Fatty acids are rapidly delivered to and extracted from membranes by methyl-β-cyclodextrin

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Abstract We performed detailed biophysical studies of transfer of long-chain fatty acids (FAs) from methyl-β-CD (MBCD) to model membranes (egg-PC vesicles) and cells and the extraction of FA from membranes by MBCD. We used i) fluorescein phosphatidylethanolamine to detect transfer of FA anions arriving in the outer membrane leaflet; ii) entrapped pH dyes to measure pH changes after FA diffusion (flip-flop) across the lipid bilayer; and iii) soluble fluorescent-labeled FA binding protein to measure the concentration of unbound FA in water. FA dissociated from MBCD, bound to the membrane, and underwent flip-flop within milliseconds. In the presence of vesicles, MBCD maintained the aqueous concentration of unbound FA at low levels comparable to those measured with albumin. In studies with cells, addition of oleic acid (OA) complexed with MBCD yielded rapid (seconds) dose-dependent OA transport into 3T3-L1 preadipocytes and HepG2 cells. MBCD extracted OA from cells and model membranes rapidly at concentrations exceeding those required for OA delivery but much lower than concentrations commonly used for extracting cholesterol. Compared with albumin, MBCD can transfer its entire FA load and is less likely to extract cell nutrients and to introduce impurities.—Brunaldi, K., N. Huang, and J. A. Hamilton. Fatty acids are rapidly delivered to and extracted from membranes by methyl-β-cyclodextrin. J. Lipid Res. 2010. 51: 120–131.

Supplementary key words flip-flop • desorption • unbound fatty acid • albumin • cholesterol

The very low aqueous solubility of long-chain fatty acids (FAs) is one of the major limitations in studies of FA transport in vitro and in vivo. A common misconception is that FA can form micelles at physiological pH because the pKa values for monomeric FA are typically 4.8. Instead, they form insoluble structures similar to phospholipid bilayers, in which ~50% of the FA is ionized. The aqueous solubilities of the monomeric forms of the common 16- and 18-carbon dietary FA are <10 µM (2).

A frequently used approach for adding FA at higher concentration is to complex the FA with albumin, which allows preparation of FA that is solubilized at millimolar concentrations. Although albumin is a physiologically relevant carrier of FA, careful consideration must be given to the facts that albumin can deliver impurities to cells and can extract FA and other nutrients from cells, which could alter their metabolic state (3). In addition, there is continuing disagreement about the rates of FA dissociation from albumin and whether this affects biophysical measurements of FA transport (4, 5). Furthermore, the binding properties of albumin are complex: different FA binding sites have different relative affinities, and the kinetics of desorption are dependent on acyl chain length (6). The high affinity of albumin for FA may result in very little delivery to membranes (7).

A promising new vehicle for solubilization of FA is the family of cyclodextrins (CDs), which are cyclic oligosaccharides formed by bacterial degradation of starch. These molecules typically contain six (α), seven (β), or eight (γ) glucose residues linked by (1→4) glycosidic bonds. They have a polar surface and a hydrophobic cylindrical cavity that can bind and solubilize a wide variety of hydrophobic molecules, such as cholesterol and FA, while remaining soluble in water (8). The number of CD molecules required to solubilize one FA molecule increases with an increase in the hydrocarbon chain length of FA (9), although the exact stoichiometry is difficult to determine.

In pharmaceutical applications, CD is widely used to solubilize hydrophobic drugs and enhance drug absorption in the gastrointestinal tract (8). It is used as a lipid-binding agent in the culture media of bacteria and animal cells (10, 11) and as a substitute for albumin for intrave-
These results confirm our previous estimates of flip-flop in associates from MBCD very rapidly compared with albumin. Concentration of unbound FA throughout the delivery of FA with a focus on the common dietary FA, myristic (MA; (MBCD) as a FA donor to model membranes and cells with a concentration of unbound FA in the aqueous phase would avert the current uncertainty about how fast FAs dissociate from albumin and how this affects measured transport rates. Furthermore, a low concentration of unbound FA in the aqueous phase would be maintained.

In this study, we tested the usefulness of methyl-β-CD (MBCD) as a FA donor to model membranes and cells with a focus on the common dietary FA, myristic (MA; C14:0), palmitic (FA; C16:0), stearic (SA; C18:0), and oleic acids (OA; C18:1). We employed different fluorescence assays developed in our laboratory for monitoring the binding and transbilayer diffusion of FA (17–19). The transmembrane diffusion of FA (flip-flop) typically is measured directly using a pH-sensitive fluorophore, such as pyranine or 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein (BCECF). The FA adsorption step can be measured using the soluble fluorescent-labeled FA binding protein [acyloylan-labeled intestinal fatty acid binding protein (ADIFAB)] in the external medium (18). A newer assay measures adsorption of FA by the fluorescence of FPE, a phosphatidylethanolamine phospholipid molecule covalently linked to fluorescein in the headgroup region (17, 19). FPE added in the external buffer inserts into the outer leaflet of membranes and detects binding of ionized FA to the lipid bilayer. Furthermore, when FA is complexed with a donor, all of the above assays also include the kinetics of desorption of FA from the donor.

