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Topical Review

Lipid Phase Fatty Acid Flip-Flop, Is It Fast Enough for Cellular Transport?

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Abstract. The mechanism by which fatty acids are transported across cell membranes is controversial. The essence of the controversy is whether transport requires membrane protein mediation or whether the membrane's lipid phase provides a pathway so rapid that a protein is not needed. This review focuses on the mechanisms of fatty acid transport across lipid bilayer membranes. These results for lipid membranes are used to help evaluate transport across cell membranes. Within the context of this analysis, a lipid phase mediated process is consistent with results for the transport of fatty acids across erythrocytes but provides a less adequate explanation for fatty acid transport across more complex cells.

Introduction

Fatty acids (FA)[1] supply a major portion of metabolic energy needs and are important building blocks for a variety of macromolecules. Most of this FA is produced in adipocytes, transported into the serum and eventually makes its way to the FA metabolizing tissues throughout the body. In the course of moving from the adipocyte to the target cell, the FA must cross the plasma membranes of several cells. Also central to the adipocyte's function is the reverse process in which FA are transported from the serum into the cell, where they are esterified and stored in triacylglycerol depots. Because traversing the membrane may be the rate-limiting step in FA utilization, the mechanism by which FA cross membranes has been the subject of considerable interest [for reviews see 2, 5, 9, 17, 18, 20, 29].

Actual transport of FA across membranes involves the movement of FA from the aqueous phase on one side to the aqueous phase on the other side of the membrane. This process can be viewed (Fig. 1) as requiring 3 separate kinetic steps, desolvation of the FA from the aqueous phase and insertion into the outer hemileaflet of the membrane (k_{on}), flip-flop of the FA between outer and inner leaflets (k_{ff}), and finally the dissociation from the inner leaflet into the inner aqueous phase (k_{off}). Although considerable effort has been devoted to understanding the mechanism of FA transport across membranes, the nature of the transport mechanism is controversial because of a lack of agreement about, (i) which kinetic step is rate limiting, (ii) the magnitude of the

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[1] Fatty acids as referred to here are monocarboxylic acids with unbranched hydrocarbon chains. The longer (>8 carbons) chain FA are relatively insoluble in the aqueous phase at neutral pH and are therefore found predominately in noncovalent association with membranes, proteins, or aggregates with other FA or FA salts (soaps). That fraction found in monomeric form in the aqueous phase we term free fatty acids (FFA) or occasionally, for emphasis, unbound free fatty acids. Because the pK of the monomer is about 4.5, most of what we designate as FFA is in fact the FA ion (carboxylate). Increasing the concentration of aqueous phase FA, increases the concentrations of the acid, which limits solubility at a given pH and therefore solubility increases with pH. The corresponding nomenclature as used in the literature is not uniform. Often, the term "fatty acids" is used to designate an esterified molecule or fatty acid residue (for example, as part of triacylglycerols, phospholipids, etc.). In this case the molecule is no longer an acid. To emphasize this distinction, the term unesterified FA or unesterified FFA is sometimes used in place of FA as used here. Because detection of the aqueous monomer (for the long chain FA) was not done with much frequency prior to the ADIFAB probe, no specific term has been developed for this quantity. The term free FA (FFA) is consistent with the usage for the aqueous phase concentrations of other small hydrophobic molecules such as free thyroid hormones, etc.

