Rapid Flip-flop of Oleic Acid across the Plasma Membrane of Adipocytes*

Nonesterified long-chain fatty acids may enter cells by free diffusion or by membrane protein transporters. A requirement for proteins to transport fatty acids across the plasma membrane would imply low partitioning of fatty acids into the membrane lipids, and/or a slower rate of diffusion (flip-flop) through the lipid domains compared to the rates of intracellular metabolism of fatty acids. We used both vesicles of the plasma membrane of adipocytes and intact adipocytes to study transmembrane fluxes of externally added oleic acid at concentrations below its solubility limit at pH 7.4. Binding of oleic acid to the plasma membrane was determined by measuring the fluorescent fatty acid-binding protein ADIFAB added to the external medium. Changes in internal pH caused by flip-flop and metabolism were measured by trapping a fluorescent pH indicator in the cells. The metabolic end products of oleic acid were evaluated over the time interval required for the return of intracellular pH to its initial value. The primary findings were that (i) oleic acid rapidly binds with high avidity in the lipid domains of the plasma membrane with an apparent partition coefficient similar to that of protein-free phospholipid bilayers; (ii) oleic acid rapidly crosses the plasma membrane by the flip-flop mechanism (both events occur within 5 s); and (iii) the kinetics of esterification of oleic acid closely follow the time dependence of the recovery of intracellular pH. Any postulated transport mechanism for facilitating translocation of fatty acid across the plasma membrane of adipocytes, including a protein transporter, would have to compete with the highly effective flip-flop mechanism.

Adipocytes are highly differentiated cells specialized in handling large quantities of un-esterified long-chain fatty acids (FA). During lipid storage in the fed state, FA are released in

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the blood from chylomicros by lipolysis or from albumin, move through the endothelium, bind to the outer leaflet of the plasma membrane, and cross the membrane bilayer. FA are trapped in the cytoplasm by conversion to acyl-CoA and stored primarily as triglycerides in lipid droplets (reviewed in Glatz et al. (1)). During lipolysis of stored triglycerides in the fasting state, FA are released from intracellular lipid droplets, move to and across the plasma membrane, and are released into the interstitial space, where they bind to albumin. Subsequently, the FA pass through the endothelial cells or diffuse through the spaces between them to reach the blood.

These large bidirectional fluxes could occur by several postulated mechanisms, both complex and simple. Cytological changes observed in adipocytes during release and deposition of intracellular lipid led to the complex model that large intracellular fluxes of FA occur by formation of vesicles from the plasma membrane of adipocytes and endothelial cells (2, 3). It has also been postulated that caveolin, a protein present in invaginations of the plasma membrane (caveolae), plays a role in FA uptake (4). There are several other candidate proteins for enhancement of FA fluxes into adipocytes and other cells; for a detailed review see Ref. 5. These include the adipocyte fatty acid-binding protein (aP2) located in the cytosol (1, 6, 7) and membrane-bound “transport proteins” (8–10), such as “FA translocase” (FAT (FATP)/CD36) (11, 12), adipose differentiation related protein (ADRP (13)), and “FA transport protein” (14, 15). It is not clear whether these proteins enhance the transmembrane movement of FA or catalyze some other step in the overall process of FA uptake (16–19).

The simplest mechanism is that FA diffuse freely and rapidly as monomers from the blood plasma to the cytoplasm (and the reverse) and cross cell membranes by permeation of their unionized form. For decades, this has been the paradigm for short-chain FA and other weak electrolytes (20–25). Membrane permeability coefficients for short- (e.g. acetic acid) and medium-chain (e.g. octanoic acid) FA are high and increase with increasing chain length (hydrophobicity), in accord with the Overton rule, up to a chain length of 12 carbons (24, 26, 27).

Long-chain fatty acids (LCFA) have much lower water solubility (~6 μM for oleic acid (28)) but bind to albumin in a transitory manner to enable large fluxes. Therefore, their permeability coefficients cannot be measured in the same way as those for short- and medium-chain FA (24). However, because LCFA bind more avidly than short-chain FA to phospholipid membranes, the Overton rule would predict that the membrane permeability of long-chain FA should be higher than that of medium-chain FA, as long as association, flip-flop, and desorption are not dramatically hindered by the longer alkyl-chain lengths (16, 25, 29–39). In cell membranes, transmembrane fluxes of long-chain FA would be limited primarily by diffusion.
of FA monomers through the unstirred water layers adjacent to the membrane, a limitation that might be overcome by albumin (24, 31).

