The effect of intracellular pH on long-chain fatty acid uptake in 3T3-L1 adipocytes: evidence that uptake involves the passive diffusion of protonated long-chain fatty acids across the plasma membrane

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To understand the mechanism of long-chain fatty acid permeation of the plasma membrane in mammalian cells, the effects of changes in the cytoplasmic pH on the internalization of physiologically relevant, submicromolar concentrations of uncomplexed long-chain fatty acids were investigated in 3T3-L1 adipocytes. The acidification of the cytoplasm upon NH_4Cl prepulsing of intact cells was accompanied by a rapid reduction of cellular long-chain fatty acid uptake (measured as the total accumulation of $[9,10\text{-}^3\text{H}]\text{oleate}$). This was followed by a slow recovery to normal levels of uptake as the cytoplasmic pH recovered. Conventional filtration assays do not distinguish between fatty acid movement across the plasma membrane and intracellular steps, such as binding to cytoplasmic fatty acid-binding proteins or metabolism. While the *in vitro* binding of a photoreactive fatty acid, 11-*m*-diazirino-phenoxy $[11\text{-}^3\text{H}]\text{undecanoate}$, to a cytoplasmic fatty acid-binding protein was insensitive to changes in pH from pH 7.5 to 5.5, the *in vitro*

conversion of oleate into oleoyl-CoA by cellular acyl-CoA synthetase decreased dramatically. Therefore, the labelling of the 15 kDa cytoplasmic fatty acid-binding protein in intact cells by the photoreactive fatty acid was used as a more direct measure of the permeation of the probe across the plasma membrane. Acidification of the cytoplasm resulted in an immediate reduction in the labelling of this protein in intact adipocytes. Its photolabelling recovered, however, upon the recovery of the cytoplasmic pH to normal levels. This was due to effects of the cytoplasmic pH on the permeation of the photoreactive fatty acid across the plasma membrane rather than its binding to the 15 kDa protein or metabolism *in vivo*. This is the first demonstration that the movement of physiologically relevant, submicromolar concentrations of uncomplexed long-chain fatty acids across the plasma membrane of intact cells is coupled to the cytoplasmic pH and suggests that it occurs by the diffusion of the protonated long-chain fatty acid through the lipid bilayer.

INTRODUCTION

Long-chain fatty acids are important metabolic substrates for both energy production and lipid synthesis. They are also involved in a variety of crucial cell signalling cascades [1–6]. The involvement of long-chain fatty acids in these processes requires their uptake by and trafficking within cells. The mechanisms by which mammalian cells take up long-chain fatty acids remain poorly understood. It has been proposed that the cellular uptake of long-chain fatty acids consists of a number of steps [7]. These include long-chain fatty acid delivery to the cell surface by carriers such as serum albumin [8], their movement across the plasma membrane, binding to cytoplasmic fatty acid-binding proteins (FABPs), delivery to intracellular sites and conversion into long-chain fatty acyl-CoA [7]. Oleate uptake by 3T3-L1 adipocytes is saturable and exhibits a high affinity for uncomplexed oleate, indicating that it is protein mediated [8–11]. However, the step(s) responsible for this saturability remain unknown.

It has been proposed that the saturability of the uptake of long-chain fatty acids reflects the involvement of proteins in their transbilayer movement [11–15]. At least three distinct plasma membrane proteins have been implicated in long-chain fatty acid uptake in 3T3-L1 adipocytes [10,11,14,15]. Furthermore, a unique protein has been identified as the sole, high-affinity FABP in 3T3-L1 adipocyte plasma membranes [9,16]. These proteins have been proposed to participate in the transbilayer movement

of long-chain fatty acids in 3T3-L1 adipocytes. However, it is equally possible that they may be involved in one or more of the other steps in the uptake process (mentioned above) or in the regulation of uptake [14].

Alternatively, the saturability of long-chain fatty acid uptake has been proposed to reflect protein-mediated events within the cell; these include binding to cytoplasmic FABPs and metabolism [7,17]. In part, the controversy over the nature of the saturable step in long-chain fatty acid uptake results from the fact that long-chain fatty acids are lipophilic, and readily partition into membranes [18,19]. This has led to the proposal that the movement of long-chain fatty acids across plasma membranes should not require the involvement of proteins [7]. Studies have shown that while long-chain fatty acids are non-permeant when ionized, they can diffuse rapidly across model membranes when protonated [20,21]. Consistent with this, long-chain fatty acids have been found to distribute across model membranes in response to imposed pH gradients [22].

It has long been appreciated that at very high concentrations of long-chain fatty acids, uptake becomes non-saturable [23,24]. Recently, Hamilton and co-workers have shown that, at such high long-chain fatty acid concentrations, uptake involves the permeation of the protonated species across the membrane by passive diffusion [25]. However, the high concentrations (micromolar) of long-chain fatty acids at which the non-saturable process becomes significant are considered to be physiologically irrelevant [23,24]. Therefore, the relevance of passive diffusion as

Abbreviations used: ACS, acyl-CoA synthetase; ALBP, adipocyte lipid-binding protein; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; 11-DAP- $[11\text{-}^3\text{H}]\text{undecanoate}$, 11-*m*-diazirino-phenoxy $[11\text{-}^3\text{H}]\text{undecanoate}$; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; FABP, fatty acid-binding protein; pH_C , cytoplasmic pH.

