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Research Article

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Fatty Acid Uptake by Isolated Rat Heart Myocytes Represents a Carrier-mediated Transport Process

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Abstract

The mechanism by which fatty acids enter cardiomyocytes is unclear. Therefore, the influx kinetics of [^{3}H]oleate into isolated rat heart myocytes were examined. Cells were incubated at 37°C with [^{3}H]oleate bound to albumin in various molar ratios and the initial rate of uptake (V_0) was determined as a function of the unbound oleate concentration in the medium. V_0 was saturable with increasing oleate concentrations incubated (K_m 78 nM; V_{max} 1.9 nmol $\times$ min^{-1} per 10^6 cells) and temperature dependent with an optimum at 37°C. Furthermore, binding of [^{3}H]oleate to isolated plasma membranes of cardiomyocytes was saturable, revealing a K_D of 42 nM, and was inhibited by heat denaturation or trypsin pretreatment of the membranes. From these membranes a single 40-kD protein with high affinity for a variety of long chain fatty acids was isolated. With a monospecific antibody to this membrane protein, binding as well as cellular influx of [^{3}H]oleate was selectively inhibited. These data indicate that at least a portion of myocardial fatty acid uptake is mediated by a specific membrane protein.

Introduction

Although fatty acids are the preferred substrates for energy production of the heart, their cellular uptake mechanism is still unclear. The lipophilic character of fatty acids suggests that they might diffuse directly through the phospholipid bilayer of the plasma membrane. However, the recent identification of a membrane-associated fatty acid transport system in hepatocytes (1–3) led to the hypothesis that myocardial fatty acid uptake might also be mediated by such a carrier. Therefore, in the present study the influx kinetics of a representative long chain fatty acid, [^{3}H]oleate, into isolated cardiomyocytes was determined to evaluate whether uptake occurs by a passive diffusional process or reveals criteria of a carrier-mediated transport mechanism. Furthermore, it was examined whether myocardial plasma membranes also contain a specific fatty acid binding protein, which mediates the influx of fatty acids into the cells. The identification of such a carrier mediated uptake system could be of biologic significance, since it might represent a site of metabolic and hormonal control of fatty acid metabolism.

Methods

Materials. [9.10-^{3}H]Oleic acid; [1-^{14}C]arachidonic acid; [9.10-^{3}H]-palmitic acid; [^{14}C]linoleic acid; L-α-1-palmitoyl-2-[1-^{14}C]oleoyl-phosphatidylcholine; cholesteryl [1-^{14}C]oleate; 3-O-[^{14}C-methyl]-D-glucose; D-[3-^{3}H]glucose; and Aquasol were from New England Nuclear, Dreieich, West Germany.

Collagenase (type I) was from Worthington Biochemicals, Freehold, NJ. Bovine serum albumin (fraction V, essentially fatty acid free), trypsin (type XIII), phloridzin, oleic acid, Hepes, ouabain, and Triton X-100 were from Sigma Chemie GmbH, München, West Germany. SDS, polyacrylamide and Bio-Beads SM2 were from Bio-Rad, München, West Germany; AH-Sepharose 4B from Pharmacia, Freiburg, West Germany; rabbit antiserum against whole rat serum and rat albumin from Cappel Laboratories, Cochranville, PA. Fluorescein isothiocyanate-conjugated swine anti-rabbit antiserum was from Dako, Copenhagen, Denmark. All reagents were analytical grade and doubly distilled, deionized water was used in all experiments. Glassware was acid washed.

Sprague-Dawley rats were fed a standard Altromin 1314 diet and were obtained from the Zentralinstitut für Versuchstiere, Hannover, West Germany.

Preparation of isolated cardiomyocytes. Calcium tolerant cardiomyocytes were isolated from 12-wk-old female Sprague-Dawley rats following the procedure of Powell et al. (4) with minor modifications (5). The cell pellets obtained were washed three times in the incubation medium containing 125 mM NaCl, 2.6 mM KCl, 1.2 mM KH$_2$PO$_4$, 10 mM Hepes, and 5.5 mM glucose (pH 7.4, 20°C). After dilution to 2 $\times$ 10^6 cardiomyocytes/ml, the cell suspensions were immediately used for uptake studies.