Our major findings were i) MBCD maintains a low concentration of unbound FA throughout the delivery of FA to cell membranes and lipid membranes, and ii) FA dissociates from MBCD very rapidly compared with albumin. These results confirm our previous estimates of flip-flop in the low millisecond range. Our results show also that the MBCD concentration required to solubilize and deliver FA to cells is low (<0.1 mM) but that higher concentrations used to extract cholesterol from the plasma membrane of cells (millimolar) will likely extract FA and other amphipathic nutrients from cells.

EXPERIMENTAL PROCEDURES

Preparation of FA solutions, FA:MBCD, and FA:BSA complexes

Stock solutions of MA (C14:0), FA (C16:0), SA (C18:0), and OA (C18:1) (10 mM) were prepared in ethanol or DMSO or by dissolving FA in 0.1 mM KOH to make the K⁺ salt. FA:MBCD complexes were prepared in two ways: i) purchased FA:MBCD compound (Sigma-Aldrich) was dissolved in water at 10 mM final concentration in FA (only OA, molar ratio ~1:6 OA:MBCD); ii) aliquots of a MBCD solution in water were added to a microcentrifuge tube containing pure FA followed by incubation at 70°C for 1 h and sonication for 5 min. The molar ratios of FA:MBCD were 1 MA:10 MBCD, 1 PA:12 MBCD, and 1 SA:22 MBCD. All stock solutions of FA:MBCD were clear at room temperature. A stock solution of MBCD alone was made in water at 100 mM. BSA complexed with OA was prepared as described before (20) at OA:BSA molar ratios of 4:1 and 8:1. For stopped-flow experiments, appropriate volumes of OA, MBCD, and OA:MBD stocks were dissolved in 50 or 20 mM HEPES-KOH (pH 7.4).

Preparation of cultured cells

The 3T3-L1 preadipocytes and HepG2 cells were cultured in high-glucose DMEM supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 units/ml penicillin. BCECF was incorporated into cells by incubating cells for 20 min with 2 µM BCECF-AM at 37°C followed by three washes in PBS buffer. Cells were resuspended in albumin-free Krebs-MOPS buffer (118 mM NaCl, 5 mM KCl, 1.1 mM KH₂PO₄, 20 mM MOPS, 2.5 mM CaCl₂, and 5.1 mM glucose, pH 7.4) and then treated with nonenzymatic cell dissociation solution for 5 min.

Preparation of lipid vesicles

Small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) of egg-PC were prepared by sonication and extrusion, respectively, as described previously (21). Briefly, the pH probe pyranine (0.05–0.5 mM) was trapped inside the vesicles by placing it in the buffer when the lipids were hydrating. The surface potential probe, FPE, resuspended in 95% ethanol (2 mg/ml), was added to vesicles in suspension (1 mol% FPE relative to egg-PC) and incubated for 1 h in the dark at room temperature to label the outer bilayer leaflet. For fluorescence experiments with pyranine or FPE, the buffer used was 20 or 50 mM HEPES-KOH (pH 7.40). In the presence of ADIFAB, the buffer was 20 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM Na₄HPO₄ (pH 7.40). In stopped-flow experiments, in which FA are delivered as a complex with SUV, an aliquot of FA in ethanol solution was added to a suspension of donor SUV.

pH calibration with nigericin

The relationship between internal pH and pyranine fluorescence was calibrated by permeabilizing SUV to H⁺ with 1 µM nigericin. The external pH was adjusted with aliquots of KOH and H₂SO₄, and the pH was measured with a mini-pH electrode.

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Materials

3T3-L1 and HepG2 cell lines were purchased from American Type Culture Collection (Manassas, VA). Cell culture supplies were purchased from Fisher Scientific (Agawam, MA) or Gibco Life Technology (Long Island, NY). Cellstripper™ nonenzymatic cell dissociation solution was purchased from Mediatacc (Manassas, VA). Pyranine (8-hydroxypropene-1,3,6-trisulfonic acid), BCECF-AM (acetoxymethyl ester), and fluorescein phosphatidylethanolamine (FPE) were purchased from Molecular Probes (Eugene, OR). ADIFAB was from FFA Sciences (San Diego, CA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL). MBCD, oleic acid-methyl-β-cyclodextrin complex (oleic acid-water soluble), BSA, all fatty acids (MA, PA, SA, and OA) and all buffer materials were purchased from Sigma-Aldrich (St. Louis, MO).
Fluorescence measurements