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a means of permeation of long-chain fatty acids across the plasma membrane under normal physiological conditions (namely, submicromolar concentrations of uncomplexed long-chain fatty acid in the presence of serum albumin) is not clear.

If the movement of long-chain fatty acids across plasma membranes in intact cells is not protein-mediated, but occurs by diffusion of the protonated fatty acid through the lipid bilayer, then the cellular uptake of long-chain fatty acids should also be affected by the pH gradient across the plasma membrane in much the same way as the distribution of long-chain fatty acids across artificial lipid bilayers is affected by the pH gradient across the bilayer [22]. Furthermore, this should be the case regardless of the concentration of uncomplexed long-chain fatty acid. To test this, we have measured the effects of the reduction of the cytoplasmic pH (pH_c) on the cellular uptake of physiologically relevant concentrations of long-chain fatty acids. Uptake was measured either as the total accumulation of [9,10- 3H]oleate within cells or as the labelling of a cytoplasmic FABP in intact cells using a photoreactive long-chain fatty acid. 3T3-L1 adipocytes express a 15 kDa cytoplasmic FABP, called the adipocyte lipid-binding protein (ALBP) [26]. We use its labelling in intact cells by the photoreactive fatty acid, 11-*m*-diazirinophenoxy[11- 3H]undecanoate (11-DAP-[11- 3H]undecanoate) as a direct measure of the level of fatty acid in the cytoplasm and therefore the amount of fatty acid which has crossed the plasma membrane ([9]; B. L. Trigatti and G. E. Gerber, unpublished work). We report that the reduction of pH_c by NH_4Cl prepulsing resulted in a decrease in the level of oleate uptake as well as the photoaffinity labelling of the cytoplasmic FABP in intact cells. When the pH_c was allowed to recover with time, both oleate uptake and the level of photoaffinity labelling of the cytoplasmic FABP were restored. This is the first direct demonstration that the cellular uptake of physiologically relevant concentrations of long-chain fatty acids is responsive to the pH gradient across the plasma membrane, and suggests that it occurs by the passive diffusion of the protonated species through the hydrophobic portion of the bilayer.

EXPERIMENTAL

Materials

All reagents for cell culture and SDS/PAGE were obtained from GIBCO-BRL except for BSA (fraction V, essentially fatty acid free), dexamethasone, insulin and 3-isobutyl-1-methylxanthine, which were purchased from the Sigma Chemical Company. The acetoxymethyl ester of 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF) was purchased from Molecular Probes Inc. 11-DAP-[11- 3H]undecanoic acid (3.34 Ci/mmol) was synthesized as described previously [27]. Atomlight scintillant, [9,10- 3H]oleic acid (10 Ci/mmol) and Solvable tissue and gel solubilizer were obtained from NEN Research Products. Non-radioactive oleic acid was from Fisher Scientific. Aqueous counting scintillant and Amplify fluorography reagent were purchased from Amersham.

Cell culture

3T3-L1 preadipocytes were grown to confluence and stimulated to differentiate to adipocytes in the presence of fetal bovine serum, insulin, dexamethasone and 3-isobutyl-1-methylxanthine as described previously [9]. Cells were released from plates by treatment with 1.0 mM EDTA in PBS (0.14 M NaCl, 2.7 mM KCl, 15 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 , pH 7.4) as described previously [9].

pH_c manipulation by acid loading using NH_4Cl prepulsing

Cells were loaded with 45 mM NH_4Cl for 60 min in PBS containing 10 mM glucose [28–30]. NH_4Cl was removed by centrifugation (5 min at 600 *g*) for cells in suspension, or by aspiration for monolayers. Immediately thereafter, PBS containing 10 mM glucose was added and cells were used.

Measurement of pH_c

3T3-L1 adipocytes grown on 7 mm × 25 mm microscope slides [29,30] were loaded with the acetoxymethyl ester of BCECF (10 μ M in 2.0 ml of serum-free Dulbecco's medium) in 35-mm-diam. culture dishes at 37 °C for 1 h in a humidified atmosphere containing 5.0 % CO_2 [29–32]. The monolayers were washed and NH_4Cl prepulsing was done as described above. Slides were placed in 2.0 ml of PBS in disposable 3.0 ml fluorescence cuvettes to which test solutions were added. Fluorescence emission at 530 nm, with excitation at 500 nm [30], was monitored in an Aminco Bowman series 2 luminescence spectrometer. Fluorescence was calibrated to internal pH by setting the internal and external pH values equal with nigericin in medium in which NaCl was replaced with KCl [29,30] as well as by lysis of the cells with 0.10 % Triton X-100 and titration with Mes [33].