The viability of the cell preparations were routinely tested before and after each series of incubation experiments. Morphologic evaluation was performed using a phase-contrast microscope (Zeiss, Oberkochen, West Germany) at magnifications of 50- and 128-fold. The preparations were considered suitable when > 90% of the cells excluded trypan blue and > 70% of these trypan blue negative cells revealed a rod-shaped morphology with a clear transparent stroma and distinct cross striations (6). Additional criteria for viability were based on the intracellular K$^+$-concentration estimated by atomic absorption spectroscopy, the ATP content (7), as well as the release of lactate dehydrogenase (LDH)[1] (8) and creatine phosphokinase (CPK) (9) into the medium.

Preparation of [^{3}H]oleate working solutions. Tracer amounts of [^{3}H]oleate were mixed with measured quantities of nonradioactive oleic acid in chloroform, dried under a N$_2$ gas stream, and dissolved in 0.2 ml of 0.1 N NaOH. Bovine serum albumin dissolved in incubation medium was added to the oleate/NaOH solution to obtain the desired oleate/albumin molar ratio, the pH was immediately adjusted to 7.4, and the oleate/albumin solution was diluted to its final working concentration. In such [^{3}H]oleate/albumin solutions the concentration of unbound fatty acids was calculated by the stepwise equilibrium constant method of Wosilait and Nagy (10), using the dissociation constants for the oleate/albumin complex reported by Spector et al. (11).

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1. *Abbreviations used in this paper:* CPK, creatine phosphokinase; LDH, lactate dehydrogenase.

Cellular uptake of [³H]oleate. 125 μl of the isolated cardiomyocyte suspension (2 × 10⁶ cells/ml) were incubated with [³H]oleate bound to albumin in polypropylene tubes at a final volume of 1 ml incubation medium (shaking waterbath, 37°C). After certain incubation periods 200-μl sample aliquots were pipetted into 3 ml of an ice-cold 0.5% albumin solution to stop cellular influx and efflux, and to remove surface bound fatty acids. After gentle mixing for 2 min on ice the sample was pipetted onto the center of a GF/C filter (24 mm; Whatman Inc., Clifton, NJ) at a rate equal to the rate of filtration under 50 mmHg vacuum pressure using a filtration apparatus (model 7H; Hoefer Scientific, San Francisco, CA). The cells were washed with 5 ml of the 0.5% albumin solution (4°C) and, thereafter, with 20 ml incubation medium. Washing with more of the stop solution did not change the radioactivity remaining on the filter. The filters were placed in scintillation vials, 10 ml of Aquasol were added, and the radioactivity was determined in a 1217 Rackbeta liquid scintillation counter (LKB-Wallac, Turcu, Finland). Nonspecific association of radioactivity to filters and cells was determined for each experimental circumstance by adding the cold stop solution before the addition of corresponding aliquots of cells and [³H]oleate working solutions. After vacuum filtration of the samples and washing the filters with the stop solution, radioactivity was measured as described. [³H]Oleate nonspecifically absorbed to filters and cells was routinely determined and subtracted from values measured in the corresponding incubation experiments. This blank always constituted < 2% of the incubated radioactivity. All incubations were performed in triplicate and all observations were confirmed with at least three separate cell preparations.

The fraction of [³H]oleate incorporated into cellular lipids and the amount oxidized to CO_2 during the course of uptake was determined as earlier described (1).

Further characterization of [³H]oleate transport. In studies in which the effect of sodium depletion on uptake was examined, NaCl was isoosmotically replaced by sucrose, choline chloride, LiCl, or KCl in the incubation medium. First, isolated cardiomyocytes were three times washed with the corresponding Na-free incubation medium. Thereafter the cells were incubated for 20 min at 37°C in the same Na-free buffer, before 173 μM [³H]oleate/albumin (2:1) was added and the initial rate of uptake was determined as described.