Online fluorescence measurements were performed with a Spex Fluoromax-2 fluorometer (Yvon Jobin, NJ). The sampling time was set to 1.0 s (the mixing time of the cuvette). Rapid kinetics were measured with a High Tech stopped-flow apparatus (Tgk Scientific, UK) attached to the fluorometer (dead time of 10 ms). The ratioimetric fluorescence of BCECF was measured using excitation at 439 and 505 nm \( (R = F_{505}/F_{439}) \) and emission at 535 nm (sampling time, 2.0 s; band-pass 3–5 nm). Pyranine was measured with excitation at 455 nm and emission at 509 nm (3–3 nm band-pass minimum). FPE was measured with excitation at 490 nm and emission at 520 nm (5–10 nm band-pass). The ratioimetric fluorescence of ADIFAB was measured with excitation at 390 nm and emission at 505 and 432 nm \( (R = F_{505}/F_{432}) \) (sampling time, 2.0 s; band-pass 3–5 nm). Experiments with vesicles and cells were carried out at room temperature and 37°C, respectively.

Measurement of aqueous OA concentration

The fluorescent FA indicator ADIFAB was dissolved in buffer (0.2 µM) and used for measurements with vesicles. The concentration of unbound aqueous OA \( ([OA]_a) \) was calculated from the following equation: 
\[
[OA]_a = K_d \cdot 19.5 \cdot (R \times R_0)/(11.5 - R),
\]
where \( K_d \) is the dissociation constant \( (K_d = 0.23 \mu M \text{ for OA at 25°C} ) \), and \( R \) and \( R_0 \) are the fluorescence ratios of ADIFAB in the presence and absence of FA, respectively. The value of \( R_0 \) was either used as provided by FFA Sciences or measured before adding FA to the suspension of vesicles.

Data analysis

Analysis of fluorescence data was performed to determine the \( t_{1/2} \) for FA transfer. The observed rate constant of the fluorescence change was obtained by fitting the fluorescence trace to a first-order exponential decay function: 
\[
F(t) = F(\infty) + F(0) \exp(-tk_{obs}),
\]
where \( t \) is time, \( F(0) \) is the initial fluorescence intensity, and \( F(\infty) \) is the fluorescence at \( t = \infty \). The rate constant is related to the half-time of fluorescence change \( (t_{1/2}) \) by the following equation: 
\[
t_{1/2} = \ln2/k_{obs}.
\]

RESULTS

Delivery of FA to lipid vesicles by MBCD

Our initial experiments aimed to find conditions under which MBCD would deliver FA to lipid vesicles (SUV and LUV) and to determine the kinetics of the partitioning. For these aims we used \( i \) the surface potential probe FPE, which was inserted in the outer leaflet of the membrane to measure the adsorption of FA that dissociated from MBCD, and \( ii \) the pH probe pyranine, which measures the release of \( H^+ \) to the intravesicular compartment that results from the transbilayer movement and achievement of ionization equilibrium of FA at the inner leaflet of the lipid membrane. Therefore, pyranine measures the combined steps of adsorption and flip-flop.

As shown in Fig. 1, sequential additions of 1OA:6MBCD complexes (1.2 µM OA:7.2 µM MBCD) to SUV (100 µM egg-PC) labeled with FPE (Fig. 2A) or containing entrapped pyranine (Fig. 2B) resulted in a rapid decrease in the fluorescence of both probes. These results indicate that OA rapidly dissociated from MBCD, bound to the membrane, and underwent flip-flop within 1–2 s, the time resolution of the online fluorescence measurements.

To complement the above data with OA, we measured the delivery of other FA by MBCD to LUV containing entrapped pyranine. As shown in Fig. 2, the addition of 1MA:10MBCD (Fig. 2A), 1PA:12MBCD (Fig. 2B), and 1SA:22MBCD (Fig. 2C) complexes to SUV (400 µM egg-PC) also produced a fast reduction in pyranine fluorescence, similar to that observed with FA added in the unbound form (22). Note that higher ratios of MBCD to FA were required to solubilize longer-chain FA. Subsequent addition of an excess of MBCD (FA-free) produced a fast increase in pyranine fluorescence that can be attributed to extraction of the added FA. MBCD was not able to extract the delivered SA when added at the concentration used for extraction of MA, PA, and OA. Furthermore, MBCD did not extract SA after its addition in the unbound form (data not shown). These results indicate a much higher affinity of SA for the lipid membrane relative to MBCB.
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and pyranine, indicates rapid dissociation of OA from MBCD; moreover, it does not depend on previous information or assumptions about flip-flop.

As also shown in Fig. 3A, subsequent addition of 1OA:6MBCD produced smaller incremental changes in the ADIFAB fluorescence. This does not reflect true saturation of binding to the membrane but the partition of FA between donor (MBCD) and acceptor (SUV).

As observed before by our group with the addition of unbound FA or FA:albumin complexes (19, 22), subsequent additions of FA:MBCD resulted in progressively smaller changes in pH_in (Fig. 1A). These results are explained by the interfacial ionization of FA and flip-flop: as pH_in decreases, the ionization of FA at the inner leaflet is suppressed and the net delivery of H^+ per dose of added FA is reduced.