Previously, we developed a method to monitor the transbilayer movement of FA that uses a fluorescent pH probe (pyranin) trapped inside phospholipid vesicles (32, 33). This method was designed to test the hypothesis that fatty acids in their un-ionized form will cross the phospholipid bilayer, reach ionization equilibrium in the membrane interface, and release protons that are detected by a water-soluble pH dye. Binding of FA at one side of the membrane is not sufficient to cause pH changes at the opposite site of the membrane, and dissociation of the proton from the FA (but not the desorption of FA from the membrane) is measured. Furthermore, natural FA instead of synthetic derivatives can be studied by this method.

The changes in pH measured for several FA by stopped-flow fluorescence were extremely rapid (t/suboff/ < 50 ms (34)) and matched quantitatively the expected changes based on the vesicle diameter and buffer strength of the internal volume of the vesicles (33, 34). We concluded that LCFA flip-flop across model phospholipid bilayers readily in their un-ionized form (32–34). The desorption step from the vesicular membrane is slower than flip-flop but still fast (K/suboff/> 1 s⁻¹) for FA with an acyl-chain length of up to 18 carbons (35, 36). On the basis of these properties in model membranes, the diffusion mechanism can be considered a viable mechanism for transport of FA across cell membranes (16, 25, 29, 37). We also extended our measurements of the transmembrane movement of FA to cells with a trapped pH dye (BCECF), both in suspension (38, 40), and as single cells (41). Decreases in cytosolic pH occurred immediately after the addition of FA to the external medium, supporting the hypothesis that FA diffuse across the plasma membrane by the flip-flop mechanism.

Despite these new approaches and results from other laboratories supportive of rapid spontaneous flip-flop of FA in membranes (39, 42–47), several areas of contention remain. Some investigators have proposed that the unfacilitated transmembrane movement (flip-flop) of FA is the rate-limiting step for FA transport in model membranes (30, 48, 49). Moreover, some have argued that the flip-flop of FA in model membranes might be much faster than in biological membranes, where the different curvature, lipid composition, and the presence of membrane proteins might slow down FA diffusion (9, 10, 30, 50). Specific FA-binding proteins in the membrane might then catalyze the slow diffusion of FA across the membrane. Although mechanisms have not been established for the candidate transporters, one postulated mechanism is transport of the FA anion, as described in detail recently (51). It has been variously suggested that free diffusion does not compete well with protein-mediated transport of FA anions at low FA concentrations (9, 10, 51, 52), or alternatively at high concentrations (50), or plays no significant role in FA uptake at any concentration (14, 15).

In this study we focus on the adsorption and transmembrane movement steps of FA transport in the plasma membrane of adipocytes. We use a dual fluorescence approach to monitor the adsorption step at the same time as the flux across the membrane is measured. We present new evidence favoring fast adsorption and flip-flop of FA in vesicles prepared from the plasma membrane of adipocytes and in the plasma membrane of intact adipocytes.

**EXPERIMENTAL PROCEDURES**

**Preparation of Small Unilamellar Vesicles (SUV)—**Protein-free SUV with trapped 2,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (1 mM) were prepared by sonication as previously described (32).

**Preparation of Adipocytes—**Intact adipocytes were prepared by collagenase digestion of the epididymal fat pads of 2 to 4 male rats (180–200 g) as described (40). BCECF was trapped by incubating for 30 min with 1 µM BCECF-AM at 37 °C under gentle shaking and washing the cells with albumin-free Krebs buffer (40).

**Preparation of Plasma Membrane Vesicles (PMV)—**Epididymal fat cells were isolated as above from 20 male rats as described (40) and subsequently homogenized in buffer (20 mM HEPES-NaOH, 5 mM EDTA, 250 mM sucrose, pH 7.40) containing 0.5 mM of the fluorescent probe pyranin. The plasma membrane fraction was isolated by differential centrifugation according to Simpson et al. (53) as modified by Kublaoui et al. (54), and the untrapped pyranin was removed by gel filtration (Sephadex G-25). The phospholipid and protein contents of the obtained PMV and adipocytes were determined with standard assays (55, 56). The protein/phospholipid ratio was high (1:1, w/w). Homogenization of the vesicles by extrusion was not successful, possibly because of the high protein content. Electron micrographs (negative stain) showed that the vesicle population of the preparation was heterogeneous in size and lamellarity yet included a significant fraction of large unilamellar vesicles with a diameter of 100 to 500 nm. Adipocyte PMV made using this protocol have the right-side-out orientation, according to independent analyses of insulin receptor orientation (57).