The effect of ouabain on initial rate of oleate uptake was tested by incubation of 125 μl of the isolated cardiomyocyte suspension (2 × 10⁶ cells per ml) with 125 μl 4 mM ouabain in incubation medium (37°C). Controls were incubated with 125 μl of incubation medium alone, instead of ouabain. Thereafter 173 μM [³H]oleate/albumin (2:1) was added in presence or absence of 1 mM CaCl₂ (final concentration in the incubation system). After each preincubation procedure in Na-free buffer or with ouabain, viability of the cells was newly determined.

Cellular uptake of 3-O-[¹⁴C methyl]-D-glucose (3-O-[¹⁴C]MeG). For evaluation of the glucose membrane transport competence of isolated cardiomyocytes, uptake kinetics of the nonmetabolizable glucose analogue 3-O-[¹⁴C]MeG was examined at 20°C. 125 μl of the cardiomyocyte suspension were incubated in 1 ml incubation buffer in absence of glucose with increasing concentrations (1-15 mM) of 3-O-[¹⁴C]MeG. At 5-s intervals 200-μl sample aliquots were pipetted into 3 ml of a stop solution containing 0.2 mM phloridzin in incubation buffer before measurement of the cell associated radioactivity by vacuum filtration (see above).

Isolation of enriched cardiac sarcolemma fractions. Enriched cardiac sarcolemma fractions were prepared by the procedure of van Alstyne et al. (12) using ventricle tissue of Sprague-Dawley rats collected from several laboratories over a period of 6 mo (stored in liquid nitrogen). For each experiment 100 g of ventricular tissue was used. The final membrane pellets were resuspended in 0.15 M NaCl/0.02 M Na-phosphate buffer (PBS; pH 7.4) and diluted to a protein concentration of 5 mg/ml as determined by the method of Lowry et al. (13). The sarcolemma fractions were stored at −70°C until use. In aliquots of five representative preparations purity and contamination with other cell organelles was assessed by transmission electron microscopy (14) and marker enzyme determination. As membrane markers

Na⁺/K⁺-ATPase was measured with and without 1 mM ouabain according to Schwartz et al. (15); K⁺-p-nitrophenyl phosphatase was determined as described by Skou (16) and the activity was given as the fraction that is ouabain (1 mM) sensitive; 5'-nucleotidase was determined according to Mitchell and Hawthorne (17). Following other enzyme activities were determined as earlier described (18): acid phosphatase for lysosomal contamination, glucose-6-phosphatase for the microsomal fraction, and succinate-cytochrome c reductase for mitochondria.

Binding of [³H]oleate to isolated plasma membranes. 4.54 μM [³H]oleate bound to albumin in various molar ratios (0.1:1 to 2:1 oleate/albumin) were incubated with isolated plasma membranes (100 μg membrane protein in 20 μl) in a final volume of 400 μl PBS (pH 7.4) at room temperature. Heat denatured plasma membranes were prepared by incubation of a sarcolemma-fraction aliquot of 5 mg protein in 50 ml PBS at 65°C for 16 h while continuously stirring. Thereafter this membrane suspension was concentrated by ultrafiltration (Diaflow membranes PM-10; Amicon Corp., Witten, West Germany), before again aliquots of 100 μg protein per 20 μl were prepared. The effect of trypsin digestion on [³H]oleate binding was determined by preincubation of 100 μg plasma membrane aliquots (20 μl) with 180 μl of 0.1 mg/ml trypsin and subsequent incubation with 1.816 nmol [³H]-oleate/albumin (1:1) in 200 μl PBS. After incubation for 10 min, binding to the plasma membranes was assessed by a vacuum filtration assay as described above. 200 μl of the incubation mixtures were rapidly vacuum filtered on Whatman GF/C glass filters (24 mm), which were quickly washed with 20 ml ice-cold PBS. Washing with > 20 ml did not change the amount of radioactivity remaining on the filter. After addition of 10 ml Aquasol, radioactivity was measured as described above. [³H]Oleate absorption to the filters in the absence of plasma membranes was very reproducible for any given set of experimental circumstances, and constituted < 2%. The [³H]oleate absorbed to the filters without plasma membranes was routinely determined and subtracted from the experimental values. To ensure that the filters did not allow significant quantities of plasma membranes to pass through, aliquots of 100 μg plasma membrane protein were applied to the filters and washed with 20 ml of PBS. As controls, identical aliquots were applied to other filters and placed directly into a test tube without washing. Protein content of both samples was found to be identical (0.97±0.05 mg); even after trypsin pretreatment of the membranes 0.94±0.04 mg protein were retained on the filter.