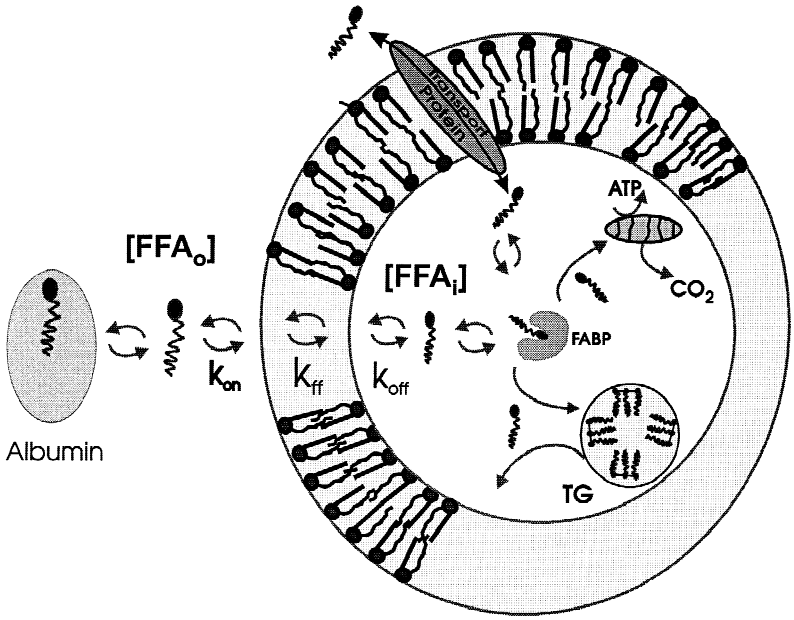

Fig. Steps in the pathway of FA metabolism. This illustrates the steps involved in the movement of FA between the extracellular medium and intracellular space. In the extracellular medium FA are released from albumin into solution and, at steady state, are present at a concentration of $[FFA_o]$. They then bind to the outer hemileaflet of the membrane with a rate constant k_{on}, flip across the lipid phase at a rate k_{ff}, and finally dissociate at the inner surface with a rate k_{off}. In addition, FA transport across the membrane may be facilitated by a protein which might, for example, involve a "single step" through the protein, or binding to the lipid phase followed by protein-mediated flip-flop. Once in the inner aqueous phase at a steady state concentration of $[FFA_i]$ the FA may bind to intracellular FABP, an intracellular membrane, or other hydrophobic phase. All these processes are reversible, as indicated by the double arrows. In contrast, FA are irreversibly modified, by CoA activation from which they may be metabolized to ATP and CO_2 or esterified, for example, to triacylglyceride. The effect of esterification can be reversed when lipase activation releases FA from TG storage.

transport rate, and (iii) the role of membrane proteins in cellular transport. The previous reviews of this subject have emphasized some of these issues. The current review extends the discussion to more recent results, emphasizing how transport through the lipid phase contributes to our understanding of cellular transport, and discussing what some of the basic issues are that underly the controversies.

Although a number of studies of cellular transport of FA have provided evidence suggesting that transport is protein mediated [1, 3, 6, 15, 16, 23, 34, 41, 42, 45, 51, 52, 54], results of other studies are consistent with FA transport mediated by the lipid phase of the membrane [10, 11, 21, 26, 27, 33, 36]. Implicit in a protein-facilitated process is that FA transport through the lipid phase of the membrane occurs at a rate too slow to support the level of FA metabolism required by the cell. Considerable effort has therefore been devoted to understanding transport across pure lipid membranes. Studies of such membranes indicate that transport occurs spontaneously, without protein facilitation [12, 13, 27, 31, 32, 47, 50]. However, at issue even for these simplest of membranes is the identity and magnitude of the rate-limiting step as well as the overall rate, for transport across lipid bilayers.

The notion that FA transport, and in particular that flip-flop, through lipid membranes should be fast is based on the view that, as hydrophobic molecules, FA should diffuse rapidly across the bilayer, after binding to the membrane from the aqueous phase. The interior of the bilayer in this view is equivalent to an isotropic hydrocarbon liquid and diffusion through such a liquid is rapid. Indeed, the rate of lateral diffusion of lipids in the plane of the lipid bilayer membranes is equivalent to diffusion through a liquid with the viscosity of about 1 Poise [14], and this predicts that diffusion of FA across the membrane should occur in about 3 μsec ($t \sim 2x^2/D$ for a slab [58] of thickness $x = 40$Å, and diffusion coefficient $D = 1 \times 10^{-7}$ cm^2/sec). The rate-limiting step for transport in this case would either be the rate of insertion into the membrane from the aqueous phase or dissociation of the FA from the membrane into the aqueous phase.

As will be discussed below, this predicted rate of transbilayer diffusion is much faster than observed and, given actual properties of membranes, FFA diffusion across the bilayer may not be represented accurately by diffusion through an isotropic solvent. Reasons for this may include the anisotropic FA-lipid interaction which exhibits polarity and packing gradients as a function of distance into the bilayer [28, 43] and that to execute flip-flop the FA must undergo a 180° rotation, in addition to translation. More generally, permeation of small non-electrolyte molecules through the bilayer appears to be better described as diffusion through a soft polymer, rather than through a low viscosity solvent such as hexadecane [49, 57, 58]. Thus it is not apparent what the rate limiting barriers should be for FA flip-flop across lipid bilayers.

