Evidence in Favor of a Facilitated Transport System for FA Uptake in Cultured L6 Cells

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ABSTRACT: In this manuscript we report a study of the transport of FA in L6 muscle cells. Cultured L6 cells took up labeled FA (C_{10} to C_{20}) as a linear function of time up to 15 min. Thereafter, the rate of uptake gradually declined although it persisted for at least 12 h after the addition of the substrate. Kinetic parameters (K_m, V_m, and k_o) were determined from a fitted Michaelis–Menten-type equation modified by a term for a saturable (linear) component of the measured total uptake. V_m values were different for some of the FA studied, and K_m data showed significant differences between saturated and unsaturated FA. The maximal rate of uptake was observed at pH 7.40 for decanoate, palmitate, and eicosatrienoate. Uptake was significantly influenced when the pH of the incubation medium was changed. Experiments designed to study the influence of FA/albumin molar ratio indicated that V_m was dependent on the total (bound and free) concentration of the FA. A concentrative uptake was demonstrated in short-term experiments with an apparent plateau of 20 and 40 μM for palmitate and eicosatrienoate, respectively. A competitive inhibition was also observed between palmitate as substrate and the other FA. From our results we can postulate that the uptake of FA in L6 cells is the sum of passive diffusion plus a saturable component and that the rate of uptake is dependent on one (or more) protein structures, although their precise characteristics and functions remain to be elucidated.

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FA bound to albumin and serum lipoproteins represent the most suitable form in which to transport fat for its utilization. Different types of cultured cells are able to carry out the uptake and metabolism of FA. It was previously demonstrated that in cardiac myocytes the oxidation of long-chain FA is the predominant source of energy necessary for proper electrochemical function (1). As the capacity of this kind of cells to store FA in lipid pools is very limited, myocytes are dependent on a continuous supply of these substrates from the blood stream (2). The L6 rat skeletal muscle cell line would share similar metabolic characteristics with cardiac myocytes; as a consequence, it may be a useful experimental model to investigate FA uptake.

Prior to their cellular uptake, FA must be dissociated from albumin, then they go through the plasma membrane. The driving force for this process is considered to be the concentration gradient existing across the biomembrane (1–4). Previous papers support the conclusion that transmembrane transport is governed by simple, nonfacilitated diffusion (2–6). However, it was also suggested that the plasma membrane fatty acid-binding protein (FABP), an FA transport protein, and a membrane translocase found in myocytes (2,7–11) would be involved in the FA uptake. However, the role of FABP remains a matter of discussion since transfection of L6 muscle cells with FABP-cDNA does not modify FA uptake (12). In addition, it was reported that high-affinity albumin-binding sites would participate in this process (13,14). Luiken *et al.* (2) recently discussed the idea that FA uptake in isolated cardiac myocytes would be the result of both passive and protein-mediated processes. At present no general agreement exists on this matter, and the exact mechanism of transmembrane transport of FA is still a topic of controversy. Contrary to previous results, which supported the idea of passive diffusion of FA into transformed cells (15), our findings favor a facilitated transport system operating in these skeletal muscle cells.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]FA (98–99% pure, 50–60 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Unlabeled FA were provided by Nu-Chek-Prep (Elysian, MN). All acids were stored in benzene under an atmosphere of nitrogen at −20°C. Concentrations and purities were routinely checked by both gas–liquid radiochromatography and liquid-scintillation counting. Mass determinations were performed by GLC of FAME prepared in the presence of internal standards. FAME mixtures, HEPES delipidated serum albumin (BSA; fraction V from bovine), minimum essential medium Eagle (MEM) with Earle's salts, nonessential amino acids, L-glutamine, choline chloride, fetal bovine serum (FBS), Earle's balanced salts, and improved

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Abbreviations: FABP, fatty acid-binding protein; IMEM-Zo, improved minimal essential medium-zinc optional; MEM, minimal essential medium; UFA, uncomplexed FA.

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minimal essential medium-zinc-optional (IMEM-Zo) were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were RPE grade and provided by Carlo Erba, Milano, Italy. Silicagel G-60 plates for TLC were provided by Fluka-Riedel-de Häen, Darmstadt, Germany. Other chemicals used were reagent grade and obtained from commercial sources.

