vesicles contain lateral membrane domains or if the phospholipid asymmetry present in these vesicles is consistent with that observed in native cells.

*Effect of NEM on the Kinetics of Cholesterol Efflux.* Many studies have shown that the thioalkylating agent *N*-ethylmaleimide (NEM) can interfere with vesicular-mediated intracellular transport between various cellular compartments (*35*−*41*). NEM is also a potent inhibitor of v-ATPases (*42*) and has recently been implicated in preventing the selective uptake and subsequent internalization of low-density lipo-

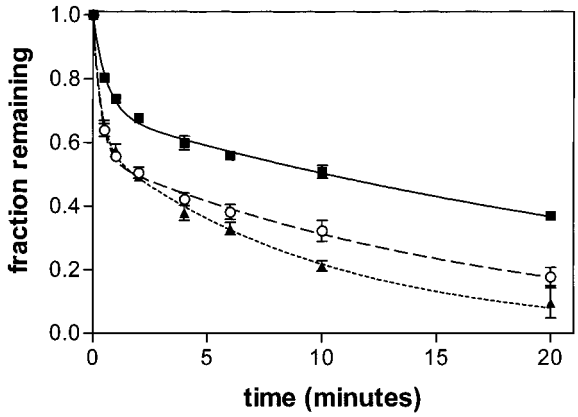


FIGURE 3: Changes in kinetic pool size as a result of NEM and heat treatments. Monolayers of CHO-K1 cells were radiolabeled, equilibrated, and placed in suspension as described in Experimental Procedures. Cells were then incubated in the absence (■) or presence (○) of 2 mM NEM for 40 min at 37 °C or heated to 60 °C (▲) for 10 min. The cells were then added to 50 mM 2OHp$\beta$CD efflux medium that was 50% saturated with cholesterol and time points were taken at 37 °C. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means ± SD for triplicate determinations.

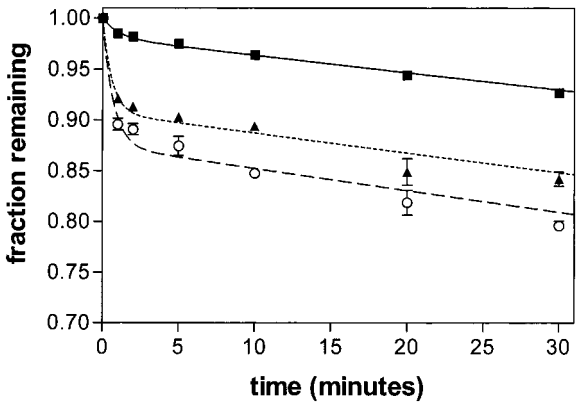


FIGURE 4: Effects of NEM and heat treatments on the efflux of [$^3$H]cholesterol to $HDL_3$. CHO-K1 cells were radiolabeled, equilibrated, and placed in suspension as described in Experimental Procedures. Cells were then incubated in the absence (■) or presence (○) of 2 mM NEM for 40 min at 37 °C or heated to 60 °C (▲) for 10 min. The cells were then added to 1.3 mg/mL $HDL_3$ and time points were taken at 37 °C. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means ± SD for triplicate determinations.

protein free cholesterol (*43*). For these reasons, we examined the effect of NEM on the kinetics of cholesterol efflux.

CHO-K1 cells radiolabeled with [$^3$H]cholesterol were incubated with 2 mM NEM for 40 min in suspension. The cells were then added to the 2OHp$\beta$CD efflux medium and the kinetic pools of cholesterol were determined. Pretreatment of CHO-K1 cells with NEM resulted in a dramatic increase in the size of the fast kinetic pool of cholesterol from 25% ± 5% to 50% ± 8% (Figure 3). This increase in the size of the fast pool of cholesterol as a result of NEM treatment was also seen in FU5AH and GM3468 cells (data not shown). NEM had the same effect in the fast pool replenishment system where the fast pool is initially depleted and the radiolabeled cholesterol in the slow pool moves into the fast pool. In these experiments, 2 mM NEM was added to the cells during the replenishment period (after depletion of the fast pool). This treatment resulted in not only a replenishment of the fast pool but also a large increase in the size of the fast pool. The fast pool in control cells was 25% after regeneration and increased to 40% when NEM was present during the regeneration period.