FA Transport and Flip-Flop in Lipid Membranes

Short Chain FA

In the framework of Overton's rule the rate of transport across the membrane should increase with the lipid/water

partition coefficient (K_p) and therefore with increasing FA chain length. Measurements of the permeation (P^m) of the series of 2–6 carbon saturated FA through planar lipid bilayers reveal a near perfect correlation between P^m and K_p [56]. Although these steady-state measurements do not identify the rate-limiting step, they support the notion that the rate of permeation is limited by the solubility of the FA in the membrane rather than the rate of diffusion through the interior (flip-flop) or the rate of dissociation. Extrapolating from these results with short ($\leq$8 carbons) chain FA whose K_p values are $\leq$0.1, to longer ($\geq$18 carbons) chain FA with K_p values $>10^5$ [4], predicts permeation rates for the longer chain FA that are more than 10^5 faster than for the shorter chain FA [29]. A direct measurement, using temperature jump fluorescence, of octanoic acid (8:0) flip-flop in small unilamellar vesicles composed of soybean lipid, yields a rate constant of about 1×10^3 sec^{-1} [47]. This suggests, by extrapolation, that if solubility is also the rate limiting step for long chain FA, the time for diffusion of long chain FA across the bilayer would be less than about 10^{-8} sec. A time of 10^{-8} sec is faster than the 1 μsec predicted for diffusion through a simple hydrocarbon solvent and, as discussed below, with observed transport and flip-flop times for longer chain FA.

Medium to Long Chain FA

These results suggest that solubility may not represent the rate-limiting step for transport of long chain FA. Unlike short chain FA whose relatively large aqueous solubilities allow FA to be measured accurately in the aqueous phase, the high lipid/aqueous partition coefficients (>10) of the medium to long chain FA ($\geq$9 carbons) has meant that, until recently, it was not possible to measure directly the aqueous phase molecules with sufficient sensitivity and temporal resolution. As a consequence, earlier studies were done using fluorescently labeled FA, which allowed monitoring of the kinetic steps involved in transport across lipid vesicles. Measurements of the transfer of medium chain fluorescent FA, up to 12 carbons, between lipid vesicles were used to infer k_{off} values and to place limits on the rate of flip-flop ([13, 50] and *unpublished results*). These results are consistent with dissociation as the rate limiting step for transport of these medium chain FA and with flip-flop rate constants of greater than 5 sec^{-1} for the 9 carbon pyrene and greater than 3 sec^{-1} for the 12 carbon anthroyloxy FA (AOFA).

Initial studies of the transport of long chain fluorescent FA across lipid vesicles were done by monitoring the kinetics of transfer of the 16 and 18 carbon AOFA between lipid vesicles [32, 50]. In this configuration the time course of transfer allows one to resolve each of the kinetic steps involved in transport. The results of these studies indicated that: (i) flip-flop is the rate limiting step for transport (about 10-fold slower than dissociation), (ii) k_{ff} are on the order of 0.005 sec^{-1} for small unilamellar vesicles (SUV ~200 Å diameter) and about 10-fold slower (0.0006 sec^{-1}) for large unilamellar vesicles (LUV ~1000 Å diameter), (iii) k_{ff} decrease with increasing chain length, increase with increasing double bond number, and increase as the site of AO attachment moves from the 2 to the 16 position along the FA's hydrocarbon chain, and (iv) k_{ff} decrease with increasing concentrations of cholesterol (Table 1).

Table. Long chain FA transport rates across lipid vesicles and cells[a]

Membrane	Native FA	AOFA
SUV[b]	>20	0.005
LUV[c]	3–15	0.0006
GUV[d]	0.1	
RBC[e]	0.3	
Myocytes[f]	>1.5	

[a] Rate constants (sec^{-1}) are for native FA, oleate or palmitate, and for AOFA, 12-AO-stearate (18:0), for either k_{ff} or the overall rate contant k_t, measured at about 20°C (unless specified otherwise).
[b] Small unilamellar vesicles, native [27] and AOFA [32].
[c] Large unilamellar vesicles, native [27, 30] and AOFA [31, 32].
[d] Giant unilamellar vesicles [30].
[e] Human red cell ghosts [33].
[f] Estimated at 37°C, using the myocyte cell geometry and rates of metabolism as described [30].

A more direct method to determine the flip-flop rates of these long chain AOFA has been developed [26, 27]. In this method k_{ff} values are determined by monitoring the acidification of the internal aqueous phase of lipid vesicles as the protonated FA flips from the outer to the inner hemileaflet of the bilayer. The results of these studies yield flip-flop rates for the AOFA that are more than 10^4 faster than those determined from intervesicle transfer measurements. In contrast, more recent measurements, using refinements of this same technique, have obtained flip-flop rates for the long chain AOFA consistent with the slow values observed in the intervesicle transfer studies [31]. For reasons detailed in [31], flip-flop is most likely the rate limiting step for the longer chain fluorescent FA (AOFA) and the rate limiting step, may shift to dissociation for the medium chain fluorescent FA.