Cell culture and experimental procedure. L6 muscle cells were grown in surface cultures in approximately 30 cm^2 flasks at 37°C with 5 mL MEM supplemented with 10% (vol/vol) FBS, 0.30 g/L glutamine, and 25 mM HEPES. When the cells were at the logarithmic phase of growth (approximately 72 h after seeding), the culture medium was replaced by IMEM-Zo minus linoleic acid and containing HEPES (25 mM final concentration). The cells were maintained in this medium for 24 h. Then the medium was aspirated and replaced by fresh medium at 37°C supplemented with different FA at various final concentrations. The FA were added as sodium salts bound to delipidated albumin according to Spector *et al.* (15). The uptake process was stopped following the method of Samuel *et al.* (16). After aspiration of the medium, the cells were washed twice with 5 mL of cold Earle's balanced salt solution containing 0.5% BSA. All operations were performed within 20 to 25 s. Control flasks were supplemented with defatted BSA at the same concentration used in the experimental ones. Radioactivity recovered in the last wash routinely represented 4 to 7% of the total radioactivity remaining bound to the cells. Cells were washed and then treated with 2 mL of 0.1 N NaOH and immediately shaken to produce cellular lysis. The resulting lysates were transferred to ice-cold tubes, and the culture flasks were washed twice with 1 mL each of 0.1 N NaOH. After stirring of the pooled solutions, an appropriate aliquot was directly transferred into scintillation vials containing 10 mL of ACS II Scintillation Cocktail for aqueous samples from Amersham Pharmacia Biotech (Buckinghamshire, England). A Beckman LS-5801 Liquid Scintillation Counter with 95% efficiency for ^{14}C was used. Counting of cellular-associated radioactivity by this method gives results similar to those obtained by extraction of total cellular lipids following the procedure of Folch *et al.* (17). Another aliquot of the cellular lysates was taken for protein measurement following the method of Lowry *et al.* (18). Differences in the uptake values of flasks from the same experimental group did not exceed 5%. Lipid analyses were performed on cellular pellets obtained from surface cultures of L6 cells during the logarithmic phase of growth. The attached cells were mechanically scraped off from the growing surface through the use of a rubber-tipped spatula. After centrifugation at 500 × *g* for 10 min in the cold, the pellet was washed again with 10 mL of Earle's balanced saline solution and centrifuged as described before. The final pellet was extracted according to the method of Folch *et al.* (17). Neutral and polar lipids were further isolated from the total lipid extract by silicic acid microchromatography (Bio-Rad Laboratories, Richmond, CA) according to the method of Hanahan *et al.* (19). The total amount of lipids in each fraction was determined gravimetrically (20). Samples for GLC were transesterified and analyzed as previously described (21). Lipid analyses performed on culture medium were carried out after filtering through a 0.22 μ SM-11307 Sartorius Membranefilter (Göttingen, Germany) to remove cell debris. The filtrates were lyophilized, and the residual components extracted by the method of Folch *et al.* (17). The procedures employed for TLC analysis were described previously (22). In some experiments the initial rate of FA uptake was determined in modified incubation media. To study the effect of sodium concentration on uptake, we followed the procedure of Stremmel, Strohmeyer and Berk (23). A buffer containing 0.4 mM KH_2PO_4, 0.3 mM K_2HPO_4, and 15 mM HEPES at pH 7.40 was employed instead of IMEM-Zo medium. The desired concentration of NaCl or substitutes, such as choline chloride, KCl, $CaCl_2$, or $MgCl_2$, was added and, in order to perform the incubations at the same osmolarity (285 ± 5 mOsm/kg H_2O), all media were checked by a Semi Micro Osmometer A-800 from Knauer GmbH (Berlin, Germany). Cell viability was assessed by the exclusion test of trypan blue (>96%) (24).

Software and calculations. Data are reported as the mean ± SE calculated from three or four independent analyses. The software used for statistical studies (correlation coefficient for nonlinear curve fitting, linear regressions, Student *t*-test, and ANOVA) and other calculations was Systat (version 8.0 for Windows) from SPSS Science (Chicago, IL). Data were also plotted and analyzed using Sigma Scientific Graphing Software (version 8.0) from Sigma Chemical Co. Graphics were constructed considering the amount of uncomplexed FA (UFA) in the presence of BSA. The UFA concentrations were calculated on the association constants determined by Spector *et al.* (25–27) for 12:0, 16:0, 18:0, and 18:2n-6 FA using a personal computer. In the case of the UFA, 18:3n-3 and 20:3n-6, the UFA concentrations were measured in separate experiments (in the absence of cells) by the two-phase partitioning method using heptane as the organic phase as described by Spector *et al.* (27–29). These measurements were performed with a fractional standard error among independent determinations of 2.5% or less. Uptake data were fitted to a modified hyperbolic function of the calculated UFA concentrations using the software mentioned before. During the data fitting, care was taken to find both global minima in the sum of squares and the best nonlinear regression coefficient. We found that, for all the FA studied, our results were best fitted by the following modified hyperbolic equation:

$$UT = V_m[UFA]/(K_m + [UFA] + k_o\,[UFA]) \qquad [1]$$

where UT is the global uptake expressed in nmol FA/min/mg cellular protein, [UFA] is the UFA concentration in the presence of a specific BSA concentration (nM), K_m is the UFA concentration at a half-maximal uptake rate (nM), V_m is the maximal uptake rate of the saturable uptake component (nmol/min/mg cellular protein), and k_o is the rate constant for the linear (nonsaturable) component (μL/min/mg cellular protein). The same equation can be expressed as the sum of two components, one of them with a saturable behavior

(rectangular hyperbola) and the other as an unsaturable component (a linear function of UFA concentration):

$$UT = \left(V_m\,[UFA]/(K_m + [UFA])\right) + k_o'\,[UFA] \quad [2]$$

Both equations were essentially identical to those employed by Trigatti and Gerber (14) and Stump, Fan, and Berk (30), and both of them gave statistically identical results for calculation of the kinetic parameters. Nonlinear correlation coefficients (R^2) were within the range 0.980 to 0.997 depending on the FA studied.

RESULTS

Owing to the significant modifications of the FA composition evoked by the culture time in complete medium (data not shown), the experiments described in this work were carried out using cultured monolayers of L6 muscle cells during the early logarithmic phase of growth (approximately 72 h after seeding). We chose for our experiments various FA of different chain length and degree of unsaturation. Experimental parameters such as the FA concentration, specific activities, and incubation times were determined by testing the initial rate of uptake under different assay conditions. When the time course of FA uptake was studied under physiological conditions, i.e., at exogenous concentrations of FA and albumin of 20 and 5 μM (FA/BSA equal to 4:1), a rapid initial phase was observed for all substrates studied (Fig. 1A). This uptake was measurable within 30 s after FA addition (Figs. 1B and 1C) and linear with time for at least 15 min (Fig. 1A). Thereafter, the rate of uptake gradually declined for those FA from C_{14} to C_{20}. In contrast, the period of linear uptake was longer for