The effect of NEM on cholesterol efflux kinetics was specific in that other common sulfhydryl-modifying agents such as dithionitrobenzoic acid (20 mM), iodoacetic acid (2 mM), and iodoacetamide (2 mM) did not alter the kinetics of cholesterol efflux or the sizes of the fast and slow pools. When cells were heated to 60 °C for 10 min prior to exposure to the 2OHp$\beta$CD efflux medium, a significant increase in the size of the fast pool was observed (from 25% ± 5% to 53% ± 12%) (Figure 3). This effect was similar to that seen with pretreatment of the cells with NEM.

The increase in the size of the fast kinetic pool of cholesterol after NEM treatment and heat treatment was not specific for the nature of the extracellular acceptor of cholesterol. A suspension of CHO-K1 cells radiolabeled with [$^3$H]cholesterol was exposed to efflux medium containing $HDL_3$ as an acceptor of cellular cholesterol. $HDL_3$ is a much less efficient acceptor of cholesterol when compared to the 50 mM OHp$\beta$CD solution and the resulting $t_{1/2}$ for both the fast and slow pools of cholesterol were much longer. However, the cellular changes induced by either incubation with NEM or heating to 60 °C still resulted in a increase in the size of the fast kinetic pool of cellular cholesterol when $HDL_3$ was the extracellular acceptor (Figure 4).

Table 1: Decrease in Cellular ATP Levels after Treatment with Cellular Inhibitors

| inhibitor | decrease in ATP |
|---|---|
| Agents That Affect Cholesterol Efflux | |
| 2 mM NEM | 99 ± 0.2% |
| 60 °C, 10 min | 98 ± 0.4% |
| Agents That Do Not Affect Cholesterol Efflux | |
| 2 mM KCN | 88 ± 3% |
| 2 mM $NaN_3$ | 73 ± 3% |
| 3 mM NaF | 17 ± 2% |
| 10 nM bafilomycin $A_1$ | 12 ± 0.6% |
| 2 mM iodoacetamide | 66 ± 7% |

*Relationship between Cellular ATP Levels and Cholesterol Efflux.* The previous data suggests that the maintenance of the kinetic pools of cholesterol may require an intact cell, cellular energy, and/or functional proteins. To determine if there is a correlation between the cellular ATP levels and the change in kinetic parameters, the amount of ATP present in the treated or control cell suspensions was measured. Cells in suspension were incubated for 40 min with various agents that either do or do not have an effect on cholesterol efflux. The amount of ATP present in the cells at the end of the incubation period was determined as described in Experimental Procedures. Table 1 shows the percent decrease in cellular ATP after incubation with a particular agent relative to the amount of ATP present in untreated cells. The two treatments that modified cholesterol efflux, 2 mM NEM and heating to 60 °C, produced a 99% decrease in the cellular ATP levels. Other compounds such as potassium cyanide and sodium azide decreased the amount of ATP present in the cell by 88% and 73%, respectively. However, these agents did not have any effect either on the size of the fast and slow kinetic pools of cholesterol or on the $t_{1/2}$ of efflux for either pool. This indicates there is no direct correlation

between the depletion of cellular ATP levels and alterations in cholesterol efflux kinetics.

Due to the dramatic decrease in cellular ATP levels seen with either NEM or heat treatment, it was necessary to establish that the cells were not dying. A commonly used measure of cellular toxicity and membrane integrity is the leakage of cellular adenine (*18*, *19*). This method has been found to correlate very well with other measures of cell injury, such as leakage of lactate dehydrogenase (LDH) and the reduction in cellular ATP (*18*). Monolayers of CHO-K1 cells were radiolabeled with [$^3$H]adenine for 2 h and equilibrated for 10 min. In this time period the [$^3$H]adenine is rapidly incorporated into ATP (*18*). Incubation of CHO-K1 cells with a 50% saturated solution of 2OHp$\beta$CD solution for 2 h does not result in the leakage of [$^3$H]adenine above that released to EMEM/HEPES (data not shown; *14*). The amount of leakage of cellular adenine was determined after incubation of cell suspensions with either 2 mM NEM, 20 mM DTNB, or 100 mM 2-deoxyglucose and 100 mM sodium azide. None of these treatments induced any significant release of cellular adenine; 9.6% $\pm$ 0.4%, for NEM, 9.9% $\pm$ 0.02% for 2-deoxyglucose and sodium azide, and 5.6% $\pm$ 0.4% for DTNB treatment. The leakage of adenine was similar among all the treatments, although NEM was the only agent to have any effect on the kinetics of cholesterol efflux. This suggests the NEM-induced increase in the size of the fast pool is not a result of an increase in the permeability of cell membrane or of cellular injury in the time period analyzed.