Measurement of oleate uptake and acyl-CoA synthetase (ACS) activities

Cells (0.5 mg of cellular protein/ml in PBS) were incubated with 0.25 mM [9,10- 3H]oleate in the presence of 0.50 mM BSA at 37 °C. [9,10- 3H]oleate uptake was measured by a filtration assay as described previously [8,9]. Long-chain ACS activities were measured in homogenates by following the conversion of [9,10- 3H]oleate into [9,10- 3H]oleoyl-CoA as described previously [34].

Photoaffinity labelling with 11-DAP-[11- 3H]undecanoate

Cells in suspension, or cytoplasm isolated from cells, were labelled with 11-DAP-[11- 3H]undecanoate as described previously [9]. Monolayers in 1.5-cm-diam. wells of 4-well cell culture trays (Nunc) were incubated for 5.0 s at 37 °C with 200 μ l of PBS containing 75 μ M 11-DAP-[11- 3H]undecanoate and 50 μ M BSA. Photolysis was for 2.0 s using a beam of light from a 1000 W Xe–Hg arc lamp. The beam of light was directed on to the monolayer using a mirror positioned at an angle of 45° at the focal point of the light beam. After photolysis, labelled cells were washed twice at 37 °C with 1.0 % BSA in PBS, and once at 37 °C with PBS. They were suspended in 300 μ l of ice-cold 2.0 mM $MgSO_4$, DNase I and RNase A (3.0 μ g each) and PMSF (to a final concentration of 0.10 mM) were added and samples were incubated for 10 min at 37 °C.

Subcellular fractionation

All procedures for subcellular fractionation were carried out in the cold, unless otherwise indicated. Cytosol, for *in vitro* photoaffinity labelling, was prepared from cells (1.0 ml of 5.0 mg of protein/ml in PBS) homogenized by passage (10 times) through a 23-gauge needle. Homogenates were subjected to centrifugation at 100000 rev./min for 20 min using a TLA 100.2 rotor in a Beckman TL-100 ultracentrifuge. The supernatant minus the fat cake was recovered and delipidated by passage through packed Lipidex 1000 (1 ml per ml of cytosol at a concentration of 1.0 mg of protein/ml) at 37 °C in the barrel of a 3-ml syringe [35].

Subcellular fractions from prelabelled cells were prepared

essentially as described previously [9], with some minor modifications. Briefly, 3T3-L1 adipocytes from one dish of 10 cm diameter were suspended in 1.0 ml of homogenization buffer (1.0 mM Hepes, 1.0 mM EDTA, pH 7.4, containing 0.25 M sucrose) and were homogenized in a motor-driven Potter–Elvehjem tissue grinder (moderate speed, five up-and-down strokes). The homogenate was subjected to centrifugation for 20 min at 10000 *g*. The pellet obtained was washed with homogenization buffer, resuspended in 1.0 ml of the same, again using a Potter–Elvehjem tissue grinder (2 strokes at moderate speed), and layered on to a discontinuous sucrose gradient consisting of 1.0 ml each of 0.87 M, 1.12 M and 1.50 M sucrose in 10 mM Hepes and 1.0 mM EDTA, pH 7.4. The sucrose gradient was subjected to centrifugation at 23000 *g* for 90 min. The fraction labelled PM (plasma membrane) was recovered from the 0.25 M/0.87 M sucrose interface, while the fraction labelled M (mitochondrial) was recovered from the 1.12 M/1.50 M sucrose interface. Each was diluted 5-fold with hypotonic medium (20-fold diluted PBS) and subjected to centrifugation at 100000 *g* for 60 min. The 10000 *g* supernatant (from the initial centrifugation step, above) was subjected to centrifugation at 100000 *g* for 60 min to obtain a low-density microsomal (denoted LDM) pellet and a cytosolic supernatant (called fraction C). Isolated subcellular membrane fractions were homogenized in ice-cold hypotonic medium (20-fold diluted PBS) by passage (five times) through a syringe fitted with a 23-gauge needle and then pelleted by centrifugation at 100000 *g* for 1.0 h at 4.0 °C. Membrane pellets were resuspended to approx. 2–10 mg of protein/ml in the presence of 0.1 mM PMSF and were stored at –80 °C.

Miscellaneous methods

The concentrations of uncomplexed fatty acids in equilibrium with BSA were determined as described previously [8]. Assays were performed as described for cytochrome *c* oxidase [36], NADH dehydrogenase [37], 5'-nucleotidase [38], inorganic phosphate [39] and protein [40]. Labelled proteins were analysed by SDS/PAGE on 15 % polyacrylamide gels as described previously [9]. Gels were treated for 15 min in 4 times their volume of fluorography reagent. Fluorographed gels were dried and exposed to Kodak XAR-5 film at –80 °C. The amount of radioactivity in labelled bands was determined by scintillation counting of the bands eluted from the rehydrated gel as described previously [16].