The method of monitoring the acidification of the internal compartment of lipid vesicles has also allowed the determination flip-flop rates of long chain *native* FA [25–27, 30]. These measurements have been done using the pH sensitive fluorophore pyranine, trapped within the inner aqueous compartment of lipid vesicles of increasing size and different compositions. Measurements of bilayer conductivity in the presence of long chain FA indicate that flip-flop rates of the anionic form of the FA are about 3 orders of magnitude slower than the protonated FA, and therefore the rate of the protonated species limits transport in lipid membranes [19]. The results for the protonated FA in lipid vesicles indicate that: (i) k_{ff}

are >200 sec^{-1} for SUV (faster than stopped-flow resolution), (ii) k_{ff} values for LUV range between 3 and 15 sec^{-1} (slower rates occur in cholesterol containing vesicles at lower temperatures), and (iii) in giant unilamellar vesicles (GUV, diameter ⩾2000 Å) formed by detergent dialysis, k_{ff} values ranged between 0.1 and 1.0 sec^{-1}. These studies of long chain native FA indicate that the rate of FA flip-flop across lipid vesicles is a sensitive function of vesicle size and lipid composition.

Actual transport of native FA between membrane separated aqueous phases is possible using the fluorescent probe ADIFAB to monitor the concentration of aqueous monomers of long chain native FA. ADIFAB is the acrylodan labeled intestinal fatty acid binding protein and undergoes a shift in fluorescence from blue (432 nm) to green (505 nm) upon binding FA [30, 33, 39]. Rates of FA binding to and dissociation from ADIFAB are rapid (>4 sec^{-1}), ADIFAB can detect [FFA] at about 1 nM, and under the conditions of most studies, ADIFAB itself has little effect on the free concentration [30, 33, 40]. Rate constants for the kinetic steps that characterize transport were determined using ADIFAB trapped within lipid or membrane vesicles to measure transport, and in the extravesicular space to measure dissociation. Results for LUV and GUV reveal that flip-flop is rate limiting for transport of palmitate (16:0), oleate (18:1), and linoleate (18:2). Transport across these membranes requires times that range from 0.5 to 10 sec, slower rates corresponding to larger vesicles, longer chain and more saturated FA, and lower temperatures. These measurements have been extended to saturated FA with chain lengths of 12, 14, and 16 carbons and to the unsaturated 18:3 and 20:4 FA. The results reveal an exponential increase in transport rate with decreasing chain length chain length (for the same double bond number) and with double bond number (for the same chain length) (A.M. Kleinfeld, *unpublished results*).

Description of FA Transport Across Lipid Membranes as Movement Through a Polymer

The results of the studies summarized above indicate that the kinetic steps that govern FA transport across lipid bilayer membranes are functions of membrane structure and composition, and the molecular species of FA. These results, just as for the lipid bilayer permeability of other nonelectrolytes, are described better in terms of a polymer rather than a homogeneous solvent model of the membrane [35, 49, 57]. Studies of lipid bilayers and biological membranes have revealed that nonelectrolyte permeability decreases more steeply with increasing size of the nonelectrolyte molecules than expected if the bilayer were equivalent to a homogeneous nonpolar solvent such as hexadecane [49, 57]. These results are consistent with a soft polymer model of the bilayer core which predicts an exponential or power law dependence on diffusant size [35, 49].

If FA transport/flip-flop also reflects the characteristics of movement through a polymer it may be worthwhile to use results for other nonelectrolytes to predict FA transmembrane diffusion times. For example, from Fig. 2.3 of [49], the diffusion coefficient of nonelectrolytes ($M_w \leqslant 110$) through red cell membranes is reasonably fitted by $\log D = -4.9–0.033*M_w$. This predicts, using estimates for diffusion through a slab [58], that the transbilayer diffusion time for octanoic acid is about 6 msec. This represents quite good agreement with the ~1 msec value measured for SUV [47], given the tighter packing of red cell membranes. For the larger FA, this same expression predicts about 40 sec for oleate (M_w 283) and more than 10^9 sec for AO-oleate (M_w 504). Although these are respectively about 10 and 10^6-fold slower than the measured values [30, 32, 33], the discrepancy may not be that serious. Better agreement would be obtained for these larger molecular weight FA if the slope (0.033) in the expression for log *D*, which was obtained from results for smaller molecular weight molecules, were about 20–30% smaller. In fact results in soft polymers yield steeper slopes for smaller as compared to larger molecular weight diffusants [57].