*Effect of Agents That Alter Either the Phospholipid Content or Asymmetry of the Plasma Membrane*. Many studies have shown a preferential association of cholesterol with certain classes of phospholipids. Cholesterol has been reported to have the strongest interaction with sphingomyelin, followed by phosphatidylcholine and phosphatidylethanolamine (*44*−*48*). To determine the effect of phospholipid degradation on the kinetics of efflux, suspensions of CHO-K1 cells were treated with either 0.1 unit/mL sphingomyelinase or 0.2 unit/mL PC-PLC for 60 min prior to the exposure to the 50 mM 2OHp$\beta$CD efflux medium. Both of these enzyme treatments resulted in an increase in the size of the fast pool of cholesterol (Figure 5). The size of the fast pool of cholesterol increased from 25% in control cells to 34% after incubation with 0.1 unit/mL sphingomyelinase (Figure 5A) and 50% after incubation with 0.2 unit/mL PC-PLC (Figure 5B). There was no increase in the release of [$^3$H]adenine above control after either of these two treatments (data not shown). A 10-fold higher concentration of either sphingomyelinase or PC-PLC was also used. The higher concentration of sphingomyelinase did not cause any further increase in the size of the fast pool (Figure 5A). In contrast, more than 90% of the cholesterol was released in less than 30 s when the 10 fold higher concentration of PC-PLC was used (Figure 5B). Under these conditions (2 unit/mL PC-PLC), a significant amount of [$^3$H]adenine was released into the medium, 30% more than that released in control cells (data not shown). Replicate experiments were performed exhibiting similar increases in the size of the fast pool of cholesterol as a result of exogenous lipase addition.

Recently, a phospholipid scramblase was shown to mediate the rapid transbilayer movement of plasma membrane phospholipids in response to an elevation in intracellular $Ca^{2+}$

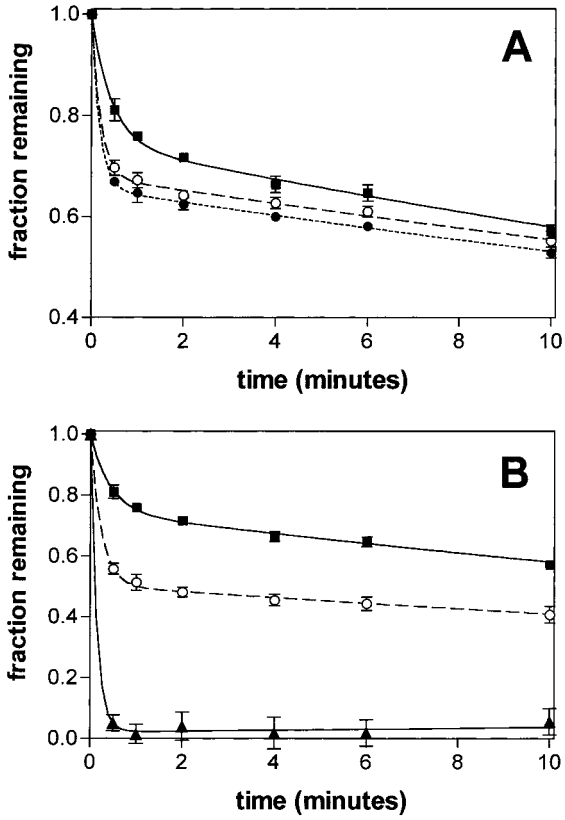


FIGURE 5: Effect of sphingomyelinase and PC-PLC on [$^3$H]-cholesterol efflux. Monolayers of CHO-K1 cells were radiolabeled, equilibrated, and placed in suspension as described in Experimental Procedures. (A) Cells were then incubated at 37 °C in the absence (■) or presence of 0.1 unit/mL sphingomyelinase (○) or 1 unit/mL sphingomyelinase (●) for 60 min. (B) Cells were then incubated at 37 °C in the absence (■) or presence of 0.2 unit/mL PC-PLC (○) or 1 unit/mL PC-PLC (▲) for 60 min. The cells were then added to 50 mM 2OHp$\beta$CD efflux medium that was 50% saturated with cholesterol and time points were taken at 37 °C. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means $\pm$ SD for triplicate determinations.