RESULTS

To investigate the role of pH_c on cellular long-chain fatty acid uptake by 3T3-L1 adipocytes, the pH_c was manipulated by the NH_4Cl prepulsing technique [28–30]. This involved loading the cells with NH_4^+ by incubating them with 45 mM NH_4Cl , followed by the removal of NH_4Cl , leading to the efflux of NH_3 from the cell. The loading of cells with NH_4^+ has been reported to result from the influx of NH_3 , followed by its protonation in the cytoplasm to produce NH_4^+ , causing an initial increase in the pH_c followed by a gradual recovery brought about by the action of the cellular homeostatic mechanisms [28]. The subsequent removal of NH_4Cl then results in a rapid drop in the pH_c due to the dissociation of NH_4^+ within the cell, and efflux of NH_3 from the cell; this is followed by a gradual recovery of pH_c to normal levels due to the cellular homeostatic mechanisms [28]. During this procedure, the pH_c was monitored with the fluorescent indicator, BCECF (Figure 1). Prior to prepulsing, the pH_c was 7.3. After prepulsing, the cytoplasm rapidly became acidified to a pH of 6.7. The pH_c recovered within 10 min, reaching a level

Figure 1 Effect of NH_4Cl prepulsing on pH_c

3T3-L1 adipocytes, grown on glass coverslips, were loaded with BCECF as described in the Experimental section. Cells were incubated with 45 mM NH_4Cl in PBS containing 10 mM glucose. The internal pH prior to removal of NH_4Cl (vertical arrow) was 7.3, as measured by the fluorescence emission at 530 nm (excitation at 500 nm) as described in the Experimental section. External NH_4Cl was removed and fluorescence emission at 530 nm was monitored immediately thereafter. Shown is the change in pH_c upon removal of NH_4Cl after the conversion of fluorescence emission to pH as described in the Experimental section. The external pH was maintained at 7.4 throughout the experiment.

of almost 7.6, overshooting the normal resting value of 7.3. The external pH was maintained at 7.4 throughout the procedure.

To determine the effects of decreasing the pH_c on long-chain fatty acid uptake, [9,10- 3H]oleate accumulation within the cell was monitored using a conventional filtration assay. The rapid acidification of the cytoplasm upon prepulsing was accompanied by a 70 % reduction in the rate of [9,10- 3H]oleate accumulation within cells (measured in cells 30 s after the washout of NH_4Cl , relative to cells carried through the loading and washout procedure in the absence of NH_4Cl ; Figure 2a). The recovery of the pH_c to more alkaline levels was also accompanied by the recovery of normal levels of [9,10- 3H]oleate accumulation (relative to cells carried through the loading and washout procedure in the

Figure 2 The effect of reduction of pH_c on [9,10- 3H]oleate accumulation in 3T3-L1 adipocytes

3T3-L1 adipocytes, in suspension, were prepulsed with (+) or without (–) NH_4Cl as described in the Experimental section. Recovery was for either (a) 0.50 or (b) 30 min at 37 °C prior to the measurement of the cellular accumulation of [9,10- 3H]oleate as described in the Experimental section. Typical results obtained are presented as the amount of oleate taken up, normalized for cellular protein content.

Figure 3 Effect of pH on *in vitro* photoaffinity labelling of the 15 kDa, cytoplasmic fatty acid binding protein

Fatty acid binding by the 15 kDa, cytoplasmic FABP (arrow in **a**) was measured using 11-DAP-[11-³H]undecanoate. Cytoplasm, prepared from 3T3-L1 adipocytes, was delipidated with Lipidex 1000 at 37 °C as described in the Experimental section. Incubation with 10 μ M 11-DAP-[11-³H]undecanoate was for 15 min at 37 °C at 0.50 mg of cytoplasmic protein/ml in 20 mM sodium phosphate, at the indicated pH. Samples were analysed by SDS/PAGE as described in the Experimental section. **(a)** The labelling pattern obtained when cytoplasm was photolabelled after incubation with 11-DAP-[11-³H]undecanoate at pH 7.4. Numbers on the left indicate the migration of marker proteins of the specified molecular masses. The arrow indicates the location of the 15 kDa protein. **(b)** The level of incorporation of the 11-DAP-[11-³H]undecanoate into the 15 kDa cytoplasmic FABP upon photolysis, as a function of pH. The level of incorporation was measured by extraction of the radioactivity in labelled bands from the gel, followed by scintillation counting as described in the Experimental section. The results of a typical experiment are shown.

absence of NH_4Cl ; Figure 2b). In this experiment, oleate uptake was measured under physiologically relevant conditions; the concentration of uncomplexed [9,10-³H]oleate in equilibrium with BSA was 47 nM. It was not clear, however, whether the decreased oleate uptake accompanying the reduction in pH_c resulted from a decreased level of permeation of oleic acid across the plasma membrane or a pH dependence of other steps in the uptake process.