The polymer model provides a reasonable explanation for the large differences in rates observed for short and long chain native FA and also suggest that, at least in part, the large difference in transport rates between the AOFA and native FA can be ascribed to their molecular weight differences. Studies currently in progress have extended the transport studies of egg phosphatidylcholine-cholesterol GUV and human red cell ghosts to the series of saturated FA with chain lengths between 12 and 18; preliminary results are consistent with an exponential decrease in transport rate with increasing chain length (A.M. Kleinfeld, *unpublished observations*). Further evidence consistent with the notion that flip-flop is limited by the polymer character of the bilayer is provided by thermodynamics activation potentials for lipid membranes and red cells [30, 32, 33]. The results of studies with native and AOFA indicate that increases in barrier energies, for the different FA and membranes, primarily reflect decreases (less favorable) in the activation entropy. Presumably, this reflects a diffusion barrier resulting from the tortuosity of the polymer network.

Summary of Lipid Vesicles Studies

Results from a variety of studies indicate that flip-flop and/or transport times range from about 1 μsec to >1000 sec, depending upon the FA type, vesicle size and vesicle composition (Table 1). For the physiologically important long chain FA, flip-flop times for vesicles approaching cell size and compositions, are about 1–10 sec. The

results, moreover, reveal a strong dependence on FA size, consistent with the notion that the bilayer core has the properties of a complex polymer and diffusion through this polymer represents the rate limiting step for transbilayer transport of long chain FA. The very different flip-flop/transport rates observed with different model membranes raises the possibility that the complexity of the polymer and therefore the magnitude of the barrier is a sensitive function of the composition and organization of the lipid bilayer. Thus rates of flip-flop/transport for an arbitrary lipid bilayer may not indicate whether transport across a specific cell's lipid phase is sufficient to support metabolic requirements. Only by direct measurement of cellular transport characteristics will it be possible to determine if transport through the cell's lipid phase is adequate.

FA Transport Across Cell Membranes

To address the issue of whether transport across lipid vesicles provides an accurate model of cellular transport it is necessary to compare lipid vesicle transport rates with those across cell membranes. Direct measurements of the actual transport of the native FA between the aqueous phases on either side of a cell membrane have been reported for human red blood cells [33]. Measurements of intracellular pH (pH_i), analogous to the method used in lipid vesicle studies, have also provided information about the rate of flip-flop across plasma membranes [10, 21, 38]. However, most information about FA transport across cell membranes has been obtained from measurements of the amount of FA that associates with the whole cell (uptake). Results of such measurements have provided evidence for protein-mediated transport pathways in a variety of cells [1, 15, 16, 24, 34, 41, 42, 54].

Transport Across Red Cell Membranes

Because the issue of protein *vs.* lipid-mediated transport is controversial, it seems reasonable that using a simple system like the red cell should help clarify methodological issues and address the mechanism of transport across the red cell's membrane. However, even for these simplest of cell membranes there is no consensus concerning the protein *vs.* lipid role in FA transport. Studies which measured the rate of radioactive FA associated with whole cells or ghosts suggested both protein-mediated as well as lipid-phase mediated transport pathways [7, 8, 37, 44]. Direct transport measurements, done using ADIFAB trapped in human red blood cell ghosts, reveal transport properties that are quite similar to GUV composed of egg phosphatidylcholine and cholesterol [33]. Transport times at 37°C were about 1 sec for oleate, all the steps involved in the transport were reversible, and flip-flop was rate limiting. Although some disagreement remains concerning the exact role of the membrane's lipid phase [7], similar transport rates, when extrapolated to equivalent temperatures, were obtained in most studies [7, 8, 33]. Because FA transport is unaffected by a variety of protein reagents and the characteristics (magnitude of rate constants, dependence on FA type, and temperature) of FA transport across red cell ghosts are similar to those for lipid vesicles, transport across the red cell probably occurs via the lipid phase of the membrane [8, 33].