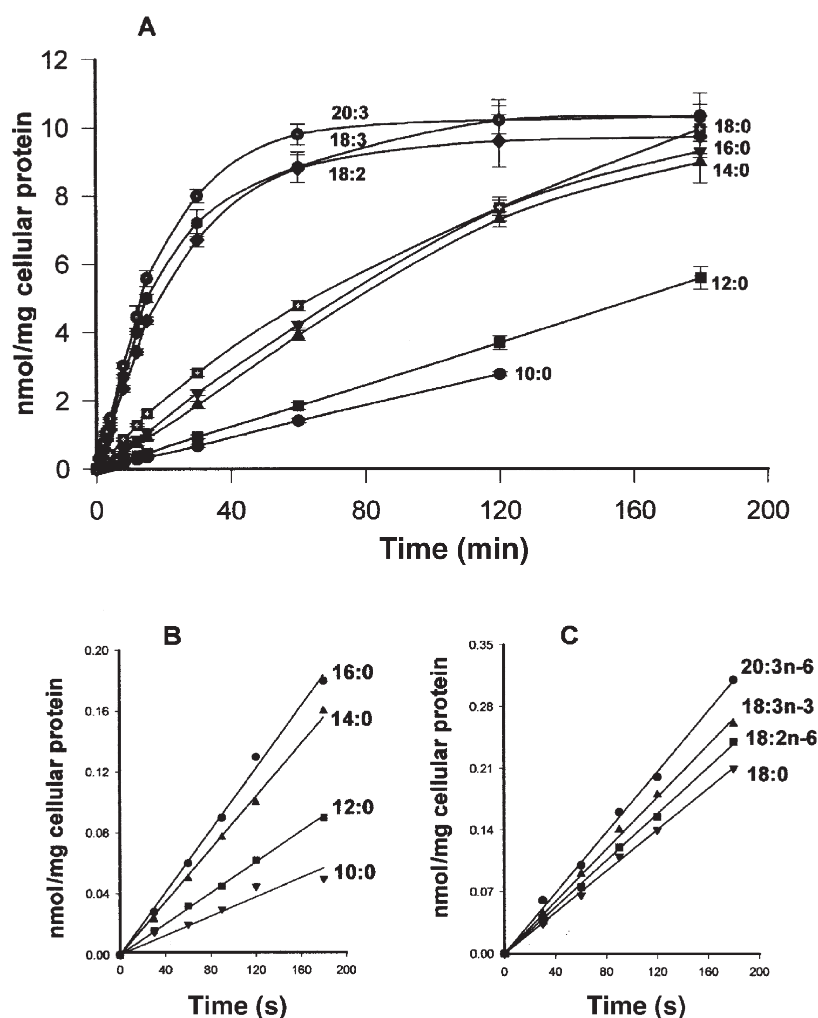

FIG. 1. The time course of FA uptake was determined in L6 cells using confluent monolayers of myocytes at an early logarithmic phase of growth. They were incubated in improved minimal essential medium-zinc optional (IMEM-Zo) medium with different FA (fixed initial concentration 20 μM) bound to delipidated albumin (FA/BSA molar ratio 4:1) and analyzed on cellular uptake at the times indicated. Samples were taken from 0 to 180 min (A) or registered during the initial 180 s at 30-s intervals (B and C). For details see Materials and Methods section. Data are the mean ± SEM of three experiments.

shorter-chain FA (C_{10} and C_{12}). For all the substrates studied, uptake was observed for at least 8–12 h (data not shown). It may be suggested that the fall-off in the rate of uptake could be the result of a toxic effect of the FA on the cells (3). After taking into account this possibility, the kinetic data were determined in the 0–3 min period after the FA addition. Exclusion of trypan blue was routinely performed for each determination, leading to the observation that more than 96% of cells were viable in all assays. Figure 2 shows the initial uptake rates for various FA as a function of the UFA concentration in the incubation medium calculated as mentioned in the Materials and Methods section. We chose the initial concentration of FA and BSA to obtain similar sets of UFA concentrations within the range 0 to 900 nM for each FA studied. Calculations for initial uptake rates were made from the slopes of the cumulative linear regressions curves over the first 3-min incubation period. As explained before, the uptake rates were fitted best (R^2 within the range 0.980 to 0.997) by the sum of the saturable (hyperbolic) plus nonsaturable (linear) components for all the FA studied. Computed values for the kinetic parameters obtained from these plots are presented in Table 1. V_m values varied from 0.55 ± 0.03 to 1.80 ± 0.11 nmol·min^{-1}·mg cellular protein^{-1}, and they were different from each other for the saturated FA studied. Results indicate that, in the case of the saturated FA, V_m values increased with the length of the carbon chain. V_m data for the UFA were very similar to each other, and these values were between those of palmitic and stearic acids. In the case of the K_m data, we also found significant differences between unsaturated FA (133 ± 7, 130 ± 8, and 128 ± 6 nM for linoleate, γ-linolenate, and dihomo-γ-linolenate, respectively) and saturated FA (75 ± 4, 71 ± 3, and 68 ± 3 nM for C_{12}, C_{16}, and C_{18}, respectively) (Table 1). From these kinetic parameters, it is possible to calculate the k_o constant and the percentage contribution of the uptake that is ascribed to the saturable component of the global kinetic behavior (Table 1). When the FA/BSA molar ratios