Filtration assays measure the net accumulation of radioactively labelled long-chain fatty acids within cells and, therefore, reflect both the permeation of radioactive long-chain fatty acids across the plasma membrane and intracellular events; these include binding to cytoplasmic FABPs and conversion into long-chain

Figure 4 Effect of pH on the activity of long-chain ACS in cell homogenates

Long-chain ACS activities in homogenates of 3T3-L1 adipocytes were measured at various pH values as described in the Experimental section. Results are presented as the rate of formation of [9,10-³H]oleoyl-CoA from [9,10-³H]oleate and are normalized for protein content. Results are the means $\pm$ S.E.M. of three determinations.

fatty acyl-CoA by long-chain ACS [7]. 3T3-L1 adipocytes express ALBP, a 15 kDa cytoplasmic FABP [26], which could be labelled almost exclusively by 11-DAP-[11-³H]undecanoate, a photo-reactive long-chain fatty acid, in cytoplasm obtained from 3T3-L1 adipocytes (Figure 3a; B. L. Trigatti and G. E. Gerber, unpublished work). Typical results of the effect of pH on the photoaffinity labelling *in vitro* of the 15 kDa cytoplasmic FABP in cytoplasm isolated from 3T3-L1 adipocytes are shown in Figure 3(b). The level of 11-DAP-[11-³H]undecanoate incorporated into the protein upon photolysis was relatively constant when the pH was varied from 5.5 to 7.5. This is in agreement with studies on the binding of oleic acid to the rat liver FABP, which found little effect of pH on the level of binding from pH 6.4 to 8.5 [41].

The effect of pH on the activity of long-chain ACS in homogenates from 3T3-L1 adipocytes was measured using [9,10-³H]oleate as the substrate (Figure 4). ACS activities were maximal above pH 7.5, but dropped off rapidly as the pH was decreased. The activity of ACS was virtually zero at pH 6.5. Therefore, the reduction of pH_c from 7.3 to 6.7 during the prepulsing procedure (Figures 1 and 2a) could have resulted in a substantial decrease in the activity of long-chain ACS *in vivo*, and therefore in the activation of oleate for further metabolism. The activation of long-chain fatty acids, catalysed by long-chain ACS, is believed to be the rate-limiting step in long-chain fatty acid uptake in *Escherichia coli* [42], and has been proposed to represent an important step in long-chain fatty acid uptake in mammalian cells [7]. This suggests the possibility that the decrease in oleate uptake accompanying the drop in pH_c upon prepulsing with NH_4Cl (Figures 1 and 2a) may have resulted from a decrease in the activity of long-chain ACS. This demonstrates the requirement for a more direct assay for long-chain fatty acid permeation of the plasma membrane.

The photoaffinity labelling of the cytoplasmic FABP in intact cells with the photoreactive long-chain fatty acid could represent a more direct means of monitoring the permeation of long-chain fatty acids across the plasma membrane. The cellular uptake of the photoreactive probe by 3T3-L1 adipocytes was saturable when measured as the accumulation of radioactivity within cells

Figure 5 Rate of 11-DAP-[11-³H]undecanoate uptake by 3T3-L1 adipocytes as a function of the concentration of unbound 11-DAP-[11-³H]undecanoate

The rates of uptake of 11-DAP-[11-³H]undecanoate by 3T3-L1 adipocytes were measured at various concentrations of the photoreactive fatty acid in the presence of 0.50 mM BSA as described in the Experimental section. Results are expressed as the average rate of uptake $\pm$ S.E.M. (of three determinations) versus the concentration of uncomplexed photoreactive fatty acid at each ratio of fatty acid:BSA (determined as described previously [8,9]).

using a filtration assay (Figure 5). Uptake of the probe exhibited an affinity (K_t of 255 nM) which was lower than that observed for oleate uptake by these cells [9,10], but was similar to that observed for palmitate and linoleate uptake in rat adipocytes [11]. The V_{max} for uptake of the photoreactive long-chain fatty acid (13.3 nmol/min per mg of cellular protein) was similar to that previously determined for oleate uptake by 3T3-L1 adipocytes (16 nmol/min per mg of cellular protein) [9].

To determine the subcellular distribution of proteins labelled after photolysis of intact 3T3-L1 adipocytes with the photoreactive fatty acid, cells were fractionated by a combination of differential and sucrose-density-gradient centrifugation. The activities of different marker enzymes in each fraction, normalized

Figure 7 Subcellular fractionation of 3T3-L1 adipocytes photolabelled with 11-DAP-[11-³H]undecanoate

3T3-L1 adipocytes (1.0 mg of cellular protein/ml in PBS) were photolysed after a 30 s incubation at 37 °C with 10 μ M 11-DAP-[11-³H]undecanoate. Subcellular fractions were prepared from the labelled cells and analysed by SDS/PAGE. Shown is the radioactive labelling pattern observed when proportionate amounts of protein (10 μ g) were applied to each lane. The fraction is indicated above each lane: T, total homogenate; C, cytoplasmic fraction; and M, particulate fraction enriched in mitochondria. Numbers on the left indicate protein standards of the specified molecular masses. The arrow indicates the 15 kDa, cytoplasmic band.