**Fluorescence Measurements—**Pyranin, BCECF, and its acetoxymethylester (BCECF-AM) were purchased from Molecular Probes, Eugene, OR. Vesicles (SUV or plasma membrane vesicles) were suspended in a stirred polystyrene cuvette containing 2.5 mM of buffer at pH 7.4 and 20 °C with, in some cases, 0.2 µM of the engineered fatty acid-binding protein ADIFAB (Molecular Probes). Measurements were done with a SPEX Fluoromax fluorimeter with the sampling time set to 1.0 s (the mixing time in the cuvette). The fluorescence of pyranin was measured using excitation at 455 nm and emission at 509 nm with a 2-nm bandpass. For ADIFAB, excitation was at 390 nm and the emission at 505 nm; 432 nm was sampled alternately with 2.0-s time intervals, and the fluorescence ratio (R) was calculated (bandpass 3–5 nm). For BCECF, excitation wavelengths 439 (pH-dependent) and 505 nm (pH-independent) were used and emission at 535 nm was measured (sampling time, 2.0 s; bandpass 3 nm). Intact adipocytes (10⁶ cells/ml) were suspended in a well stirred cuvette with 2 ml of albumin-free Krebs buffer (pH 7.4) with 0.2 µM ADIFAB at 37 °C. The fluorescent signals of BCECF and ADIFAB were sampled simultaneously, with 4.0-s time intervals, and their fluorescence ratios were evaluated (3-nm bandpass). There was no interference between the signals of BCECF and ADIFAB at the chosen wavelengths.

**Calibration of BCECF—**The relationship between internal pH and the BCECF fluorescence ratio was calibrated by permeabilizing the cells or SUV to H⁺ with 1 µM nigericin (a K⁺/H⁺ exchanger) and to K⁺ with 1 µM valinomycin. The external pH was adjusted with aliquots of KOH (33), and the pH was measured with a mini-pH electrode. pH equilibration was ensured by the two ionophores and the relatively high K⁺ concentration on both sides of the membrane.

**Measurement of Aqueous Oleic Acid Concentrations—**The fluorescent FA indicator ADIFAB (FFA Sciences, San Diego, CA) was dissolved (0.2 mM) in the buffer used for measurements with PMV or cells. The concentration of unbound aqueous oleic acid ([OA]₀) was calculated from [OA]₀ = K/R - R₀/(11.5-R₀), where K/R₀ = 0.28 µM for OA, and R is the fluorescence ratio of ADIFAB. The value of R₀ was provided by FFA Sciences, or was measured when [OA]₀ was assumed to be zero (58).

**Measurement of Metabolic Fate of FA—**[9,10-³H]Oleic acid was used to monitor incorporation of exogenous oleic acid into esterified products. 50 nmol of OA* (i.e. OA with a fraction of labeled OA, 1000 dpm/nmol) was added to 2.0 ml of adipocytes in suspension (10⁶ cells/ml) at 37 °C. All reactions were stopped by mixing the cell suspension with 10 ml of chloroform/methanol (2:1, v/v) at chosen time intervals after addition of OA* (10 s, 1, 5, and 20 min). The lipid phase was extracted according to the method of Folch et al. (59) and concentrated by evaporation of the organic solvent under a stream of nitrogen gas. The mixture of products was subsequently redissolved in 100 µl of chloroform and separated by thin-layer chromatography (TLC; hexane/ethyl ether/acetic acid, 80:20:1) from unlabeled lipids (diglycerides, phospholipids, and oleic acid) were added as indicators of the minor components. The developed TLC plate was visualized in iodine vapor, and the corresponding lipid components were scraped off and quantified by scintillation counting.

**RESULTS**

**Rapid Flip-flop of Oleic Acid in the Plasma Membrane—**Changes in internal pH upon addition of external FA have been observed both in protein-free model membranes and in cells.
The rapid flip-flop of un-ionized FA in model membranes does not guarantee that FA redistribute quickly across the plasma membrane of cells by the same mechanism (30, 60). In addition, the observed internal acidification upon the addition of FA to cells might be attributed to an indirect effect on cell metabolism (9). Furthermore, the cytosol contains an abundance of intracellular membranes and FABP that could affect pH changes following exposure to FA.