Partitioning of OA between phospholipid membranes and MBCD

An independent assay used the fluorescent probe ADIFAB in the external buffer to monitor the concentration of unbound OA and to assess the delivery of OA to vesicles by MBCD. As shown in Fig. 3A, the fluorescence of ADIFAB increased rapidly upon addition of 1OA:6MBCD complexes to a suspension of SUV. Each plateau in Fig. 3A reflects the concentration of unbound OA in water and in equilibrium with the membrane, MBCD, and the ADIFAB probe. This result, together with results obtained with FPE and pyranine, indicates rapid dissociation of OA from MBCD; moreover, it does not depend on previous information or assumptions about flip-flop.

As also shown in Fig. 3A, subsequent addition of 1OA:6MBCD produced smaller incremental changes in the ADIFAB fluorescence. This does not reflect true saturation of binding to the membrane but the partition of FA between donor (MBCD) and acceptor (SUV). From the ADIFAB fluorescence ratio measured in Fig. 3A, we calculated the concentration of unbound OA ([OA]_m) in the external buffer after each addition of 1OA:6MBCD complex. For comparison with these results, we also measured the concentration of unbound OA produced by the delivery of either 1) OA not complexed with any carrier (added in the unbound form from a stock solution of FA in ethanol or DMSO); or 2) OA complexed with BSA (OA:BSA molar ratios of 4:1 and 8:1). Because albumin contains three high-affinity FA binding sites, we used OA:BSA complexes at molar ratios higher than 3FA:1BSA to enhance delivery of FA by albumin (20, 22).
than MBCD. This result reflects the higher affinity of BSA for FA than for MBCD.

In the presence of 200 nM SUV, the concentration of unbound OA ([OA]u) in the external buffer in equilibrium with the SUVs was calculated from the fluorescence ratio of ADIFAB obtained after each addition of OA added either unbound, from a stock solution in DMSO or ethanol (squares), complexed to MBCD (circles), or complexed to BSA (molar ratio 8OA:1BSA; triangles) (B). The concentration of [OA]u in equilibrium with MBCD in the absence of vesicles is also shown (C). Data in A are a representative experiment of three to four independent experiments. Data in B and C are means ± standard deviation of three to four independent experiments.

As shown in Fig. 3B in the presence of MBCD, the concentrations of unbound OA in equilibrium with vesicles were much lower than those obtained with OA delivered free of any carrier. In the presence of a fixed SUV concentration of 100 nM, the concentration of unbound OA was between 0.01 and 0.1 µM over the 1OA:6MBCD concentration range investigated, far below the solubility limit for OA (6 µM) (2).

For lower concentrations of total added OA, the reduction in the concentration of unbound OA by MBCD was comparable to levels obtained in the presence of BSA when initial mole ratios of OA:BSA were either 8:1 or 4:1 (data not shown). For instance, at a concentration of 6 µM of total OA, the concentration of unbound OA measured in the presence of MBCD and BSA was 0.035 ± 0.013 and 0.024 ± 0.01 µM, respectively. In contrast, in the absence of a delivery system, the concentration of unbound OA in equilibrium with vesicles produced by 6 µM of total OA was 0.094 ± 0.0085 µM. However, as the concentration of total OA increased together with the concentration of the FA carrier (i.e., constant FA:carrier mole ratio), BSA was more effective in buffering the concentration of unbound OA than MBCD. This result reflects the higher affinity of BSA for FA than for MBCD.

In the presence of 200 µM SUV, the concentration of unbound OA produced by the delivery of 6 µM OA complexed to MBCD was 0.017 ± 0.002 µM, which is half of that measured in the presence of 100 µM SUV. Under these conditions, the concentration of unbound FA measured in the absence of any carrier (OA added unbound) was 0.06 ± 0.015 µM.

The partition coefficient of OA added in the unbound form (Kp) was quantified from the linear relationship between the membrane-bound OA ([OA]m), calculated after every addition of OA to the vesicle suspension and [OA]u: Kp = [OA]m/[OA]u = ([([OA]t - [OA]u)/[OA]u)/ Vm/Va), where [OA]m is the FA concentration in the membrane based on the volume of the membrane (Vm); [OA]t and [OA]u are the total and unbound OA concentrations, respectively, which are based on the volume of water phase (Va). For a phospholipid bilayer, Vm/Va has been calculated as 10^{-3} mmol/l phospholipid (23).

The Kp calculated when uncomplexed OA was added to the vesicle suspension was 0.5 × 10^6, in agreement with
the $K_p$ for protein-free vesicles previously reported by our group (7).

In parallel experiments, ADIFAB was used to measure the concentration of unbound OA in equilibrium with MBCD in the absence of vesicles (Fig. 3C). These results confirmed that MBCD efficiently maintained the concentration of unbound OA in water of $0.3 \pm 0.05 \mu M$ over a wide range of concentrations.