to protein content, are shown in Figure 6. The fraction obtained at the 1.12/1.5 M sucrose interface (designated M in Figure 6; also, see the Experimental section) contained the highest level of cytochrome *c* oxidase activity, indicating that it was enriched in mitochondrial membranes (Figure 6a). In contrast, it contained relatively low levels of NADH dehydrogenase (Figure 6b) and 5'-nucleotidase (Figure 6c) activities, indicating that it was not contaminated with microsomes or plasma membranes respectively. Upon photolysis of intact 3T3-L1 adipocytes, incubated for 30 s at 37 °C with 10 μ M 11-DAP-[11-³H]undecanoate, a number of cellular proteins became labelled (Figure 7). These

Figure 6 Marker enzyme analysis of different subcellular membrane fractions obtained from 3T3-L1 adipocytes

Subcellular membrane fractions were prepared from 3T3-L1 adipocytes as described in the Experimental section. T refers to the total homogenate, while M, LDM and PM refer to the mitochondrial, low-density-microsomal and plasma membrane fractions respectively. Each fraction was assayed for (a) cytochrome *c* oxidase, (b) NADH dehydrogenase and (c) 5'-nucleotidase activities as described in the Experimental section. Cytochrome *c* oxidase and NADH dehydrogenase activities are expressed as the decrease in absorbance at either 550 or 410 nm, respectively, per min. 5'-Nucleotidase activities are expressed as the amount of inorganic phosphate liberated per min. All activities have been normalized to the protein content of each fraction. Results are the average $\pm$ S.E.M. of three determinations.

Figure 8 The effect of a decreased pH_c on the movement of the photoreactive long-chain fatty acid across the plasma membrane of 3T3-L1 adipocytes, measured by the photolabelling of the 15 kDa cytoplasmic FABP in intact cells

After having been prepulsed either in the absence (—) or the presence (+) of NH_4Cl , monolayers of 3T3-L1 adipocytes on tissue-culture dishes (1.5 cm diameter) were incubated in PBS containing 10 mM glucose for either 0.50 or 30 min (indicated at the top of each lane) prior to incubation for 5 s at 37 °C with 75 μM 11-DAP-[11- ^3H]undecanoate in the presence of 50 μM BSA in PBS. Photolysis was as described in the Experimental section. Labelled proteins were analysed by SDS/PAGE followed by fluorography. Numbers on the left indicate the migration of marker proteins of the specified molecular masses. The arrow indicates the location of the 15 kDa cytoplasmic FABP.

included proteins of 66, 40, 34, 15 and 13 kDa (lane marked T of Figure 7, representing the total cellular fraction). Upon fractionation of prelabelled cells (as in Figure 6), and analysis of proportionate samples of the fractions by SDS/PAGE, the following results were observed (Figure 7). The 15 kDa labelled band was found exclusively in the cytoplasmic fraction (lane C), while the other bands were associated with the fraction enriched in mitochondrial membranes (lane M). These fractions, together, accounted for the entire pattern of labelled bands observed in the total homogenate. No labelled plasma membrane proteins were observed (results not shown). This was due to the low abundance of this fraction, since labelled proteins could be observed when equal amounts of protein were analysed [9]. The finding that the 15 kDa cytoplasmic FABP was labelled in intact cells (Figure 7, lanes T and C) indicated that the probe was internalized and interacted with that protein. Furthermore, it suggested that the labelling of this protein could be used to monitor the internalization of the photoreactive long-chain fatty acid as an alternative to more conventional filtration assays.

In fact we have recently found that the labelling of the 15 kDa cytoplasmic FABP by the photoreactive long-chain fatty acid in intact 3T3-L1 adipocytes was sensitive to the concentration of the photoreactive probe in the cytoplasm (B. L. Trigatti and G. E. Gerber, unpublished work). The treatment of cells with 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS) reduced the internalization of the photoreactive probe and thereby resulted in the decreased labelling of the 15 kDa cytoplasmic FABP. In contrast, treatment with α -iodopalmitate, an inhibitor of long-chain ACS, resulted in a build-up of the probe in the cytoplasm, and, consequently, an increased labelling of the 15 kDa cytoplasmic FABP upon photolysis (B. L. Trigatti and

G. E. Gerber, unpublished work). This, combined with the insensitivity of the labelling of the 15 kDa cytoplasmic FABP with the photoreactive long-chain fatty acid to pH in the range of 5.5 to 7.5 (Figure 3), indicated that the labelling of the 15 kDa cytoplasmic FABP by the photoreactive long-chain fatty acid in intact cells should allow the discrimination between the inhibition of uptake due to inhibition of the permeation of the plasma membrane (expected to result in a decrease in labelling due to a decreased cytoplasmic concentration of probe) and that caused by the inhibition of long-chain ACS (expected to result in an increased labelling resulting from a build-up of cytoplasmic probe due to decreased utilization).