To address such questions, we applied our flip-flop assay (32, 33) to vesicles prepared from the plasma membrane of rat epididymal fat cells. The isolated PMV contain proteins (including putative FA transport proteins) but do not metabolize FA in the absence of essential co-factors and reactants in the cytosolic compartment. PMV with trapped pyranin were suspended in buffer, and an aliquot of oleic acid (10 nmol, corresponding to a final concentration in the cuvette of 4 μM) was added to the buffer (Fig. 1). The pyranine fluorescence intensity dropped, corresponding to a drop in internal pH. This change occurred within a few seconds, the time resolution (mixing time) of the experiment. Furthermore, as with protein-free phospholipid vesicles, the pH gradient persisted because of very slow leakage of H⁺. We attribute the pH change to fast translocation of un-ionized FA across the lipid bilayer of the plasma membrane following ionization of FA in the inner leaflet, as observed previously in protein-free vesicles (29, 32, 33). Because ionized FA translocate very slowly, as shown previously in experiments with valinomycin (33), transport of cyclic H⁺ is limited and the generated pH gradient dissipates very slowly.

**Acid Partition of Oleic Acid in the Lipid Phase of the Plasma Membrane**—To test further our hypothesis that the movement of un-ionized FA through the plasma membrane of adipocytes occurred through the lipid domains (phospholipid bilayer) of the plasma membrane, we addressed whether the added FA bind primarily to the lipid domains or to membrane proteins. The partitioning of FA between the membrane and adjacent water phases could be qualitatively and quantitatively different for plasma membranes compared to protein-free model membranes because of the high protein content of the former.

Using the fluorescent FA indicator ADIFAB (58, 61), we investigated whether a single partition coefficient can describe FA distribution between the aqueous phase and PMV in the same way as for protein-free model membranes (58). Plasma membrane vesicles were suspended in buffer containing 0.2 μM ADIFAB. The concentration of unbound oleic acid in the water phase outside the vesicles ([OA]₁) was evaluated (“Experimental Procedures”) using

\[ R_p = \frac{[OA]_m}{[OA]_p} \]

Before oleic acid was added to the suspension of PMV, [OA]ₐ was already 0.24 μM (Fig. 2), most likely because of FA present in the plasma membrane, some of which might have been produced by hydrolysis during the preparation of PMV. When oleic acid was added to aliquots of 2 μM, the ADIFAB fluorescence ratio increased, and the calculated [OA]ₐ increased linearly with the total concentration of OA. Because the solubility limit of oleic acid is about 6 μM (28), we assumed that after each addition the added oleic acid bound to the PMV, and that no oleic acid precipitated or bound to the walls of the cuvette (58). The concentration of oleic acid in the membrane phase, [OA]ₐ, is then equal to the difference between the total added oleic and the unbound oleic acid in the water phase after equilibration. Independently, we determined the amount of phospholipid in the PMV preparation (76 μM phospholipid for each experiment illustrated in Figs. 2 and 3) by chemical analysis (“Experimental Procedures”). The volume of the phospholipid phase (Vₐ) was calculated assuming

\[ Vₐ = \frac{10^{-3} \times \text{mM phospholipid}}{N_a} \]

where N_a is the volume (2.5 ml) of the water phase (61). The data in Fig. 2 encompassed a wide range of concentrations of OA, up to 25 mol% with respect to phospholipid. Partitioning was quantitated from the linear relationship between the membrane-bound oleic acid calculated after each addition of oleic acid (Fig. 2) and [OA]ₐ (not shown). The apparent partition coefficient (Kₚ = [OA]ₐ/[OA]ₐ) evaluated from the slope was 1.0 × 10⁶, similar to the Kₚ for protein-free vesicles (58, 61). When the experiment was repeated with PMV prepared from adipocytes preincubated with insulin, we obtained results (not shown) very similar to those shown in Fig. 2.

**Dual Fluorescence Measurements in Adipocytes**—In our previous studies, the pH changes measured with fluorescent pH probes were faster in model membranes (t₁/₂ < 1 s) than in cells (t₁/₂ = 8 to 1–2 min). It was necessary to add higher concentrations of FA to the cells to observe changes in pH in at least in part because of the lower surface-to-internal volume ratio in cells compared with vesicles. In our experiments adding FA without albumin, if some of the added FA precipitated, the association of the FA to the cells would be limited in part by desorption of FA from the precipitates. The resultant complex kinetics could have produced the biphasic changes noted in some of our previous studies (40). In the experiments reported herein, we overcame such potential artifacts by using a more sensitive fluorimeter that yielded reliable measurements with a 10-fold lower concentration of FA and permitted simultaneous measurement of changes in unbound external FA and pH₁₉. Our previous studies also used pyranin for phospholipid ves-
icles and BCECF for cells, because intact cells cannot internalize pyranin. To ensure that the BCECF provides a quantitative measure of protons transported into cells, we calibrated the pH in changes as measured by BCECF. The relationship between the ratio of the pH-dependent and pH-independent fluorescence of BCECF was the same whether the probe was dissolved in buffer, trapped in SUV, or trapped in cells (Fig. 3).