(*49*−*51*). The activation of this scramblase results in rapid transbilayer movement of aminophospholipids and choline phospholipids with displacement of phosphatidylserine and phosphatidylethanolamine to the outer leaflet of the plasma membrane and compensatory movement of phosphatidylcholine and sphingomyelin to the inner leaflet. It was of interest to determine if this perturbation of the normal asymmetrically distributed plasma membrane phospholipids would result in an alteration of the kinetic pools of cholesterol. CHO-K1 cells were preincubated for 30 min with the calcium ionophore A23187 (2 $\mu$M) in EMEM/HEPES (free $Ca^{2+}$ concentration 1.8 mM) prior to the exposure to the 50 mM 2OHp$\beta$CD efflux medium; this condition activates the phospholipid scramblase in various cell types (*49*−*51*). The size of the fast pool of cholesterol increased from 25% to 36% after incubation with A23187 (Figure 6). This increase in the size of the fast kinetic pool of cholesterol was similar to that seen with preincubation of cells with sphingomyelinase.

*Effect of NEM on Phospholipid Degradation.* We observed that treatment of the cells with either 2 mM NEM or exogenous sphingomyelinase or PC-PLC increased the size of the fast pool of cholesterol. It is thought that NEM inhibits

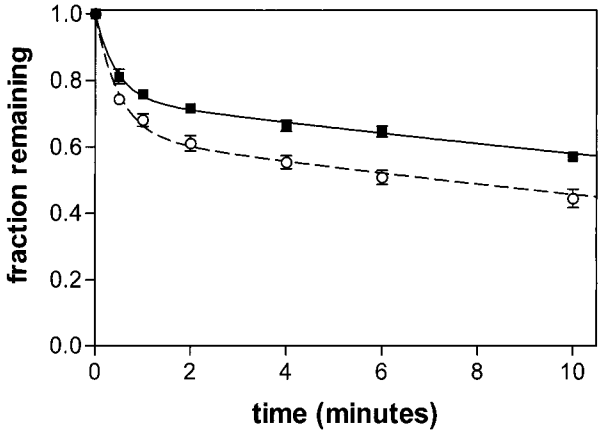


FIGURE 6: Effect of calcium ionophore A23187 on cholesterol efflux. Monolayers of CHO-K1 cells were radiolabeled, equilibrated, and placed in suspension as described in Experimental Procedures. Cells were then incubated in the absence (■) or presence (○) of 2 $\mu$M calcium ionophore A23187 for 30 min at 37 °C. The cells were then added to 50 mM 2OHp$\beta$CD efflux medium that was 50% saturated with cholesterol and time points were taken at 37 °C. The efflux of cholesterol was expressed as the fraction of initial [$^3$H]cholesterol that was remaining in the cells. Data points are means ± SD for triplicate determinations.