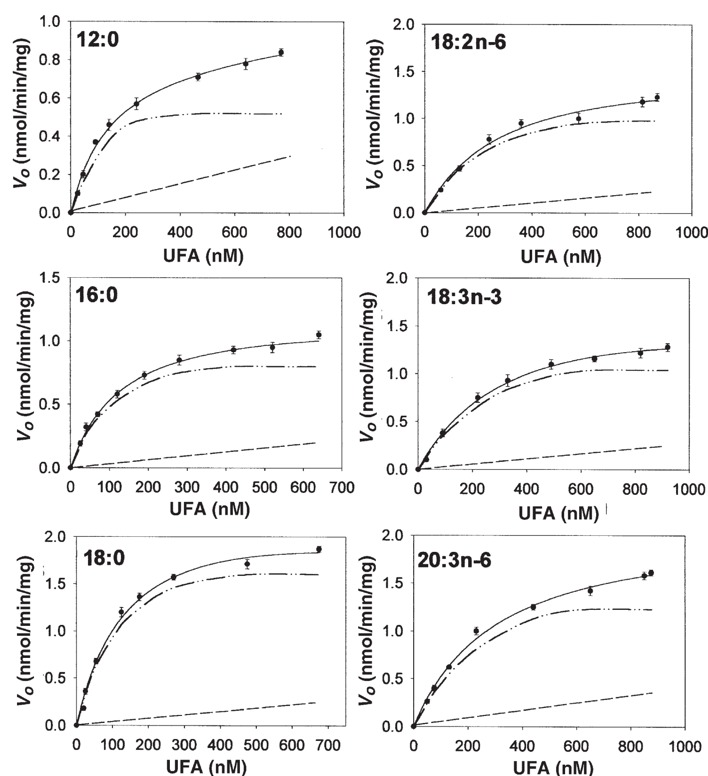

FIG. 2. Initial uptake rates of various [1-^{14}C]FA as a function of the uncomplexed FA (substrate) concentration in the incubation medium. Monolayers of cultured L6 cells in IMEM-Zo medium were incubated at 37°C for 3 min with various concentrations of the indicated FA (fixed initial concentrations from 0 to 500 μM) bound to delipidated BSA. The resulting FA/BSA molar ratios varied from 0.2:1 to 4:1. The resultant uncomplexed FA (UFA) concentrations were within the range 0 to 1000 nM; they were calculated, or experimentally determined, as indicated in the Materials and Methods section. The initial uptake rates were obtained from the linear regression slopes of the cumulative uptake curves over the initial 3-min incubation period and were fitted by a computer program to a nonlinear hyperbolic curve containing a term for nonsaturable uptake. (●), Experimentally measured total FA uptake; (—), computer-fitting of experimental data; (–··–··), saturated uptake component; and (-----), nonsaturated uptake component. Values are the means ± SEM of three independent measurements. For abbreviation see Figure 1.

TABLE 1
Kinetic Parameters Obtained from the Uptake of FA in L6 Cells[a]

FA	V_m [b]	K_m [b]	k_o [c]	%NSC[d]
12:0	0.55 ± 0.03	75 ± 4	0.38 ± 0.02	34 ± 2
16:0	0.91 ± 0.05	71 ± 3	0.35 ± 0.03	20 ± 1
18:0	1.80 ± 0.11	68 ± 3	0.37 ± 0.03	14 ± 1
18:2n-6	1.11 ± 0.10	133 ± 7	0.25 ± 0.02	17 ± 2
18:3n-3	1.22 ± 0.13	130 ± 8	0.26 ± 0.03	17 ± 3
20:3n-6	1.40 ± 0.08	128 ± 6	0.37 ± 0.04	20 ± 2

[a]The kinetic parameters (V_m, K_m, and k_o) for each curve from Figure 2 were generated from a weighted least-squares calculation fitted from the individual data points for each FA by means of a Michaelis–Menten-type equation containing the term k_o·[UFA] for nonsaturable (linear) uptake.
[b]$nmol \cdot min^{-1} \cdot mg$ cellular $protein^{-1}$.
[c]nM.
[d]$\mu L \cdot min^{-1} \cdot mg$ cellular $protein^{-1}$.
[e]Percentage contribution of the nonsaturable uptake component. UFA, uncomplexed FA; NSC, nonsaturable component.

were maintained below 4:1, the linear component contributed 14 to 34% of the observed (total) uptake. The contribution observed for C_{18} FA was of lesser extent than those from the other ones (Table 1). Within the saturated FA, the contribution of the nonsaturable uptake correlates inversely with the chain length, whereas in the case of the unsaturable FA this parameter remains almost constant (Table 1).

A series of experiments was designed to study the influence of FA/BSA molar ratio on the rate of uptake. To understand the role of BSA in the uptake of a saturated (palmitate) and an unsaturated (eicosatrienoate) FA, we performed some experiments at various concentrations of UFA in the incubation medium. The FFA concentration was selected to avoid exceeding its solubility (31,32). The incubations were performed at variable or fixed BSA concentrations (Fig. 3). In this setting, [^{14}C]palmitate or [^{14}C]eicosatrienoate at a fixed concentration was complexed to BSA at various concentrations or a BSA at a fixed concentration was complexed to FA at increasing concentrations producing a FA/BSA molar ratio that varied between 0.25:1 and 6:1 (Fig. 3, circles). Alternatively, the FA/BSA molar ratio was maintained constant at 6:1 as the FA concentration was increased (Fig. 3, squares). This provided various calculated UFA concentrations within the range 0 to 900 nM. Another uptake curve was obtained in the absence of BSA (Fig. 3, triangles). Results indicate a clear dependence of the uptake on the presence of BSA in the incubation medium. However, no significant differences were observed at the initial rate of uptake when the FA/BSA molar ratio was varied from 0.25:1 to 6:1. The uptake in the absence of BSA was clearly decreased with respect to that observed in the presence of the protein, and in this case, results were best fitted to a linear regression (Fig. 3, triangles).

The optimal pH for initial rates of uptake was found to be around 7.40 for decanoate, palmitate, and eicosatrienoate (Fig. 4). It is important to remark that we found maximal uptake at physiological pH for the three FA studied. Changing the pH of the incubation medium had the largest influence on the uptake of palmitate.

No differences were observed for the initial rates of uptake when using a medium supplemented with glucose (10 mM). We also found that the omission in the medium of K^+ (replaced by Na^+) or Ca^{2+} (replaced by Mg^{2+}) had no significant influence on the rate of any of the FA studied (data not shown). However, when we examined the uptake of palmi-