Applying our improved methodology to cells, we added K





-octanoate at final concentrations below the solubility limit of oleic acid at pH 7.4 (6 μM) to a suspension of adipocytes. We also performed dual fluorescence measurements to discriminate the adsorption step of FA transport (ADIFAB) from the combined steps of adsorption and transmembrane movement (BCECF).

BCECF was trapped inside the adipocytes, and ADIFAB was present in the external buffer. Changes in pH in and external [OA]o were evaluated simultaneously in 4.0-s time intervals (see “Experimental Procedures”). When 10 nmol of oleic acid (4 μM final concentration) was added to a suspension of adipocytes (10^6 cells/ml), the concentration of unbound oleic acid, [OA]u, increased only to 20 nM in the first 4-s time interval (Fig. 4). This result from ADIFAB fluorescence indicates that >99% of the added oleic acid immediately partitioned to the plasma membrane but did not provide information about whether the added oleic acid crossed the bilayer. The latter was monitored by the BCECF fluorescence, which showed a drop in pH in of 0.01 units immediately after the addition of oleic acid and the completion of this drop within the time resolution (4 s) of the experiment. We increased the time resolution to 1.0 s by repeating the experiment in the absence of ADIFAB and measured only the fluorescence of BCECF. The decrease in pH in was complete within 1 to 2 s (the time required for mixing the cell suspension) after the addition of oleic acid (not shown). Although the fast initial pH in drop after addition of FA to cells is probably because of flip-flop of un-ionized FA as in protein-free model membranes and in PMV, it may also be because of some indirect effect of the added FA on cellular metabolism. To exclude the latter, we performed additional quantitative studies, repeating the experiment of Fig. 4 for different amounts of oleic acid and examining the metabolic fate of oleic acid, as described below.

Concentration Dependence of pH Drop—Establishment of the mechanism of FA movement through membranes requires quantitation of the relationship between the concentration of FA added to cells and the amount actually taken up by the cells. In most studies this has been done by using albumin as vehicle for FA delivery and calculating the concentration of unbound FA in equilibrium with albumin. The amount of FA taken up by the cells is measured by stopping the incubation at chosen time intervals after the addition of the albumin/FA aliquot and separating the cells from the incubation medium. Unless the cell contains high levels of endogenous FA, the actual amount of FA delivered to the cells is not equal to but exceeds the unbound pool of FA, because some of the FA bound to albumin is also transferred to the cells. In fact, the FA/albumin ratio changes continuously until equilibrium is reached, and, depending on the experimental conditions, the concentration of unbound FA can change as well (37). In experiments without albumin, the concentration of unbound FA added to cells is fixed and known precisely. Furthermore, essentially all of the added FA binds to the cell, so that there is always a net flux of FA into the cell. In experiments using the protocol of Fig. 4 and aliquots of oleic acid below its solubility limit, the drop in pH in was proportional to the amount of oleic acid added (Fig. 5A) within the error of the measurements. With the addition of ~10-fold lower concentrations of oleic acid, the pH decrease was ~10-fold lower than in our previous study (40).

Partitioning of Oleic Acid into the Plasma Membrane of Intact Cells—As with PMV, we addressed whether the added FA binds mainly to the phospholipid domains of the plasma membrane or to proteins in the plasma membrane. From the ADIFAB fluorescence we evaluated the immediate rise (i.e. after 4 s, the first reading) in unbound [OA]u after adding different concentrations of oleic acid. An aliquot of 10 nmol of oleic acid is the equivalent of a total OA concentration of 4 μM. We found that the increase in ADIFAB fluorescence, corresponding to the increase in unbound oleic acid, was proportional to the amount of oleic acid added (Fig. 5B). Assuming that none of the added OA became esterified in the first 4 s (see below) and that the plasma membrane provided the major pool of cellular fatty acid-binding sites, at least initially (e.g. Ref. 25, 62, and 63), we calculated the equilibrium distribution (Kp) between the amount of unbound oleic acid in the external water phase ([OA]o, measured with ADIFAB) and the amount bound to the plasma membrane ([OA]m), calculated from the total added...
[OA] minus unbound [OA]a. As with PMV, the volume ratio between the aqueous phase and membrane phase was estimated by quantifying the phospholipid content of fat cells (150 nmol of phospholipid/106 cells). The concentration of oleic acid in the plasma membrane was calculated for each addition of oleic acid and the apparent partition coefficient from the slope ([OA]a/[OA]m) (Fig. 5B). We found Kp = 1.4 × 106, close to the Kp found for model phospholipid membranes (0.5 × 106 (61)) and for plasma membrane vesicles (1.0 × 106). The quantitative similarity of these results to those for the PMV is additional evidence that the initial drop in pH in adipocytes upon addition of FA is probably because of the flip-flop mechanism.