**Kinetics of OA transfer between MBCD and lipid membranes: stopped-flow experiments**

The preceding online fluorescence assays with FPE and pyranine showed rapid equilibration of binding of OA and saturated long-chain FA to model membranes when MBCD was a donor. However, the changes in fluorescence were completed within the mixing time of the online experiments (1–2 s). To improve on the time resolution and accurately measure the kinetics of FA transfer between MBCD and lipid membranes, we used stopped-flow fluorimetry to achieve a time resolution of milliseconds.

Stopped-flow fluorescence assays were carried out with OA and with lipid vesicles (SUVs and LUVs) containing entrapped pyranine or labeled with the surface potential probe FPE. To minimize possible artifacts from osmotic changes, we prepared both vesicles and 1OA:6MBCD solutions in HEPES buffer. We defined two populations of egg-PC vesicles: FA donor vesicles containing OA and FA acceptor vesicles with no added OA. The fluorescence probes were placed in either donor or acceptor vesicles depending on the goal of the experiment.

**Kinetics of OA transfer from MBCD to phospholipid membranes.** To measure the kinetics of OA transfer (or delivery) from MBCD to lipid membranes, 1OA:6MBCD complexes were mixed in a stopped-flow apparatus with acceptor vesicles containing either FPE (Fig. 4) or pyranine (Fig. 5).

As shown in Fig. 4A, the mixing of 1OA:6MBCD complexes with FPE-labeled SUV produced a very fast dose-dependent decrease in FPE fluorescence with a $t_{1/2}$ of 30 ms. The maximal fluorescence change was dose dependent but not the kinetic rate constants. The rate of change in FPE fluorescence corresponds to the kinetics of OA dissociation from MBCD because, as reported recently by our group (19) and confirmed here, the change in FPE fluorescence produced by OA delivered without carrier, a protocol that reports only adsorption of OA to the lipid membrane is faster (Fig. 4B; $t_{1/2} < 10$ ms). As control, mixing SUV with MBCD that was not complexed with OA (empty MBCD) did not significantly affect FPE fluorescence (Fig. 4C).

To compare the delivery of OA by MBCD with other delivery systems, such as lipid vesicles under the same conditions, we measured the transfer of OA from donor vesicles to acceptor vesicles labeled with FPE. As shown in Fig. 4D, donor vesicles delivered OA at a slower rate than MBCD. The $t_{1/2}$ of OA transfer from vesicle to vesicle was 128 ms, which is within the range of vesicle-to-vesicle transfer rates recently reported by our group ($t_{1/2} = 100$ ms) (20).

For comparison with the FPE experiments, we conducted parallel experiments with pyranine. As shown in Fig. 5, mixing 1OA:6MBCD complexes with either SUV or LUV (100 µM egg-PC) both containing entrapped pyranine produced a very fast dose-dependent reduction in pyranine fluorescence. As observed for FPE, the maximal fluorescence changes were dose dependent but not the kinetic rate constants. We did not perform a curve-fitting analysis for the data with SUV because the drop in pyranine fluorescence from the initial value (SUV mixed with buffer) was almost complete before the first time point was measured. Instead, we estimated an upper limit for the $t_{1/2}$ of 50 ms, which falls within the same range reported by FPE (Fig. 4; $t_{1/2} = 30$ ms). Therefore, we conclude that both probes present in the SUV are measuring the same kinetic step of OA dissociation from MBCD. For the data with LUV, the mixing of 1OA:6MBCD with LUV produced a reduction in pyranine fluorescence with a longer estimated upper limit ($t_{1/2}$ of 100 ms). The slower rate probably reflects the slightly slower flip-flop of FA in LUV compared with SUV, as in Fig. 5B and D and as reported before (21). MBCD alone did not affect the pyranine fluorescence significantly in both SUVs and LUVs (data not shown).

In the stopped-flow experiments, the concentration of unbound OA in water after transfer reached equilibrium was estimated using the data obtained with ADIFAB. The mixing of 3, 6, and 12 µM OA complexed to MBCD with 100 µM SUV (Figs. 4A and 5A) resulted in a concentration of unbound OA of 0.02, 0.35, and 0.7 µM, respectively. In contrast, the concentration obtained with the mixing of 6 µM of OA not complexed with any carrier (Figs. 4B and 5B) was approximately 1 µM.

**The kinetics of MBCD-mediated efflux of OA from phospholipid membranes.** To supplement these OA transfer experiments, the reversibility of exchange of OA between MBCD and vesicles was examined by measuring the transfer of OA from FA donor SUV to empty MBCD (not complexed to FA) using the fluorescence probes FPE and pyranine in the donor SUV. Changes in FPE fluorescence reflect OA desorption from the outer leaflet of the SUV and binding to MBCD, whereas pyranine kinetics reflect flip-flop in addition to these two kinetic steps.