Isolation and characterization of a fatty acid binding protein from enriched cardiac sarcolemma fractions. A fraction of 300 mg membrane proteins was solubilized with 1% (vol/vol) Triton X-100 (19). After centrifugation at 100,000 g for 60 min, the detergent was removed with Bio-Beads SM 2 (19) and the solubilized protein mixture was loaded onto an oleate-agarose affinity column, which was prepared by coupling of sodium oleate to AH-Sepharose 4B with 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide as previously described (19). After charging with the protein mixture, the column was extensively washed with PBS before elution with 8 M urea was started. The eluate was dialyzed against PBS and concentrated by ultrafiltration. Aliquots of the eluted protein were used for SDS-PAGE (20) and cochromatography studies with tracer amounts of [³H]oleate, [³H]palmitate, [¹⁴C]arachidonate, [¹⁴C]linoleate, [¹⁴C]oleate ester of cholesterol, [¹⁴C]phosphatidylcholine and [³H]glucose over an HPLC column (Ultro-Pac TSK G3000 SW; LKB Instrument GmbH, München, West Germany) equilibrated with 0.1 M NaCl, 0.1 M Na-phosphate (pH 6.8) (21).

Preparation of an antibody to the cardiomyocyte fatty acid binding membrane protein. Outbred New Zealand white rabbits were immunized with the cardiac fatty acid binding membrane protein as described (19). Presence and purity of the antibody was examined by radial double-immunodiffusion on agar plates (22) as well as by immunoblot techniques (23) with the fatty acid binding membrane protein from heart and liver, Triton X-100-solubilized rat heart plasma membranes, whole tissue homogenate of heart and liver, concentrated rat heart cytosolic proteins, rat serum albumin, and whole rat serum. The

IgG fraction of the antiserum was prepared by standard techniques (24).

For indirect immunofluorescence studies frozen 5-μm sections of rat heart as well as isolated cardiomyocytes were air-dried and then incubated for 30 min at room temperature with 1:50, 1:100, and 1:200 dilutions of the antiserum to the membrane fatty acid binding protein. After washing of the slides with PBS for 30 min, fluorescein isothiocyanate conjugated swine anti-rabbit antiserum diluted 1:20 or 1:50 was added, thoroughly washed with PBS for 60 min and finally mounted with buffered glycerol. In the controls the antiserum was preabsorbed with the cardiac membrane fatty acid binding protein in equal dilution, or the preimmune serum was used instead of antiserum. Sections and cells were examined with a fluorescence microscope (Zeiss), employing the filter combination PB 485, FT 510, LP 520 (19).

Antibody inhibition studies. Binding of [^{3}H]oleate to isolated rat heart plasma membranes was examined in presence of the IgG-fraction of the rabbit antiserum to the cardiac fatty acid binding membrane protein or as control in presence of the IgG-fraction of the preimmune serum. After pretreatment of the plasma membranes with increasing concentrations of the IgG-fractions in PBS for 30 min, incubations with [^{3}H]oleate were performed as described (final [^{3}H]oleate concentration 4.54 μM).

Similarly, the effect of the antibody on influx of [^{3}H]oleate into isolated cardiomyocytes was examined. 2 ml of the cell suspension (2 $\times$ 10^6 cells/ml) were incubated for 30 min at room temperature in gently rotating polypropylene tubes with 20–400 μg of the IgG fraction of the antiserum to the cardiac fatty acid binding membrane protein or of the preimmune serum as controls. After centrifugation and washing the cells three times in medium, viability of the cells remained > 90% as determined by trypan blue exclusion and the fraction of rod-shaped cells (> 70%) did not decrease. After dilution to 2 $\times$ 10^6 cells/ml uptake of [^{3}H]oleate as well as of 3-O-[^{14}C]MeG was examined as described above.