**Esterification of FA**—In contrast to PMV, initial acidification following the addition of oleic acid to adipocytes returned to the basal value within 2 min (Fig. 4). This recovery might be because of proton leak across the membrane by the Na+/H+ exchanger, by any other H+ leakage, or by a pumping pathway. It is also possible that the recovery of pH in is due, at least in part, to esterification of FA, which offsets the drop in pH in caused by diffusion of un-ionized FA across the plasma membrane, as we postulated previously (40). The net reaction of the esterification of OA is as follows.

3 Oleate + H+ + glycerol → triglyceride + 3H2O (Eq. 1)

With this simplified overview of the contributing pathways of FA esterification, we can predict the return to initial pH in in a straightforward way. According to our hypothesis, each oleic acid that diffuses across the plasma membrane (adsorption, flip-flop, and desorption) releases one H+ in the cytosol. For every oleic acid that is esterified, one H+ is removed from the cytosol, thereby offsetting the drop in pH in caused by transmembrane FA transport.

To monitor both pH in changes and metabolism we added 50 nmol of oleic acid plus a trace of 3H-labeled OA (i.e. OA*) to five separate 2-ml aliquots of an adipocyte suspension with trapped BCECF. The fluorescence experiment (Fig. 6, top) was terminated by mixing the content of each cuvette with 10 ml of stop agent at chosen times of 10 s, 1, 5, or 20 min (see “Experimental Procedures”). In the control experiment (zero time point), cells were mixed with stop agent and OA* was added. The lipids were then extracted and different end products (unesterified oleic acid, diglycerides, and triglycerides) of OA* determined (“Experimental Procedures”). The adipocytes readily esterified the added FA, with kinetics that corresponded closely with the kinetics of recovery of the pH gradient. Fig. 6 (lower panel) shows that at 10 s, the majority of oleic acid (80%) was unesterified, whereas at 1 min, 40% of the added oleic acid remained unesterified. The esterification kinetics were slightly faster when smaller amounts of OA* were used (as low as 5 nmol), but the distribution of the end products after 1 min was the same (not shown). We found the same general trend for the kinetics of metabolism of low concentrations of oleic acid added to adipocytes as reported previously (51).

**DISCUSSION**

Our current data support the hypothesis that FA cross the plasma membrane of adipocytes rapidly by free diffusion. The adipocyte has been at the center of research and discussion about mechanisms of transport of FA through membranes in...
general. Our new results show that some of the differences in interpretation of uptake of FA into adipocytes may be a result of differences in methodologies and in the duration of measurements. In "classic" uptake studies, cells are incubated with FA-albumin complexes and initial uptake rates are evaluated by measuring traces of radiolabeled FA trapped in the cells within the first few minutes after the incubation. In these assays, separation of the FA-albumin complexes and cells, followed by washing with FA-free albumin, is necessary (16, 51). FA uptake depends on the FA/albumin ratio, i.e. the buffered $[\text{FA}]_a$, at equilibrium with the albumin (33, 64). The initial rate of FA uptake has been described as saturable when plotted against $[\text{FA}]_a$ and as diminished by certain metabolic inhibitors (8–10). Thus, several authors have argued that diffusion is a feasible mechanism at high FA concentrations in the medium but that FA are transported primarily by proteins at the low "physiological" concentrations of $[\text{FA}]_a$ in media containing albumin (i.e. $[\text{FA}]_a = 2–20$ nM) (9) or $\leq 2$ $\mu$M (10), see also Refs. 6 and 65). According to a recent detailed analysis of uptake of oleic acid into adipocytes, the diffusion mechanism is not significant below molar ratios of oleic acid/albumin of 3:1 (51).

We previously developed and employed a new fluorescence assay for uptake of FA into cells (38) that eliminated the need to separate FA donors (e.g. FA-albumin complexes) and cells and used natural FA instead of probe molecules. We carried out our present experiments without albumin as a donor of FA to avoid the complication of not knowing precisely what proportion of added FA partitions from albumin to the cells and (ii) to focus on the membrane events of FA transport rather than on issues of partitioning of FA from albumin. The concentrations of added oleic acid were in the low micromolar range, and essentially all of the oleic acid partitioned into the cells.