As shown in Fig. 6, when donor SUV (100 µM egg-PC) containing 12 mol% OA and labeled with FPE (Fig. 6A) or containing entrapped pyranine (Fig. 6B) were mixed with MBCD in buffer (350 or 700 µM MBCD), a rapid increase in the fluorescence of both probes was observed. The maximal fluorescence change but not the kinetic rate constant was dose dependent. Moreover, there was no significant difference between the rates of OA extraction detected by FPE ($t_{1/2}$ of 84 and 100 ms for 350 and 700 µM MBCD, respectively) and by pyranine ($t_{1/2}$ of 118 and 132 ms for 350 and 700 µM MBCD, respectively). Therefore, the kinetic step of flip-flop did not slow down the FA transfer from vesicles to MBCD. The rates of OA transfer from vesicle to MBCD were similar to those measured in experiments of OA transfer from vesicle to vesicle ($t_{1/2} = 100$ ms).
types of cells, one with active metabolism and the other with very slow utilization of OA. First, for comparison with cell experiments, we illustrate a standard protocol in model membranes. As shown in Fig. 7, following the addition of 1OA:6MBCD complexes to the external buffer, a rapid drop (t_{1/2} < 2s) in the fluorescence corresponding to an internal acidification was observed in protein-free LUVs containing entrapped pyranine (Fig. 7A). A slightly slower drop with a t_{1/2} of 15 s was observed when the complexes were added to cultured 3T3-L1 preadipocytes loaded with BCECF (Fig. 7B). Subsequent addition of excess MBCD (3- to 4-fold) before recovery of the fluorescence resulted in a rapid rise in fluorescence as predicted for extraction of FA from the membrane. These results mirror our published results with addition of uncomplexed OA followed by BSA in vesicles (23) and in adipocytes (24). Compared with BSA, a much higher ratio of MBCD to FA is required.

We conclude that the kinetics reported by these two probes corresponded to the desorption of OA from the donor SUV and that OA binding to MBCD was relatively rapid. Note that the concentration of MBCD needed to extract OA from the lipid membrane was much higher than the concentration of MBCD used in the delivery of OA and the concentration of FA donor vesicles. These observations confirm the preferential partitioning of OA into the lipid membrane than into the MBCD cavity.

**Delivery of oleic acid by MBCD to cells**

Because the long-term goal of our studies of FA transport in membrane models is to apply our methods to cells, we tested the ability of MBCD to deliver OA to different types of cells, one with active metabolism and the other with very slow utilization of OA. First, for comparison with cell experiments, we illustrate a standard protocol in model membranes. As shown in Fig. 7, following the addition of 1OA:6MBCD complexes to the external buffer, a rapid drop (t_{1/2} < 2s) in the fluorescence corresponding to an internal acidification was observed in protein-free LUVs containing entrapped pyranine (Fig. 7A). A slightly slower drop with a t_{1/2} of 15 s was observed when the complexes were added to cultured 3T3-L1 preadipocytes loaded with BCECF (Fig. 7B). Subsequent addition of excess MBCD (3- to 4-fold) before recovery of the fluorescence resulted in a rapid rise in fluorescence as predicted for extraction of FA from the membrane. These results mirror our published results with addition of uncomplexed OA followed by BSA in vesicles (23) and in adipocytes (24). Compared with BSA, a much higher ratio of MBCD to FA is required.
Cyclodextrins deliver fatty acids to vesicles and cells (25). Additionally, MBCD did not affect the BCECF fluorescence recovery in HepG2 cells.

DISCUSSION

Although various CDs have been used in cell studies, especially for extraction and delivery of cholesterol, few details of the kinetics and partitioning have been reported. We took advantage of the ionization properties of FA and our novel fluorescence assays developed to study the FA transport in membranes (17–19), to monitor the movement of FA without separation of donor and acceptor. We show that MBCD has ideal properties for delivering FA. MBCD rapidly releases FA to the acceptor membrane, and the FA so delivered binds instantly to outer leaflet of the membrane and undergoes fast flip-flop to equilibrate in the membrane leaflets. However, unlike our previous protocol with micromolar concentrations of unbound OA, the instantaneous concentration of unbound FA with MBCD is always much lower. Our major findings are summarized below.

Conditions for preparation of MBCD complexes with FA

Formation of FA:CD complexes is considered a true molecular dispersion and not micellization as achieved by detergents (26). Hydrogen bonds between the FA carboxyl to extract FA from lipid vesicles and cells, a reflection of the lower affinity of MBCD for FA.

As shown in Fig. 8A, 3T3-L1 preadipocytes showed dose-dependent pH drops that were complete within 60 s and were indistinguishable from those for OA added in ethanol. The magnitude of the fluorescence drop obtained with 10 μM OA complexed with 60 μM MBCD was the same as with 10 μM OA in ethanol (unbound OA), suggesting that all of the OA partitioned from MBCD to the cell membrane. The same results were observed with 20 μM OA:120 μM MBCD compared with 20 μM OA in ethanol and for 30 μM OA:180 μM MBCD compared with 30 μM OA in ethanol (data not shown). These experiments established experimental conditions for delivery of OA by MBCD that result in complete transfer of OA from MBCD. It is important to note that conditions can be modified to result in partial transfer of OA (partitioning) if this protocol is desired.