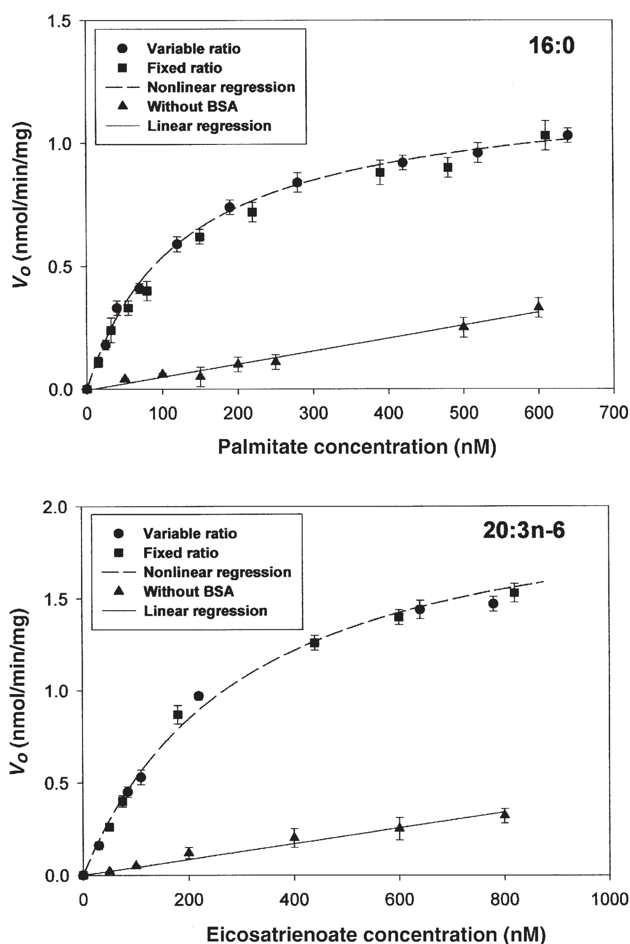

FIG. 3. L6 cells (approximately $3 \cdot 10^6$ cells in 4 mL final incubation volume) were incubated at 37°C with labeled palmitic (A) or eicosa-8,11,14-trienoic (B) acid. Incubations were conducted at variable FA/BSA molar ratios (0.75:1 to 6:1) (●) or at a fixed ratio of 6:1 (■). Other curves were obtained in the absence of albumin (▲). Uptake was measured as described in the Materials and Methods section and in the legend to Figure 1. Data are the mean ± SEM of three determinations.

tate and eicosatrienoate under the effect of isosmotic Na^+ substitution with different proportions of choline chloride to achieve 285 ± 5 mOsm/kg H_2O in all incubation flasks, the influx of FA was significantly depressed in a nonproportional fashion (Fig. 5). A major effect was observed with complete substitution of Na^+ for choline chloride added at a final concentration of approximately 135 mM. The resultant uptake values were significantly depressed within the range 0 to 50 mM Na^+ compared to the values measured at 140 mM.

Table 2 shows the specific activities of labeled palmitic and eicosatrienoic acids in the medium after the incubation of the L6 cells from 1 to 5 min. The medium of each experiment was saved, and the unesterified FA were separated by TLC, methylated, and analyzed by quantitative GLC as described in the Materials and Methods section. We found no significant changes in the specific radioactivity of the FA assayed, although up to 23% of the exogenous acid was taken up by the cells. These results are in favor of a net uptake of FA rather than an exchange with FA previously present in the cells.

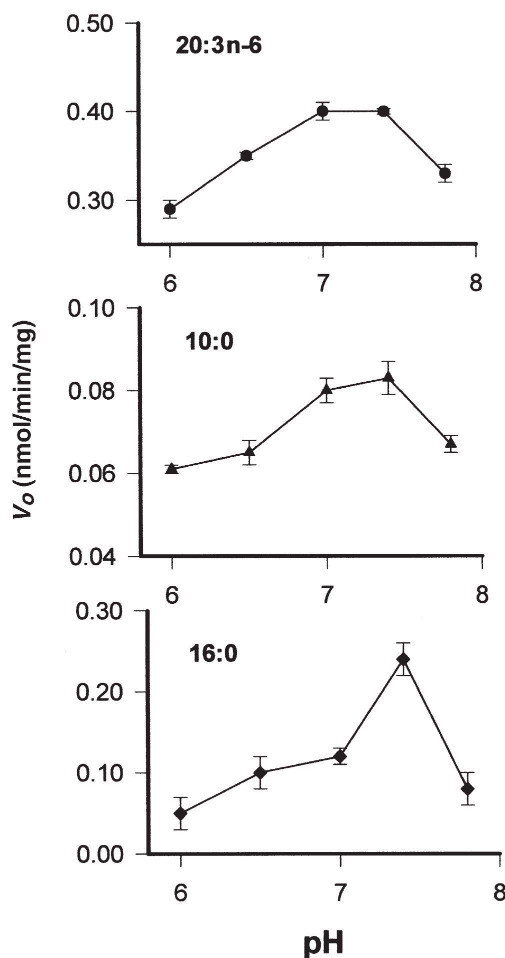

FIG. 4. Initial rates of FA uptake were determined in L6 cells incubated in IMEM-Zo medium buffered at different pH values. V_o values (nmol/min/mg of cellular protein) were determined at an FA/BSA molar ratio of 4:1 after a 3-min incubation period at 37°C. Data are the mean ± SEM of three determinations.

We carried out another type of experiment by analyzing the composition and the concentrations of the different UFA in both the culture medium and the L6 cells. Results obtained clearly demonstrated that these cells do take up FA very efficiently from the culture medium. Moreover, a concentrative uptake could be demonstrated in short-term experiments conducted at different FA concentrations of FA under conditions where the substrates were mainly present as their unesterified form (Fig. 6). As expected, this concentrative uptake declined as the concentration of the acid increased. Apparent plateaus were reached at 20 and 40 μM of external FA concentration for palmitate and eicosatrienoate, respectively (Fig. 6).

During FA uptake of L6 cells, a competitive inhibition takes place between palmitate as substrate and the other FA. In these experiments the initial rates of uptake for [1-^{14}C]palmitate were determined at total concentrations of 16:0 varying from 0 to 200 μM (UFA concentrations within the range 0 to 500 nM) with an FA/BSA molar ratio equal to 4:1, and in the simultaneous presence of different unlabeled FA as competitors added to the incubation medium at uncomplexed concentrations of 100 to 300 nM (approximately one to four times the K_m for the uptake of palmitate). Data were processed to obtain Dixon plots (21,33), and the kinetic parameters that characterized each incubation (V_m and K_i) were calculated with the aid of the graphic software mentioned before. Dixon plots were constructed as the inverse of the initial velocities vs. inhibitor concentrations (Fig. 7). These types of kinetic analysis are frequently used to identify kinetic inhibition and to determine "αK_i" and "K_i" values (33). The velocity equation for pure or mixed-type competitive inhibition may