Statistical analysis. Results are given as means±SD. The t test was used to test for significant differences among means (25). For kinetic and binding studies, a weighted least-squares fit of the individual data points to a rectangular hyperbola was performed as described previously (26). Derived kinetic parameters (K_m and V_{max}) as well as binding constants (K_D and maximal binding capacity) in presence and absence of the antibody to the membrane fatty acid binding protein were tested for significant differences with a z-test (25). P values equal to or less than 0.05 were considered significant.

Results

Uptake of [^{3}H]oleate by isolated cardiomyocytes

Validation of methods. Similar to earlier studies with isolated hepatocytes (1, 2) calcium-tolerant cardiomyocytes were washed with Ca-free incubation buffer before the incubation experiments. Preparations of these isolated cardiomyocytes revealed 76±5% of rod-shaped cells with transparent stroma and clear cross-striations. 95±4% of all cells excluded trypan blue, the intracellular ATP content was > 9 nmol/mg cell protein, and loss of cellular LDH and CPK was < 15% during 2 h after preparation of the cells. Reexposure of these cells to 1 mM CaCl₂/4% albumin did not alter their viability or morphological appearance. To maintain the physiological transcellular ion gradients, particular of Na⁺, K⁺, and Ca⁺, it was essential to use the cells immediately after preparation and prevent their exposure below a temperature of 20°C, which markedly reduces Na⁺/K⁺-ATPase (27). For determination of the functional integrity of the cells their uptake competence for glucose, which is known to be actively transported (28), was examined. For this purpose uptake of 3-O-[^{14}C]methyl-D-glucose

(3-O-[^{14}C]MeG) was determined. This compound is a nonmetabolizable glucose analogue, entering cardiomyocytes by the same transport system as glucose (29). It was shown that uptake of 1–15 mM 3-O-[^{14}C]MeG at 20°C was maximal and linear during the initial 20-s incubation period, representing cellular influx. Therefore, the cellular influx rate at each concentration of 3-O-[^{14}C]MeG was calculated from the slopes of the cumulative uptake curves over the initial 20-s incubation period. Studies of cellular influx, as a function of increasing 3-O-[^{14}C]MeG concentrations incubated, revealed saturation kinetics (criterion of carrier-mediated transport) with a K_m of 4.2±0.4 mM and V_{max} of 146±19 nmol $\times$ min^{-1} per 10^6 cells.

To determine the cellular uptake rate of a substance and not the fraction of ligand, which is merely bound to the plasma membrane, the uptake reaction has to be stopped after certain incubation periods and surface bound but not transported ligand should be removed. For all oleate/albumin solutions employed, it was demonstrated that at 4°C no translocation of fatty acids across the plasma membrane occurred. This was derived from the observation, that at 4°C no increase in cell associated [^{3}H]oleate was detectable over the entire incubation period. Accordingly, the use of phloretin as additional inhibitor of cellular transport processes (1–3, 30) was not necessary. Therefore, in this study the addition of 3 ml ice-cold 0.5% albumin to 200-μl aliquots of the incubation system was used to stop cellular influx and efflux, and to remove membrane bound fatty acids. The effectiveness of this stop/chase solution was evaluated in studies, where 25-μl aliquots of the cardiomyocyte suspension (2 $\times$ 10^6 cells/ml) were incubated with 173 μM [^{3}H]oleate/albumin (1:1) in 200 μl of the incubation medium for 30 s. Immediately thereafter (zero time) 3 ml of ice-cold 0.5% albumin in incubation buffer was added and radioactivity remaining with the cells was determined at intervals up to 15 min. During a rapid reequilibration period of maximal 1 min 47±4% of the cell-associated radioactivity observed immediately after the 30-s incubation period was removed. However, over the next 15 min no further loss of cellular [^{3}H]oleate was detectable. The suggestion that the initial loss of radioactivity observed in presence of 0.5% albumin represents [^{3}H]oleate effectively chased off membrane binding sites was supported by the observation that almost identical amounts of [^{3}H]oleate were rapidly removed when even higher concentrations of albumin were employed in the stop solution. Furthermore, incubation of cardiomyocytes with [^{3}H]oleate at 4°C, a condition where no transport but membrane binding of ligands is to be expected, only 44±3% of the values of cell associated radioactivity at 37°C were obtained. Subsequent addition of 0.5% albumin (4°C) as stop/chase solution led to a > 90% loss of this membrane bound [^{3}H]oleate. Stop of cellular transport and removal of plasma membrane bound [^{3}H]-oleate achieved by 0.5% albumin (4°C) could also be demonstrated for the other oleate:albumin complexes employed in the study.