Finally, we tested the delivery of OA by MBCD to hepatocytes (cultured HepG2 cells). These cells metabolize FA faster than 3T3-L1 preadipocytes, which is detected as recovery in the BCECF fluorescence (25). As shown in Fig. 8B, addition of OA:6MBCD resulted in a rapid fluorescence drop that was completed within 45 s, in agreement with our previous study with OA delivered in ethanol to HepG2 cells (25). Additionally, MBCD did not affect the BCECF fluorescence recovery in HepG2 cells.

Fig. 5. The kinetics of OA transfer from MBCD to lipid vesicles (SUV and LUV) measured with pyranine. A suspension of lipid vesicles containing entrapped pyranine (100 μM egg-PC) was rapidly mixed with increasing amounts of OA:MBCD complex at a fixed molar ratio of 1 OA:6 MBCD (A, SUV; C, LUV). The final concentrations of OA:MBCD complexes were 3 μM OA:18 μM MBCD, 6 μM OA:36 μM MBCD, and 12 μM OA:72 μM MBCD. As controls, the same lipid vesicle preparations were mixed with unbound OA (5 μM, B; 6 μM, D). Lipid vesicles were also mixed with only buffer as mixing control. Each fluorescence trace is the average of 5 to 10 measurements and represents one experiment.
MBCD maintains a low concentration of unbound OA in buffer

Physiological concentrations of unbound FA in the extracellular compartment are in the very low nanomolar range (7) because FA binds to cell membranes, lipoproteins, and albumin in the blood and tissue compartments. As the concentration of unbound FA in equilibrium with MBCD and lipid membranes has not been reported, we showed (Fig. 3B) that MBCD maintained a low concentration of unbound FA (OA) in buffer. The concentration of OA complexed to MBCD is shown. The dashed line is the single exponential fit (two separate fittings). The \( t_{1/2} \) values for OA delivery and extraction by MBCD in cells are 15 and 20 s, respectively. One representative experiment is shown in each panel.

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Our comparison of different methods of presenting OA to membranes showed that the concentration of aqueous unbound OA, measured after equilibration was established between the donor and the lipid membrane, depends on the vehicle used to deliver the OA (unbound, complexed to MBCD or to albumin). At lower concentrations of total OA, the reduction in the concentration of unbound OA by MBCD was comparable to very low nanomolar levels obtained in the presence of albumin (Fig. 3B). For higher

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Fig. 6. The kinetics of OA transfer from lipid vesicle (SUV) to MBCD measured with FPE and pyranine. FA donor SUVs (100 \( \mu \)M egg-PC + 12 mol% OA) either labeled with 1 mol% FPE (A) or containing 0.1 mM entrapped pyranine (B) were mixed rapidly with increasing amounts of empty MBCD (350 or 700 \( \mu \)M MBCD). The fluorescence traces from both FPE and pyranine were well fitted by a single exponential function (dashed line). The \( t_{1/2} \) values of FPE fluorescence after addition of MBCD to SUV labeled with FPE were 84 ms for 350 \( \mu \)M MBCD and 100 ms for 700 \( \mu \)M MBCD. The \( t_{1/2} \) values of pyranine were 118 ms for 350 \( \mu \)M MBCD and 132 ms for 700 \( \mu \)M MBCD. Donor SUVs were mixed with only buffer as a mixing control. Each fluorescence trace is the average of 5 to 10 measurements and represents one experiment.

Fig. 7. Delivery and extraction of OA to/from lipid vesicles (LUV) and cells by MBCD. Fluorescence changes in LUVs (50 \( \mu \)M egg-PC) containing entrapped pyranine (A) and in 3T3L-1 preadipocytes with BCECF (B) upon addition of 1OA:6MBCD complex. One representative experiment is shown in each panel.
Cyclodextrins deliver fatty acids to vesicles and cells

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cell studies using CD as a donor have not been able to
measure the rate of dissociation of FA from CD because
they typically used incubation times of hours (15, 16).
Here, for the first time, we measured the kinetics of FA
delivery by MBCD using our well-defined fluorescence as-
says with FPE and pyranine.

We conclude that the small decrease in the rate of bind-
ing to the vesicle outer leaflet (SUV and LUV) in the pres-
ence of MBCD compared with the rate with unbound FA
represents the kinetics of dissociation of OA from the
MBCD complex. These kinetics are faster than the kinet-
ics of FA dissociation for albumin (hundreds of milli-
seconds) (20).

An important conclusion of this study is that delivery of
uncomplexed OA in the concentrations that we used does
not produce an artifactual faster rate of FA flip-flop as sug-
gested by another investigator (29). They confirm the
same conclusion recently drawn from transfer studies us-
ing vesicles and albumin as FA donors (19).