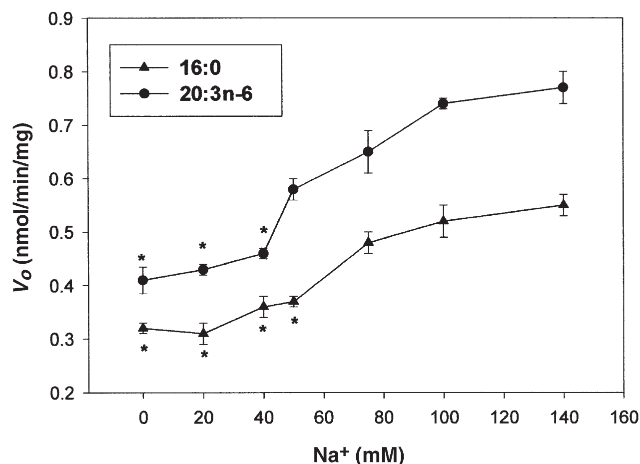

FIG. 5. The influence of sodium ion concentration on initial uptake velocity (V_o) of [1-^{14}C]palmitate (▲) or [1-^{14}C]eicosa-8,11,14-trienoate (●) was studied in a buffered IMEM-Zo medium (pH 7.40) containing the indicated Na^+ concentrations (as NaCl) plus sufficient choline chloride salt to achieve a total osmolarity of 285 ± 5 mOsm/kg H_2O in all flasks. V_o values (nmol/min/mg of cellular protein) were determined at an FA/BSA molar ratio of 4:1 after a 3-min incubation period at 37°C. Values are the mean ± SEM of three determinations. (*) $P < 0.01$ with respect to the V_o values measured at 140 mM sodium. For abbreviation see Figure 1.

TABLE 2
Specific Activity of the Incubation Medium After FA Uptake by L6 Cells[a]

Time (min)	FA (10 μM)	Uptake (nmol/mg)	Uptake (%)	Specific activity (dpm/nmol)
1	16:0	0.66 ± 0.05	4.0	3106 ± 195
5	16:0	1.75 ± 0.22	10.5	2967 ± 114
1	20:3n-6	1.50 ± 0.31	9.0	3110 ± 142
5	20:3n-6	3.80 ± 0.44	23.0	2807 ± 232

[a]FA were incubated at 10 μM initial concentration as albumin complexes (FA/BSA molar ratio equal to 4:1) with $3{\cdot}10^6$ cells (approximately 3 mg of cellular protein) at the times indicated. The incubation media were saved and analyzed as described in the Materials and Methods section. Each value is the mean ± SEM of four determinations.

be converted into a linear form in which the varied ligand is the concentration of the FA added as competitor. The regression lines obtained at different substrate (palmitate) concentrations have positive slopes that are given by $K_m/[S]{\cdot}V_m{\cdot}K_i$. Since K_m, $[S]$, and V_m are constants, the slopes are inversely proportional to K_i. By drawing a horizontal line at a height of $1/V_m$, these lines can be intercepted at the −[FA] (FA added as competitor), representing the kinetic parameter K_i. Results from Table 3 and Figure 7 indicate a competition between the FA for entry into the cells, since the presence of another FA in the incubation medium is accompanied by a decrease in palmitate uptake. The analysis of Dixon plots (Fig. 7) suggests that this inhibitory effect belongs to the type called "pure competitive" (33) with K_i values within the range 141 to 218 nM depending on the FA tested as competitor (Table 3). The values found when these FA were present either as substrates or as competitors strongly suggest that they can interact with the same molecular system as palmitate, thus displaying a similar affinity.

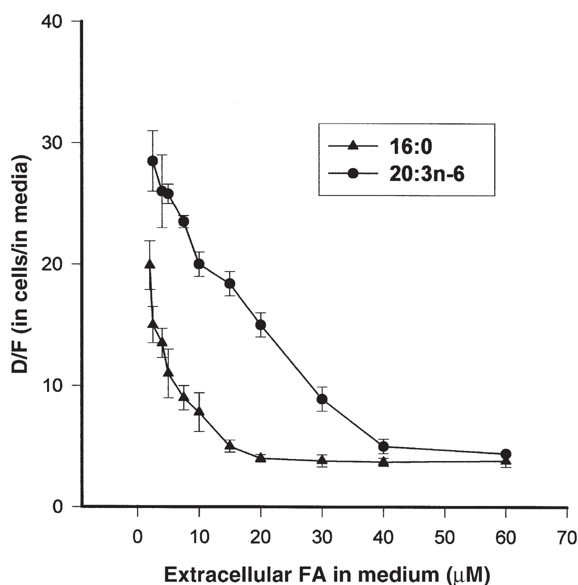

FIG. 6. FA uptake was determined in L6 cells incubated in IMEM-Zo medium supplemented with various concentrations of palmitate (▲) or eicosa-8,11,14-trienoate (●) bound to delipidated albumin (FA/BSA molar ratio 4:1). After 4 min of incubation, radioactivity was quantified in both cells (D) and media (F) as described in the Materials and Methods section. Data are the mean ± SEM of three analyses. For abbreviation see Figure 1.

DISCUSSION

It is generally assumed that the uptake of FA by mammalian cells occurs by passive diffusion (3,4,21). Active transport of FA has been described in bacteria and yeast (34). Active transport of FA in cardiac cells from chick embryo (16) or adult rats (2) has also been suggested. In addition, other authors reported a direct involvement of BSA (13,14,35), membrane proteins (23,35,36) and various cytosolic proteins (2,35,37–45) in the uptake of FA. However, up to now, the mechanism of cellular uptake of FA has been a controversial question that probably involves both a passive and a carrier-mediated transmembrane translocation (35,46,47). In the present paper, the experimental evidence indicates that in cultured myoblastoma cells a net uptake of FA occurs through a saturable transport system. Results from Figure 2 and Table 1 clearly show that the saturable component of the FA uptake exhibits a rather high affinity for C_{12} to C_{20} FA, with different initial velocities depending on the length of the carbon chain and/or unsaturation degree. After comparing results from other laboratories—recalculated using the same units—we observed that V_m values for some of the FA studied approached previously reported rates (3,7,23), and K_m for net uptake seems to be above the physiological concentrations on non-BSA-bound FA in plasma (25,35,48,49). Stearic acid has a V_m that is almost three times greater than that of 12:0; taking into account that one million L6 cells equals *ca.* 0.8 mg of cellular protein, this value is very similar to that reported by Stremmel (7) for oleic acid in cardiomyocytes. Moreover, we found that K_m data increased significantly with chain length from C_{12} to C_{18} (Table 1) in a way similar to that suggested by DeGrella and Light (3). K_m values showed clear differences between saturated FA and UFA (Table 1). Interpretation of these results may be carried out from different points of view, but we think they, may indicate the existence of a membrane-associated transport system with appropriate capacity to meet the needs of L6 cells for FA as metabolic precursors or as a source of energy.