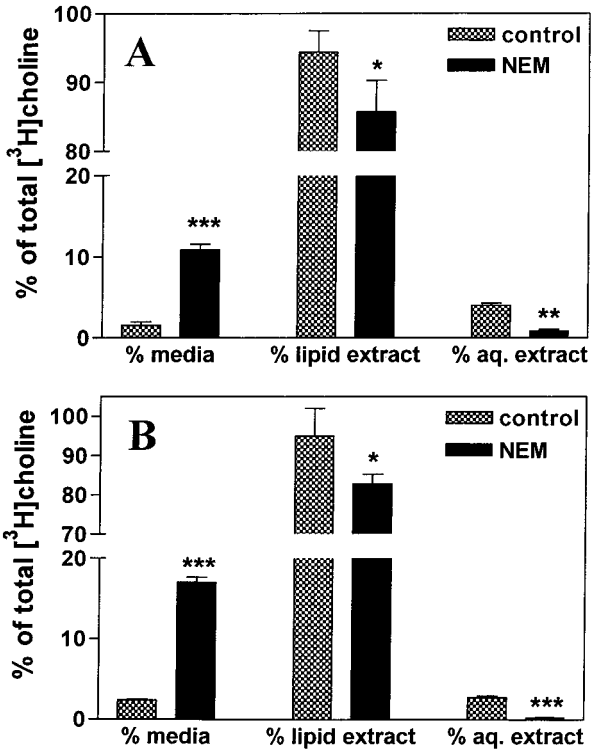


FIGURE 7: Alterations in choline containing phospholipids after NEM-treatment. Monolayers of CHO-K1 cells were radiolabeled and equilibrated in the presence or absence of 2 mM NEM for (A) 40 and (B) 120 min. Cells were extracted and the amount of [$^3$H]-choline in each fraction was expressed as a fraction of the total choline label incorporated. Data points are means ± SD for triplicate determinations. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

most cellular processes, but recently it was reported that NEM can stimulate arachidonic acid release through an activation of a signal-responsive phospholipase $A_2$ (*52*). To test if the NEM-induced changes in cholesterol efflux were secondary to an NEM-induced alteration in phospholipid content, cellular phospholipids were radiolabeled with [$^3$H]-choline and the effect of NEM on phospholipid hydrolysis was determined. After an overnight radiolabeling period the cell monolayers were equilibrated, extensively washed, and incubated for 40 or 120 min either with or without 2 mM NEM. The release of radiolabeled choline into the extracellular medium at each time point was determined. After the incubation period, the cell monolayers were extracted and the soluble cellular choline was separated from the choline-containing lipids. The amounts of radioactivity in the medium, aqueous cellular extract, and lipid cellular extract were expressed as a fraction of the total choline label incorporated. Figure 7 shows that treatment of the cell monolayers with 2 mM NEM for either 40 or 120 min resulted in an increase in the amount of [$^3$H]choline released into the medium; the value was 10.8% ± 0.6% above control at 40 min and 14.6% ± 1.3% above control at 120 min. This release of [$^3$H]choline was paralleled by a decrease in cellular choline; the amount of labeled choline present in the lipid extract decreased by 7.6% ± 5.6% at 40 min and 11.7% ± 6% after 120 min. These data suggest that incubation of cells with NEM causes the degradation of cellular choline-containing phospholipids.

## DISCUSSION

*Locations of Kinetic Pools of Cellular Cholesterol.* It is apparent that cells in suspension have two readily exchangeable pools of cholesterol exhibiting different efflux kinetics, although the exact locations of the fast and slow pools are not known. A third pool of cholesterol exists in which the rate of efflux is very slow. This pool comprises 5−10% of the cellular cholesterol and is most likely cholesterol contained in internal membranes. The very rapid release of cholesterol from the fast pool ($t_{1/2}$ = 30 s) suggests that this pool is most likely present in the plasma membrane. There are several possibilities for the cellular location of the slow kinetic pool of cholesterol. The slow pool of cholesterol may also be localized to the plasma membrane. In this case, the two kinetic pools could represent either the inner (slow) and outer (fast) leaflets of the plasma membrane or separate lateral lipid domains in the plasma membrane. Another possibility is that this slow pool of cholesterol also represents cholesterol contained in internal membranes. A third possibility is that the slow pool is a combination of some cholesterol in the plasma membrane and in internal membranes.

A few lines of evidence are consistent with the slow pool of cholesterol being intracellular cholesterol. First, the transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane has been reported to have a $t_{1/2}$ = 10−20 min for CHO cells (*31*, *53*) or 1−2 h for fibroblasts (*54*). Similarly, the transport of cholesterol from lysosomes to the cell surface has been reported to occur in 40−50 min in Fu5AH cells (*55*). These data indicate that cholesterol can move through the cytoplasm to the plasma membrane in 10 min to 1 h depending on the cell type and the original intracellular location of the cholesterol. This time frame is generally consistent with the 20 min half-time we have reported for the efflux of cholesterol from the slow pool (Figure 1). In addition, we have also shown that after the fast pool of cholesterol has been depleted, it can be replenished from cholesterol in the slow pool with a $t_{1/2}$ of 25 min. Thus, there is a correlation between the $t_{1/2}$ of

intracellular cholesterol transport and both the $t_{1/2}$ of efflux of cholesterol from the slow kinetic pool and the half-time of replenishment. A second observation consistent with the slow pool representing internal cholesterol is that the efflux of cholesterol from plasma membrane vesicles appeared to be essentially in only a fast pool (Figure 2). However, some phospholipid asymmetry has been demonstrated in these plasma membrane vesicles (*32*−*34*). It is possible that the cholesterol packing has been altered since the shedding of plasma membrane vesicles has been found to be associated with membrane lipid randomization (*56*). Thus, it is possible that by isolation of plasma membrane vesicles lateral and interleaflet lipid domains may have been disrupted, resulting in the loss of the majority of the slow pool of cholesterol.