MBCD extracts OA rapidly from phospholipid
membranes

Our direct measurement of the desorption of OA from
the outer leaflet of the membrane bilayer by FPE showed
definitively that the rate limiting step for transfer of OA
from vesicle to MBCD is desorption from the membrane.
In our experimental design with empty MBCD, the con-
centration to partition OA out of the vesicles was much
higher (~3 times) than that used for delivery, another
reflection of the weak affinity of OA for MBCD. Furth-
more, a higher ratio of MBCD to lipid vesicles also had
to be used (MBCD in excess) to extract all of OA from
the lipid membrane. Thus, when designing experiments
with MBCD; it is imperative to consider that FA could be
extracted from rather than delivered to the cell mem-
brane, depending on the ratio of MBCD to lipid mem-
brane or to cell.

CD has been used widely in raft membrane studies to
enhance desorption of cholesterol from membranes (30,
31). In contrast to our results for FA desorption, CD en-
hances the rate of cholesterol desorption, [t 1/2 of hours
without MBCD (32)], probably by reducing the free en-
ergy of hydration of cholesterol (33, 34). The concentra-
tion of MBCD used in our cell studies is much lower than
the concentrations required to extract cholesterol from
cells. It is unlikely that MBCD extracted phospholipids
from the cells or vesicles in our experiments because our
MBCD concentrations never exceeded 0.2 mM for deliv-
ery or 0.7 mM in the extraction experiments. CD at a con-
centration of 10 mM extracted <0.2% of phospholipids
present in a lipid membrane, but considerable extraction
of phospholipids with perturbation of the membrane
structure has been observed at CD concentrations of 30–50
mM (35, 36). However, our study does reveal an important
pitfall in the use of MBCD to extract cholesterol and/or
phospholipids in cells: MBCD at these higher concentra-
tions also will extract FA, and possibly other cellular con-
stituents, and other single-chain amphiphiles, such as
lyso phospholipids and acyl-CoA, thus altering the nutri-

concentrations of total OA, unbound OA was 2 to 3 times
lower in the presence of albumin than in the presence of
MBCD because of the multiple binding sites and higher af-
finity of albumin. MBCD could be advantageous as a deliv-
ery vehicle for studying FA transport and uptake because
partitioning favors FA binding to membranes, and the con-
centration delivered approximates the concentration of FA
in the MBCD complex. When albumin is used as a donor,
the concentration delivered initially to cells is much lower
than the total amount of FA in the complex (7) and cannot
be determined unless the FA concentration in the cell is
measured independently, an assay that rarely is performed.

MBCD delivers FA to phospholipid membranes in
milliseconds

Another important consideration for effective delivery
of FA to membranes is the kinetics of transfer. Previous

Fig. 8. Delivery of OA to cells by MBCD. Fluorescence changes in
3T3-L1 preadipocytes containing entrapped BCECF upon addition
of 10 µM OA:60 µM MBCD, 20 µM OA:120 µM MBCD, and 30 µM
OA:180 µM MBCD. Uncomplexed OA, added from an OA stock
solution in ethanol, was used as a control (upper trace). The data
with 30 µM OA in MBCD were fitted to a single exponential decay
(t 1/2 of 20s, dashed line) (A). Fluorescence changes in HepG-2
cells containing entrapped BCECF upon addition of 25 µM OA:150
µM MBCD. The decrease in BCECF fluorescence was complete
within 45 s after addition of 1OA:6MBCD (B). One representative
experiment is shown in each panel.
MBCD delivers FA to cells rapidly and does not interfere with intracellular FA metabolism

Our cell studies were designed to investigate the utility of MBCD to deliver OA to cells with a slow rate of metabolism of exogenously added FA (3T3L1 preadipocyte cells; Figs. 7B and 8A) and cells with fast metabolism (HepG2 cells; Fig. 8B). We demonstrated in both cell types that adsorption and transbilayer movement of OA delivered by MBCD are complete within seconds and that all of the OA that had been complexed with MBCD partitioned from MBCD into the cell membrane. We also showed that MBCD did not affect the recovery of BCECF fluorescence adsorption and transbilayer movement of OA delivered by MBCD are complete within seconds and that all of the OA that had been complexed with MBCD partitioned from MBCD into the cell membrane. We also showed that MBCD did not affect the recovery of BCECF fluorescence.

We conclude that the concentration of unbound FA in equilibrium with donors and acceptors does not affect kinetic measurements of membrane transport in cell experiments, which has been a frequent reservation about the use of unbound FA as a source of exogenous FA. MBCD appears to be an ideal vehicle for delivery of long-chain FA to both model membranes and cells. Used according to our protocols, it averts several complications and artifacts that might be introduced by albumin. However, the higher concentrations of CD commonly used to extract cholesterol might be introduced by albumin. However, the higher concentrations of CD commonly used to extract cholesterol from cells are likely to extract FA and other single-chain amphiphiles. Rather than affect only the plasma membrane lipid content, addition of MBCD could result in alterations in cell metabolism and plasma membrane structure independent of changes resulting from lower levels of cholesterol.

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