Transmembrane FA transport could be linked to some ATP-consuming process such as conformational changes of membrane-associated proteins, or co-transport with—for

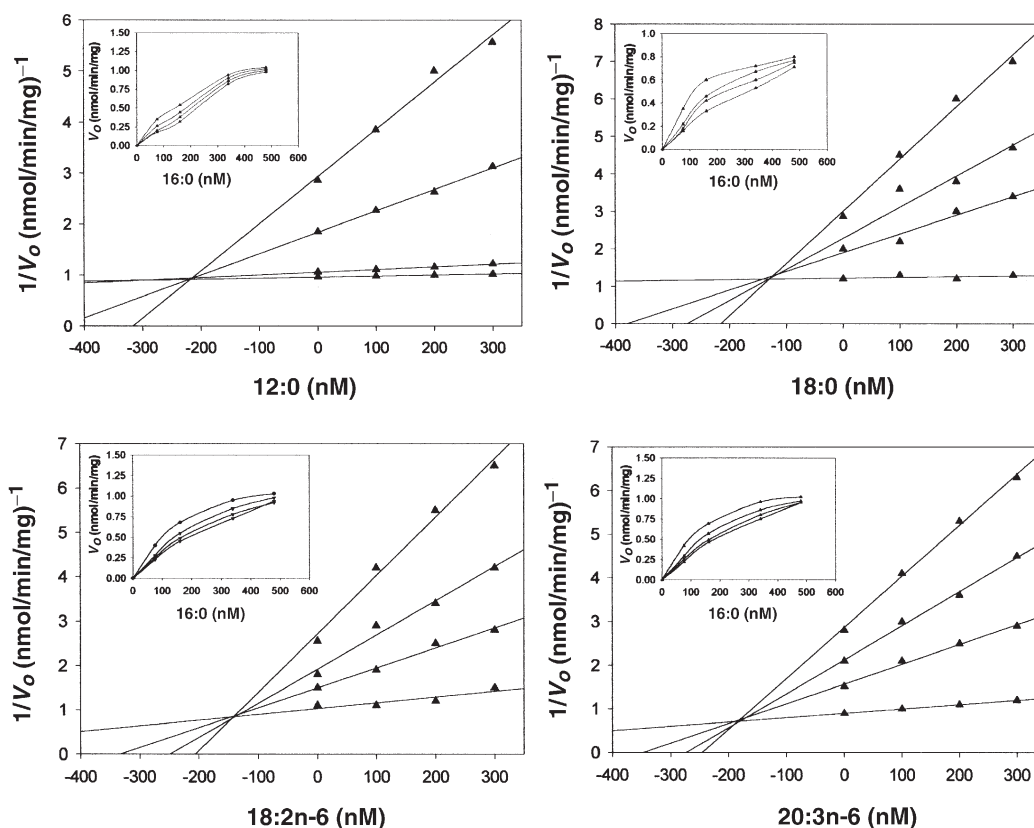

FIG. 7. Competition experiments were performed in the presence of labeled palmitate (0 to 200 μM total concentration; FA/BSA molar ratio equal to 4:1) and different unlabeled FA (12:0, 18:0, 18:2, and 20:3) as competitors at three final concentrations. Uncomplexed palmitate concentration varied from 0 to 500 nM, and the concentrations of the FA added as competitors were within the range of 100 to 300 nM (one to four times higher than the basal K_m value for palmitate). Data were processed to obtain Dixon plots, and the kinetic parameters (V_m and K_i) were calculated through a computer software program as detailed in the Materials and Methods section. The initial velocities were calculated from the slopes of the minimal-squares linear regression plots computer-generated from the 3-min initial uptake data according to the procedure described in the text. Each value represents the mean of duplicate experiments. Insert graphics show the Michaelis–Menten-type behavior for the different sets of experimental points.

instance—Na^+ (23,35,50–52). Although the available data from liver and cardiac muscle cells indicate that Na^+-dependent transport of FA represents a minor portion of total uptake, previous reports from other laboratories (23,35,50,51) and the present paper (Fig. 5) indicate a direct relationship between FA uptake and sodium availability. The fact that FA uptake is facilitated specifically in the presence of Na^+ suggests that L6 cells have a sodium-linked system for FA influx that may be dependent on Na^+/K^+-ATPase, similar to what had been found for the hepatocellular uptake of oleate previously reported by Stremmel *et al.* (23).

It is well-known that changing pH is a simple but potent strategy for altering the amount of FA partitioning into the biomembrane. The UFA is composed of anions and undissociated acid, but in practice, the total UFA concentration is considered to be the free anion concentration (27). This approach would be entirely valid if the pH of the medium is 7 or higher because the pK_a of FA is thought to be about 4.8 to 5.0 (27). For this reason, within the range of physiological pH values a considerable portion of total FA may be un-ionized. As reviewed by Spector (27), binding of FA to BSA is increased significantly when the pH of the medium is raised from 6.5 to 8.2. This would be reflected in the relative proportion between free and complexed FA. As discussed by

TABLE 3
Kinetic Parameters for the Uptake of Palmitate in the Presence of Various FA as Competitors[a]

FA added as competitor	V_m[a] (nmol/min/mg cellular protein)	K_i (nM)
None	0.91	—
16:0	0.88	218
18:0	0.78	129
18:2n-6	0.77	141
20:3n-6	0.89	178

[a]The kinetic parameters V_m and K_i were obtained from linear regression analysis of Dixon plots shown in Figure 7. For further details see the text. Data are expressed as the mean of duplicate experiments.