Immediately after they were prepulsed in the absence or presence of NH_4Cl , 3T3-L1 adipocyte monolayers were incubated at 37 °C for 5.0 s with 75 μM 11-DAP-[11- ^3H]undecanoate and 50 μM BSA in PBS (Figure 8, lanes marked 0.50 min). This corresponded to a concentration of uncomplexed 11-DAP-[11- ^3H]undecanoate of 500 nM (almost twice the K_i value, but still in the range of concentrations of 11-DAP-[11- ^3H]undecanoate in which uptake consisted primarily of the saturable process; see Figure 5). Upon photolysis, the level of labelling of the 15 kDa, cytoplasmic FABP (arrow), and of other intracellular proteins, was reduced in cells prepulsed with NH_4Cl relative to those prepulsed in the absence of NH_4Cl (Figure 8, lanes marked 0.5 min). NH_4Cl itself had no effect on the *in vitro* labelling of the 15 kDa cytoplasmic FABP by 11-DAP-[11- ^3H]undecanoate (results not shown). When monolayers were labelled 30 min after prepulsing, normal levels of labelling of the 15 kDa cytoplasmic FABP and of other intracellular proteins were observed (Figure 8, lanes marked 30 min). This indicated that the permeation of the photoreactive long-chain fatty acid across the plasma membrane was reduced 30 s after prepulsing, when the pH_c was reduced, but was restored to normal levels by 30 min after prepulsing, when the pH_c had recovered.

DISCUSSION

Hamilton and co-workers have demonstrated that the exposure of mammalian cells to high concentrations (35 μM) of uncomplexed long-chain fatty acids resulted in rapid, reversible decreases in the pH_c , as monitored by the fluorescence of BCECF trapped within the cells [25]. This was consistent with the permeation of protonated long-chain fatty acid across the plasma membrane by passive diffusion, followed by its dissociation within the cell, resulting in the acidification of the cytoplasm. While the acidification of the internal compartment upon the addition of long-chain fatty acids has been used as a sensitive measure of long-chain fatty acid internalization in artificial lipid vesicles [20,21], it was much less sensitive in intact cells [25]. This may have been due to the action of the cellular homeostatic machinery in maintaining a constant pH_c [28], necessitating the use of high concentrations of uncomplexed long-chain fatty acid to observe an effect on the pH_c [25]. It has been argued, however, that such concentrations of uncomplexed long-chain fatty acids are physiologically irrelevant [23,24]. Thus, the relevance of the results obtained by Hamilton and co-workers [25] to normal physiological conditions, in which long-chain fatty acid uptake occurs at submicromolar concentrations of long-chain fatty acid, is not clear.

We have made use of an approach which is complementary to that employed by Hamilton and co-workers. Rather than measuring the effects of long-chain fatty acid uptake on pH_c , we have investigated the effects of the reduction of pH_c on long-chain fatty acid uptake. This had the advantage of allowing long-

chain fatty acid uptake to be measured at physiologically relevant, submicromolar concentrations of uncomplexed long-chain fatty acids in the presence of BSA. We have monitored long-chain fatty acid uptake both by using a conventional filtration assay to measure the accumulation of radioactive long-chain fatty acids within cells and by using the photoaffinity labelling of a 15 kDa, cytoplasmic FABP in intact cells as a means of monitoring the transbilayer movement of a photoreactive long-chain fatty acid. The latter method had the advantage of allowing the cytoplasmic concentration of the probe to be followed directly, thus permitting a more direct measure of the plasma membrane permeation of the fatty acid probe than that provided by conventional filtration assays (B. L. Trigatti and G. E. Gerber, unpublished work).

When the pH_c was reduced by prepulsing, the photoaffinity labelling of the 15 kDa cytoplasmic FABP in intact cells by 11-DAP-[11- ^3H]undecanoate was decreased; upon recovery of the pH_c , however, the photoaffinity labelling was restored to normal levels (Figure 8). Therefore, the reduction in pH_c was accompanied by a decrease in the cytoplasmic concentration of the photoreactive long-chain fatty acid, indicating that the reduction of pH_c affected long-chain fatty acid uptake through a decrease in long-chain fatty acid permeation across the plasma membrane. Therefore, the physiologically relevant, saturable phase of long-chain fatty acid uptake, which constituted the major portion of long-chain fatty acid uptake at the concentration of uncomplexed long-chain fatty acid used (Figures 5 and 8), involved the permeation of the protonated long-chain fatty acid across the plasma membrane, presumably by means of diffusion through the lipid bilayer. Alternatively, long-chain fatty acids may traverse the plasma membrane by means of a fatty anion-proton co-transporter, which appears unnecessary in view of the lipophilic nature of protonated long-chain fatty acids and the evidence that long-chain fatty acid permeation of model membranes is fast [20,21,43].