Uptake as a function of the external fatty acid concentration. Uptake of [^{3}H]oleate was examined at various unbound oleate concentrations in the incubation medium. The unbound oleate concentration was modulated without exceeding its solubility (31, 32) by incubation of a fixed concentration of 173 μM [^{3}H]oleate bound to varying concentrations of albumin, or by incubation of a fixed concentration of albumin and increasing concentrations of [^{3}H]oleate, such that the oleate/albumin molar ratio varied between 0.25:1 up to 2:1. This

provided a range of calculated unbound oleate concentrations of 22–401 nM.

For determination of the actual translocation process of fatty acids across the plasma membranes of cardiomyocytes it is essential to analyze their unidirectional cellular influx rates, which were defined as the maximal and linear initial uptake phases. Therefore, the time course of [^{3}H]oleate uptake at all oleate/albumin molar ratios was examined at 37°C. For each oleate/albumin complex it was shown that over the initial 30-s incubation period uptake was maximal and linear, while it gradually decreased thereafter as a result of beginning cellular efflux (Fig. 1; illustrated is a representative experiment with 173 μM [^{3}H]oleate/albumin [1:1]). The maximal initial uptake phase was taken as a measure of cellular fatty acid influx that was largely independent of intracellular fatty acid metabolism, since after 30 s only 16±5% of the intracellular fatty acids were recovered in the esterified form and fatty acid oxidation was not detectable.

Determination of this cellular influx rate as a function of increasing external fatty acid concentrations can distinguish simple diffusion, which is a linear relationship, from transport, which is a saturable process. As shown in Fig. 2, with increasing oleate/albumin molar ratio and thus increasing unbound oleate in the medium initial uptake followed saturation kinetics with a K_m of 78.4±5.7 nM and a V_{max} of 1.91±0.23 nmol × min^{-1} per 10^6 cardiomyocytes. Uptake was not limited by the oleate/albumin dissociation rates, since they are at least 100-fold faster than the observed uptake rates at all oleate concentrations studied (33). Furthermore, maximal uptake over the initial 30-s incubation period never exceeded 2% of the incubated substrate, and the unbound monomeric oleate concentration in the medium was well below the critical concentration for the formation of micelles (31, 32), indicating that the apparent saturation kinetics were real and did not reflect substrate depletion. Overlap of the [^{3}H]oleate uptake curves, obtained by varying either the oleate or albumin concentration in the incubation system, demonstrates that diffusional gradients due to the differing concentrations of albumin are of no significance under the experimental conditions employed. This observation of saturable fatty acid uptake kinetics was considered to be a criterion of a carrier-mediated transport mechanism.
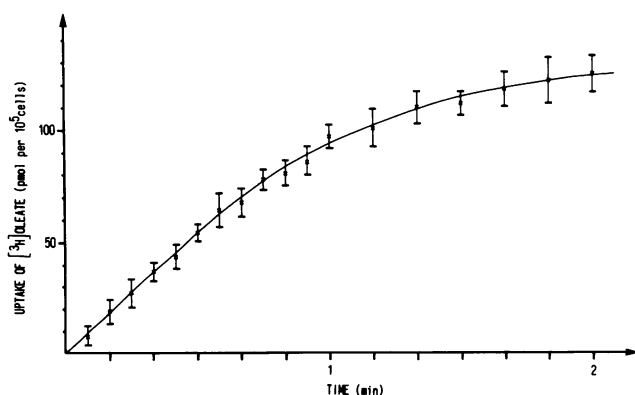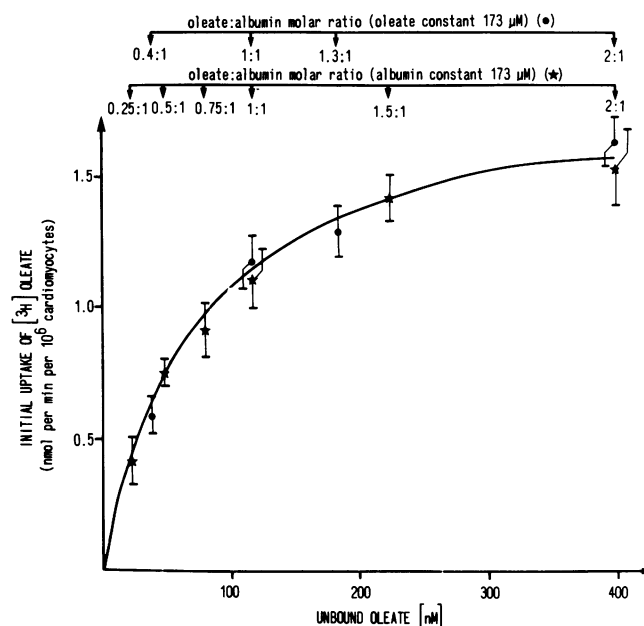
Figure 2. Initial uptake rate of [^{3}H]oleate as a function of the unbound oleate concentration in the medium. Isolated cardiomyocytes (0.25 × 10^6 cells) were incubated in 1 ml at 37°C with a fixed concentration of 173 μM [^{3}H]oleate bound to various concentrations of albumin or with various concentrations of [^{3}H]oleate (43–346 μM) bound to a fixed concentration of albumin. The unbound fatty acid concentration was calculated as described in Methods. The initial uptake rate was determinated from the linear slopes of the cumulative uptake curves over the initial 30-s incubation period. Values are means±SD of three replicate experiments. The kinetic parameters were generated from a weighted least-squares fit of the individual data points from each experiment to a rectangular hyperbola (K_m 78.4±5.4 nM; V_{max} 1.91±0.23 nmol × min^{-1} per 10^6 cardiomyocytes).