In contrast, in the presence of albumin, FA partition according to their relative affinities for binding sites on albumin and on the membrane, and the amount delivered to cells is neither the amount on the aqueous phase nor the amount bound to albumin. As an example (16), incubation of FA-free and protein-free model membranes with 0.5 mM bovine serum albumin complexed with 1.0 mM palmitic acid would result in net transfer of only $\approx 0.5\%$ of the bound palmitic acid. However, the equivalent of 5 $\mu$M unbound palmitic acid is transferred to the membrane (16), and a very similar partitioning will occur for oleic acid. Clearly, this is a large excess relative to the concentration of unbound FA (25 nM) in the presence of albumin and yields about 2.5 mol $\%$ FA with respect to phospholipid.

In the experiments reported herein, the added concentrations of oleic acid were in the same physiological range as discussed above; i.e. the binding of oleic acid to cells is similar to the transfer achieved with low ratios of LCFA/albumin at physiological concentrations of albumin. In our studies, the lowest amount of oleic acid added to cells (5 nmol) corresponded to a concentration of 2.0 $\mu$M before binding to the cells and 3.2 mol $\%$ with respect to phospholipid in the cells. In addition to measurement of oleic uptake at low concentrations by the pH assay, other novel aspects of our study included the use of two fluorescent probes to monitor the adsorption step simultaneously with the transmembrane movement of FA, and evaluation of the effects of metabolism on intracellular pH.

Several new observations support the hypothesis of membrane transport of FA by free diffusion on a time scale faster than metabolism. First, in PMV of adipocytes, we showed rapid changes in $\text{pH}_{\text{in}}$ upon addition of FA ($t_{1/2} < 2$ s) (Fig. 1). Therefore, as in protein-free model membranes, flip-flop of un-ionized FA is rapid in the plasma membrane of adipocytes. (We should emphasize that this is an upper limit for the $t_{1/2}$ of flip-flop, and an exact rate constant cannot be derived from this measurement.) Adipocyte membranes contain a large proportion of proteins, including the putative FA transport proteins FATP and FAT/CD36 (5, 9). If the added FA had been transported primarily as anions by these proteins, the changes in $\text{pH}_{\text{in}}$ would have been in the opposite (alkaline) direction. In our studies with intact adipocytes, key findings were that (i) oleic acid binds immediately to the cells (Fig. 4), (ii) changes in $\text{pH}_{\text{in}}$ are complete within seconds upon the binding of oleic acid to the cells (Fig. 4), and (iii) these changes increase proportionally with the addition of larger amounts of oleic acid to the outer medium (Fig. 5A).

A fourth key finding was that oleic acid binds avidly in the lipid phase of the plasma membrane in vesicles and cells (Figs. 2 and 5B). We quantified, for the first time, the partitioning of oleic acid into PMV and adipocytes and determined that the apparent $K_p$ of PMV is very similar to the $K_p$ of protein-free vesicles (Fig. 2). If most of the oleic acid had bound to proteins in the plasma membrane, competing with the binding to the phospholipids, the evaluated $K_p$ of PMV would have been orders of magnitude larger. Furthermore, the binding curve would have been complex, and we would not have found a single $K_p$ if binding of oleic acid to proteins was significantly greater at lower concentrations of oleic acid than at higher concentrations.

In addition, we determined that the $K_p$ for binding of oleic acid to intact cells (Fig. 5B) is the same as for PMV. Recent models of enhancement of the movement of FA through membranes by membrane-bound proteins have proposed that FA exposed to a phospholipid bilayer with protein "transporters" bind only to the proteins (8, 15). To compensate for their lower abundance and surface coverage as compared with phospholipids, such proteins would have to bind FA with considerably higher affinities than the lipid bilayer. This would be reflected in a higher partition coefficient for cell membranes compared with protein-free bilayers, which is not supported by our data.

We performed new experiments to investigate the possibility that FA-induced decreases in $\text{pH}_{\text{in}}$ in cells with active metabolism and membrane transport of other metabolites and ions are because of some secondary pH change caused by a FA-induced metabolic event, as with glucose (66). The observed fast decreases in pH in PMV indicate that the cytosolic compartment and its participation in metabolism of FA are not essential for the decrease in pH after addition of FA. In intact cells we found that changes in $\text{pH}_{\text{in}}$ were proportional to the amount of OA added (Fig. 5A) and that very little FA had been metabolized within the time course of the pH drop (Fig. 6). These observations support our conclusion that the immediate $\text{pH}_{\text{in}}$ changes upon addition of FA to cells are caused by FA flip-flop. These results are consistent with previous observations that metabolically inert FA analogues such as a FA dimer and alkylamine promote $H^+$ transport in cells by flip-flop and not by a secondary effect (40, 45).