These results can be explained by changes in the pH gradient affecting either the net flux or the equilibrium distribution of long-chain fatty acids across the plasma membrane. In the first case the net flux of long-chain fatty acids across the plasma membrane may be determined by the balance between those moving from the outer to the inner leaflet and those moving in the opposite direction. Acidification of the inside would lead to a shift in this equilibrium favouring movement to the outer leaflet. Alternatively, if long-chain fatty acid uptake was driven by the intracellular trapping of long-chain fatty acids by binding or metabolism, this would be sensitive to the long-chain fatty acid concentration on the inner leaflet of the membrane, which is determined by the distribution of long-chain fatty acids across the bilayer in response to the pH gradient.

The decrease in pH_c by 0.6 unit upon prepulsing (Figure 1) corresponded to an increase in the pH gradient across the plasma membrane from 0.1 to 0.7 (inside acidic). As demonstrated by Hope and Cullis [22], it can be calculated from the Henderson-Hasselbach equation that this increase in the pH gradient should result in a change in the ratio of long-chain fatty acids partitioned into the inner versus the outer leaflet of the plasma membrane from 0.8 to 0.2. This assumes that the level of fatty acid binding to proteins in the membrane is small compared with the concentration of fatty acid partitioned into the lipid bilayer, as has been demonstrated by Cooper et al. [44]. Thus, the long-chain fatty acid concentration in the inner leaflet of the plasma membrane should have been reduced by 75%, leading to the prediction of a 75% decrease in long-chain fatty acid uptake. This agrees well with the 70% reduction in uptake observed upon reduction of the intracellular pH (Figure 2). These results do not require that the fatty acid distribution across the plasma

membrane be rate-limiting; instead, they can be explained by changes in the pH gradient, affecting either the net flux or the equilibrium distribution of long-chain fatty acids across the plasma membrane. In the first case, the net flux of long-chain fatty acids across the plasma membrane may be determined by the balance between fatty acids moving from the outer to the inner leaflet and those moving in the opposite direction. Acidification of the inside would lead to a shift in this equilibrium, favouring movement to the outer leaflet. Alternatively, if long-chain fatty acid uptake was driven by the intracellular trapping of long-chain fatty acids by binding or metabolism, this would be sensitive to the long-chain fatty acid concentration on the inner leaflet of the membrane, which would be determined by the distribution of fatty acids across the bilayer in response to the pH gradient [22]. This suggests at least two mechanisms by which long-chain fatty acid uptake involving passive diffusion of long-chain fatty acids across the plasma membrane can be regulated by proteins and appear saturable (see Figure 5 and references [8-11,13,15,23,24,45]). Namely, long-chain fatty acid uptake can be driven by either protein-mediated binding of fatty acids within the cell, or protein-mediated conversion to long-chain fatty acyl-CoA, by intracellular long-chain ACS. In fact, Noy and Zakim have argued that long-chain ACS is rate-limiting for long-chain fatty acid uptake in mammalian cells, driving overall uptake [7]. The analogous situation appears to be the case in *E. coli* [42].

Long-chain fatty acid uptake by mammalian cells appears to differ from that by *E. coli*, which has been shown, both genetically and biochemically, to be dependent on the expression of the *fadL* gene, which encodes an outer-membrane-associated long-chain FABP [45,46]. This is due to the impermeability of the outer membrane of *E. coli* to a variety of hydrophobic compounds [47,48]. In that respect, it differs from plasma membranes of mammalian cells which are permeable to such compounds [7,47,48]. The FadL protein is, therefore, a specific requirement of *E. coli* and other Gram-negative bacteria. The inner membrane of *E. coli*, however, is analogous to the plasma membrane of mammalian cells. It has been shown to lack proteins which bind long-chain fatty acids specifically or with high affinity, suggesting that fatty acids permeate the plasma membrane of *E. coli* by passive diffusion [45].

Evidence does, however, indicate that long-chain fatty acid uptake by a variety of mammalian cells, including 3T3-L1 adipocytes, involves one or more plasma membrane proteins. These include a 43 kDa peripheral protein closely related to mitochondrial glutamate-oxaloacetate transaminase [10,12,13], a 63 kDa integral membrane protein shown to confer increased uptake of long-chain fatty acids [15] and an 88 kDa DIDS-sensitive integral membrane protein related to CD36 [11,14], but which has not been shown to bind fatty acid. Using the photoreactive long-chain fatty acid, we have previously found that a 22 kDa protein was the sole, high-affinity fatty acid receptor in 3T3-L1 adipocyte plasma membranes [9,16]. The specific functions of these proteins are unclear. While the results presented above indicate that long-chain fatty acid movement across the plasma membrane is not mediated by a transport protein, it remains possible that these proteins are involved in another step in long-chain fatty acid uptake. One such step is the delivery of long-chain fatty acids to the cell membrane by serum albumin, which appears to involve receptors for serum albumin on the cell surface [8]. Alternatively, one or more of these proteins might be involved in the regulation of long-chain fatty acid uptake [14]. The finding that long-chain fatty acid permeation across the plasma membrane is affected by the intracellular pH also suggests the possibility that uptake could be

regulated by the modulation of the pH gradient across the membrane.

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