Transport Across the Plasma Membranes of Cells for which FA Metabolism is a Major Function

That transport across red blood cells is similar to lipid vesicles may not be surprising, because FA metabolism is not a major function of these cells. However, transport might involve a more complex mechanism in cells such as, for example, adipocytes and cardiac myocytes, for which FA transport is an important function. Estimates for cardiac myoctes suggest that to maintain normal metabolic activity in these cells, rates of FA transport are required that are faster than for lipid vesicles (30). Indeed, a large number of studies, primarily involving measurements of FA uptake have provided evidence that membrane proteins play key roles in the transmembrane transport of FA across these more complex cells [1, 3, 6, 15, 34, 41, 51, 52]. The most important evidence for a protein-mediated mechanism from these studies is the demonstration of saturable uptake which can be inhibited by protein specific reagents. Both inhibitor studies and screening assays have led to the identification of candidate transport proteins [6, 22, 23, 41, 46, 53] and cells enriched in these proteins reveal increased levels of uptake [48, 55, 59].

Direct Measurements of [FFA_i] May Provide Accurate Assessments of FA Transport

These results support the notion that FA transport is mediated by a plasma membrane transport protein. However, because the rate of transport across the membrane must be infered from the rate of uptake it is possible that the plasma membrane might not be the rate limiting step in the uptake studies. This issue could best be resolved by directly monitoring actual transport of the FFA across the cell membrane. A partial step in this direction are measurements of the change in intracellular pH (pH_i) after adding extracellular FA [10, 21, 38]. These measurements, in cytotoxic T lymphocytes, pancreatic islet cells, and adipocytes, provide information about the flip-flop step and are consistent with much slower times (~100 sec) of intracellular acidification than for lipid

vesicles or red blood cells. Although these results are consistent with the cell membrane providing a substantial barrier to FA transport, they do not indicate the contribution of dissociation to the overall transport rate and the rate of change of pH_i may reflect factors in addition to FFA transport [38]. Studies currently in progress using ADIFAB microinjected into 3T3FF2A adipocytes allow the intracellular [FFA_i] distribution to be imaged by digital ratio microscopy (A.M. Kleinfeld, *unpublished results*). Preliminary results from these measurements, using physiologic levels of oleate, confirm that influx is slow and suggest that FA transport across adipocyte membranes involves a complex mechanism, not readily explicable in terms of the lipid phase.

Summary

Results of studies of FA transport in lipid membranes over the past 2 decades indicate that for systems that are most similar to cells, transport of long chain FA is limited by the rate of flip-flop and this rate is quite sensitive to the composition and organization of the lipid phase, as well as the molecular species of FA (Table 1). This suggests that depending upon the nature of the lipid phase of a cell membrane, transport might proceed rapidly through the lipid phase with rates, under physiologic conditions, that are on the order of 1 sec^{-1} or possibly faster. This is what appears to happen in human red blood cells and for these cells at least, this rate of transport is sufficient to satisfy the rate of intracellular FA metabolism. For more complex cells, especially those for which FA metabolism plays a major role in function, the situation is less clear because accurate rates of actual FFA transport and FA metabolism are lacking. For example, because the heart uses FA to provide most of its metabolic energy one would expect fast rates of FA influx into cardiac myocytes. Estimates of the rate of transport of FA across cardiac myocytes can be derived from measurements of metabolism and myocyte geometry, which yield values that range from 5 to more than 50-fold faster than obtained in lipid vesicles or red cell ghosts [30]. Thus for these cells, transport across the lipid phase may not be fast enough to support the cell's metabolic requirements, assuming that transport across the lipid phase of a cell membrane is no faster than for the slowest lipid vesicles (~1 sec for oleate at 37°C). However, given the uncertainties in the estimates, the lack of direct transport measurements, and lack of knowledge of the actual lipid phase characteristics of the cell, a lipid phase-mediated transport mechanism cannot be excluded.

The situation for the adipocyte appears to be quite distinct from cardiac myocytes. In the adipocyte, rates of flip-flop and transport across the cell membrane may be more than 100-fold *slower* than observed in the slowest lipid vesicles. This suggests that the barrier to FA transport formed by the lipid phase of the adipocyte membrane is much larger than observed in lipid vesicles or in red cell ghosts. Unlike the cardiac myocyte, the adipocyte must transport FA both into and and out of the cell under postprandial and fasting conditions, respectively. In the fasting state, serum [FFA] is low and large quantities of FA are, presumably, transported rapidly out of the cell to supply metabolic needs of the organism. Postprandially, serum [FFA] levels may increase substantially and under these conditions FA flow into the cell, where they are esterified and stored as triacylglycerol. Although this directionality might be achieved by regulation of the intra and extracellular FFA concentrations, the refractory nature of the membrane to FFA influx raises the possibility that membrane protein-mediated mechanisms may play a role in regulating this relatively complex behavior in adipocytes.

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