Hamilton (53), basic physical chemistry indicates that we expect an enhanced uptake of FA into cells when the pH of the medium is decreased. However, we found that decanoate, palmitate, and eicosatrienoate exhibited a maximal uptake at physiological pH (7.40) that was preceded by diminished uptake values in the pH range of 6.0 to 7.4 (Fig. 4). This fact suggests that a structure sensible to pH modification—other than the FA itself—may be involved in the uptake of the substrates. This is not the case when we assume that the uptake proceeds by passive diffusion, in which the driving force that determines the net uptake is the partition coefficient of FFA between medium and biomembrane (35,53). In the simple diffusion model, a very rapid rate of flip-flop of FA across the membrane is proposed (53). It has also been reported that the Na^+/K^+ antiporter may act in response to the movement of H^+ by FA flip-flop, and it may influence diffusion by local pH changes that modify the interfacial ionization of the substrate (53). Several experimental observations have described the structural changes of BSA within the pH interval we examined, including significant modifications around the physiological pH (27). Thus, BSA and/or a membrane protein could influence FA uptake by binding the substrate directly, enhancing partitioning into the membrane, or playing a role in its subsequent metabolism, for example, sequestering FA to a membrane-bound transporter. If a membrane-associated protein binds FA with high affinity in a pH-dependent manner, the rate of release from the protein will affect the rate of uptake, possibly slowing it down.

Experimental evidence from other authors indicates that only UFA enter the cells and that the rate of uptake is dependent upon the FA/BSA molar ratio, that is, upon the concentration of FFA (15,16,35,47,54). Figure 3 demonstrates that no significant differences are observed for the uptake of palmitate and eicosatrienoate at constant and variable ratios. Thus, under our experimental conditions, only the total FA concentration (bound and free) seems to be crucial for the rate of uptake. As the exogenous palmitate—or eicosatrienoate—concentrations were varied at a fixed FA/BSA ratio (Fig. 3), the uncomplexed substrate concentration, which is solely a function of the ratio (2,49,54), remained constant. Hence, the concentration of BSA in the medium was the only variable in this setting. Under these conditions, the FA uptake appeared to be saturable at increasing BSA concentration, indicating, as previously proposed by Trigatti and Gerber (14) and Glatz *et al.* (35), the possible involvement of an albumin receptor structure in the uptake mechanism. On the other hand, taking into account the high affinity of BSA for FFA, it might be assumed that these substrates cannot leave binding sites on albumin without catalysis performed by another protein. In this regard, it was reported that the rate of desorption of stearic acid from albumin may be accomplished in 70 s as determined by ^{13}C NMR studies (53). This time seems to be too long compared to the time required for observing a significant uptake of FA in our experimental system (30 s) (Figs. 1B and 1C). It is evident that the presence of living cells influences the mechanism of FA desorption. We think, in consequence, that the rate of spontaneous desorption is too slow to explain the uptake process without the intervention of another protein(s). The FA are maintained in a nonaggregated form by means of FA–albumin complexes as a source of substrate in transport studies (31,32). Under physiological conditions albumin is present in large quantities with respect to FA, strongly suggesting the existence of another structure that is involved in the uptake process. For this reason, we agree with other authors who postulate the presence of an albumin receptor in plasma membranes (13,14,35,53). This fact could explain the different results obtained for the FA uptake in experiments with artificial systems or cultured cells. Further support for the concept of a facilitated uptake for long-chain FA influx in L6 cells is found in Figure 6 and Table 2. We found a concentrative uptake of FA arising from the net incorporation of substrates rather than from an exchange with external FA. Moreover, the radioactivity was recovered exclusively in the methyl ester peak, indicating that the FA were not significantly metabolized and then released into the medium. The fact that L6 cells are able to take up FA in a concentrative fashion at all concentrations tested (Fig. 6) and that this uptake is dependent on sodium concentrations (Fig. 5) favors an active transport. We can assume therefore the existence of such a transport system in L6 cells for the uptake of FA. Notwithstanding, we should also keep in mind that FA-activating enzymes are present at the cytoplasmic level and in organelles. For this reason, a passive diffusion component cannot be ruled out. However, the fact that some FA do not compete with palmitate for activation but do compete for entry into cells (16) (Table 3) is a crucial argument against this possibility. Our results from competition studies are also in agreement with the hypothesis of a specific transport system operating in FA uptake. Similar values were found when FA were present either as substrates or as competitors, suggesting that they are recognized by the same protein structure (Table 3).

In conclusion, our results agree with previous reports (7,14,16,23,30,35) that postulate that the rate of entry of FA into L6 cells is determined by the sum of a diffusion-like (linear) component and a saturable (hyperbolic) component. As previously reported by Samuel *et al.* (16) for cultured cardiac cells, the contribution of passive diffusion in the total uptake of FA would account for no more than 30% of the total rate of uptake. In our experiments we found a contribution of 66 to 86% as saturable uptake (Table 1), which implies that 14 to 34% of the total uptake was evoked by passive diffusion. Thus, the importance of the saturable transport becomes evident. It is widely known that the cellular uptake of FA is a multistep process that involves a variety of membrane-associated events (3–5,7,8,14,23,30,35,37,52,53). Our results suggest the necessity of one or more protein structures to achieve these steps, although their precise function remains unknown. The physiological significance of this question is evident, since different tissues express a distinct set of proteins putatively involved in cellular uptake (2,5,8,10,11,13,14,23,35–45, 52,53). This phenomenon would explain the adaptation for

specific needs of each tissue and the central role related to FA availability and metabolism. These adaptive responses are complicated and not easily interpretable. For these reasons, we think they may not be the result of only passive (flip-flop) diffusion of FA through the plasma membranes.

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