The fluorescence of both BCECF and ADIFAB showed rapid changes followed by a slow recovery (Fig. 4). After addition of oleic acid, the ADIFAB fluorescence increased immediately and subsequently recovered with kinetics similar to the drop in $\text{pH}_{\text{in}}$ (e.g. Fig. 4). The cells rapidly esterified oleic acid, with kinetics similar to those of the recovery of $\text{pH}_{\text{in}}$ (Fig. 6). The ADIFAB signal reflects the FA in outer leaflet of the plasma membrane in equilibrium with the external water phase at a given time point. Because flip-flop occurs within 1 to 2 s, the maximal signal (at 4 s) represents oleic acid in the outer leaflet in equilibrium with oleic acid on the inner leaflet that has undergone flip-flop immediately after addition of oleic acid. As oleic acid becomes activated to acyl-CoA, oleic acid in the plasma membrane flip-flop rapidly across the plasma mem-
brane to replace the metabolized oleic acid, and the concentration of oleic acid on both sides of the membrane decreases. The decrease in the ADIFAB signal reveals that the added oleic acid in the membrane was activated (and probably esterified) within 2 min (Fig. 4). Because the pH_i recovered with about the same rate as the ADIFAB signal, the pH_i recovery probably reflects only FA activation and not H^+ leakage. This interpretation is supported by our previous finding that addition of FA-free albumin after the recovery of the pH_i did not cause any changes in the BCECF fluorescence (40), implying that all the FA had been metabolized.

There are several lines of additional evidence for the interpretation that the pH recovery we observe on the time scale of seconds to minutes is primarily a result of metabolism rather than of proton leakage. (i) The return of pH in PMV was very slow (Fig. 1). (ii) Replacing the medium containing Na^+ with Mg^2+ did not affect the pH change in adipocytes, suggesting the Na^+/H^+ exchanger does not play a significant role in the observed intracellular pH changes, particularly the return of the pH to the initial level (40). (iii) Addition of metabolically inert amphiphatic molecules that cause intracellular pH changes (FA dimer and alkylamine) result in sustained changes in pH with essentially no recovery (40) over the time monitored (minutes). (iv) The long-chain FA cis-parinaric, a polysaturated FA that is very slowly metabolized, causes a pH drop in adipocytes, and the recovery is very slow (67). (v) In 3T3L1 preadipocytes, which metabolize exogenously added fatty acids very slowly, there is a sustained pH drop.2 This interpretation of the pH recovery after addition of exogenous FA to adipocytes can be considered a working hypothesis worthy of additional investigation. If our interpretation is correct, the fluorescence approach offers a powerful real-time visual indicator of membrane transport and metabolism of FA in living cells.

SUMMARY

Our previous studies with adipocytes demonstrated the mechanism of flip-flop and showed that it is fast, arguing against the notion that diffusion of LCFA across the plasma membrane is intrinsically slow (40). Our present study demonstrates the flip-flop mechanism at lower concentrations of FA, which we argue represent a range of relevant physiological concentrations. The correspondence of the intracellular pH changes with the partitioning of FA into the plasma membrane indicates either that the mechanism is solely diffusion or that a parallel mechanism makes a constant contribution. The latter interpretation contradicts the prevalent idea that transporters are the only uptake mechanism at low FA concentrations and that this mechanism becomes progressively less important with increasing FA concentration (8, 50, 51). Specifically, our kinetic data showing fast rates in this study do not support the concept that diffusion of FA at low concentrations is too slow to compete with (putative) transport by anion transporters (51).

FA transport through membranes must be interpreted in the context of partitioning of FA between membranes, albumin, and the water phase (16, 25). The high avidity with which FA partition into phospholipid bilayers membranes makes it unlikely that “transport” proteins could compete with the fast flip-flop of FA at low concentrations. Even at [OA], as low as 2 nM in equilibrium with oleic acid bound to albumin (a mole ratio of oleic acid/albumin of 0.5 (64)), the oleic acid concentration in the lipid domains of the plasma membrane could be as high as 2 mM (0.2 mol % relative to phospholipid), and flip-flop would permit a transmembrane flux with which membrane proteins could not compete by any known mechanism (25).

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