Other lines of evidence argue against the slow efflux pool of cholesterol ($t_{1/2} \sim 20$ min) representing cholesterol contained in internal membranes. In CHO-K1 cells, the slow pool of cholesterol represents 70% of the cellular cholesterol which is inconsistent with the idea that 65−80% of the cellular unesterified cholesterol is present in the plasma membrane (*4*−*9*). If these estimates of the amount of cell cholesterol present in the plasma membrane are correct, the 70% of the cellular cholesterol in the slow pool could not represent cholesterol contained in internal membranes. A second line of evidence is the inability of many agents previously reported to affect intracellular cholesterol trafficking to alter either the $t_{1/2}$ of efflux or the sizes of the two kinetic pools of cholesterol (Table 1). The movement of cholesterol to and from intracellular locations has been shown to require an intact cytoskeleton, a functional Golgi network, metabolic energy, and an acidic vesicle system (*27*, *53*, *57*−*61*). Progesterone, the hydrophobic amine U18666A, brefeldin A (a Golgi disrupting agent), and cytocholasin D (a microfilament inhibitor) were all unable to change any of the kinetic parameters of efflux. These are all agents that have previously been shown to inhibit the movement of cholesterol to and from intracellular pools (*22*−*31*). Also, numerous energy poisons, such as sodium azide, potassium cyanide, sodium fluoride, 2-deoxyglucose, and bafilomycin $A_1$, were all without any effect on the kinetics of cholesterol efflux. In addition, when the absolute amount of cellular ATP was measured after incubation with a particular agent, no direct correlation between a reduction in the cellular ATP levels and an alteration in the kinetics of efflux was seen (Table 1).

As indicated above, it is possible that the slow pool comprises the plasma membrane as well as endocytic, secretory, and recycling vesicles. To assess this possibility, cells were treated with NEM. NEM has been shown to interrupt the trafficking of membrane vesicles through inhibition of the NEM-sensitive fusion protein (NSF) (*35*). This protein is required for a variety of processes including the fusion of endoplasmic reticulum-derived vesicles with the Golgi, intra-Golgi vesicular trafficking, vesicle movement from the Golgi to the plasma membrane, endosome fusion, transcytosis, and exocytosis as well as endocytosis (*36*−*40*). NEM has also been shown to inhibit regulated, stimulated, and constitutive secretion (*62*). This trafficking is mediated by the NSF, soluble NSF attachment protein (SNAP), SNAP receptor (SNARE) pathway (see ref *41* for review). If the slow pool of cholesterol was in fact cholesterol localized to internal membranes that moved to the plasma membrane in a vesicle-mediated process requiring a functional NSF protein, the result of NEM treatment would be to trap the slow pool inside the cell. This would result in the formation of a nonexchangeable pool of cholesterol. This was not the effect of NEM because NEM treatment resulted in a dramatic increase in the amount of cholesterol present in the fast pool and did not induce the formation of a nonexchangeable pool (Figure 3). These data suggest that the majority of the slow pool of cholesterol is not cholesterol present in an endosomal, secretory, or recycling vesicle population. However, the possibility exists that a portion of the slow pool might be in a vesicle compartment if the contents of the vesicle still mixed with the plasma membrane even if fusion did not occur. NEM has been shown to induce the accumulation of vesicles inside the cell (*63*). It is not known if the contents of these fusion-deficient vesicles can mix with the plasma membrane.

The NEM-induced increase in the size of the fast pool appeared to be a result of alterations in cellular lipid pools since it was not specific to the particular acceptor of cellular cholesterol occurring when either $HDL_3$ or 2OHp$\beta$CD was used. In addition, this effect was specific to NEM in that other energy poisons or common sulfhydryl-modifiying agents were found to be ineffective at modifying cellular cholesterol pools. Although NEM treatment did cause a dramatic decrease in cellular ATP levels, this was shown not to be the mode by which NEM exerts its effect on cellular cholesterol pools (Table 1) since compounds producing similar a depletion of cellular ATP had no effect on cholesterol efflux. Taken together, these data suggest that the two kinetic pools of cholesterol are present in the plasma membrane.

*Relationship between the Phospholipid Content and Asymmetry of the Plasma Membrane and Cholesterol Efflux.* Recently, it was shown that modification of the sphingomyelin content of fibroblasts as a result of sphingomyelinase treatment produced an enhancement of cholesterol efflux to 5 mM 2OHp$\beta$CD (*64*). Interestingly, in these studies treatment with PC-PLC alone did not show any significant effect on cholesterol efflux. However, when both sphingomyelinase and PC-PLC were combined there was an increase in the release of cholesterol that was more than that released with either agent alone, indicating that there is a synergistic enhancement of cholesterol efflux due to hydrolysis of both phosphatidylcholine and sphingomyelin. In our present studies with CHO-K1 cells, alterations in the phospholipid organization of the plasma membrane resulted in an increase in the size of the fast pool of cholesterol. These changes in phospholipid organization were a direct result of exogenous lipase addition or of the activation of a phospholipid scramblase. In contrast to the results of Ohvo et al. (*64*), in the present studies there was a much greater increase in the size of the fast efflux pool of cholesterol when choline-containing phospholipids were hydrolyzed by the action of PC-PLC than by sphingomyelinase (Figure 5). The differences in the effect of PC-PLC between this paper and the results of Ohvo et al. (*64*) may in part be due to differences in cell type and experimental conditions.

Given that hydrolysis of either phosphatidylcholine or sphingomyelin resulted in an increase in the size of the fast pool of cholesterol, we also tested the effect of alterations in the asymmetric distribution of the plasma membrane

phospholipids. A marked increase in the size of the fast pool of cholesterol was seen after the activation of a phospholipid scramblase (Figure 6), NEM treatment (Figure 3), heat treatment (Figure 3), and exogenous lipase addition (Figure 5). Each treatment would result in the disruption of the normal organization of lipids in the plasma membrane via either hydrolysis of the choline-containing phospholipids or rapid transbilayer movement of the plasma membrane phospholipids. This implies that phosphatidylcholine and sphingomyelin present in the plasma membrane are critical for maintaining the two kinetic pools of cholesterol; any alteration in the amount or the location of these phospholipids results in an enhancement of efflux by redistributing cholesterol into the fast kinetic pool.

In summary, it is clear that cells contain two kinetic pools of free cholesterol that can efflux to extracellular acceptors. All of the fast pool and the majority of the slow pool of cholesterol appear to be localized to the plasma membrane. Some of the slow pool of cholesterol may be present in a vesicular compartment. At this time we are unable to determine the contributions of the fast and slow pools to the inner and outer leaflet domains and lateral domains of the plasma membrane. If the cholesterol contained in the inner and outer leaflets of the plasma membrane represent the kinetic pools of cholesterol, then the $t_{1/2}$ of cholesterol transbilayer migration would be about 20 min, the $t_{1/2}$ of efflux from the slow pool of cholesterol. The $t_{1/2}$ of the transbilayer movement of cholesterol has been reported to range from seconds to days depending on the assay used to measure the appearance of cholesterol in the outer leaflet of the membrane (*3*, *65*−*69*). To determine if the slow pool of cholesterol is cholesterol present in the inner leaflet of the plasma membrane, a reliable measurement of the rate of transbilayer movement of cholesterol is needed. Also, lateral membrane microdomains such as caveolae or cholesterol−sphingolipid rafts could represent these different pools of cholesterol. In this case, the movement of cholesterol between these lateral membrane domains would have a $t_{1/2}$ of 20 min; the movement of cholesterol out of caveolae to the rest of the plasma membrane occurs on this time scale (*70*). At this time, the amount of cholesterol contained in caveolae or lipid rafts in comparison to that located in the rest of the plasma membrane is not well established (*10*, *71*). Better definition of the physical state of cholesterol in plasma membranes is required before the kinetic pools of cholesterol can be identified structurally.

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