Further characterization of oleate uptake by isolated cardiomyocytes. Temperature dependency of fatty acid transport was determined by incubation of 173 μM [^{3}H]oleate/albumin (1:1). The initial rate of oleate uptake was maximal at 37°C (1.14±0.13 nmol × min^{-1} per 10^6 cells), a significantly slower influx was observed at 22°C (0.73±0.16 nmol × min^{-1} per 10^6 cells, $P < 0.001$) and no transport was detectable at 4°C.

Since calcium is an important intracellular regulator, also affecting plasma membrane transport processes (34, 35), uptake of [^{3}H]oleate was analyzed in presence of 1 mM CaCl$_2$ in the medium. Incubation of 173 μM [^{3}H]oleate, bound to albumin in a molar ratio of 1:1 and 2:1, revealed in presence of 1 mM CaCl$_2$ uptake rates of 1.19±0.20 and 1.53±0.18 nmol × min^{-1} per 10^6 cells, respectively. These uptake values were not statistically different to those observed in absence of Ca (Fig. 2, $P > 0.05$), indicating that presence of 1 mM CaCl$_2$ has no significant influence on fatty acid transport in isolated cardiomyocytes.

Furthermore, the initial rate of oleate uptake was determined after pretreatment of cardiomyocytes with the Na$^+$/K$^+$-ATPase inhibitor ouabain. These experiments were again performed in presence and absence of 1 mM CaCl$_2$. In absence of 1 mM CaCl$_2$ incubation of 173 μM [^{3}H]oleate/albumin (2:1) with cardiomyocytes pretreated with 2 mM ouabain revealed a 38% inhibition of the initial rate of uptake (1.02±0.18 vs. 1.64±0.13 nmol × min^{-1} per 10^6 cells in controls not pretreated with ouabain, $P > 0.001$). Similarly, in the

Figure 1. Time course of [^{3}H]oleate uptake. 173 μM [^{3}H]oleate/albumin (1:1) in 1 ml PBS were incubated at 37°C with 125 μl of isolated cardiomyocytes (2 × 10^6 cells/ml). At the times indicated uptake was terminated as described. Values are means±SD of three replicate experiments.

Table I. Sarcolemma Marker Enzyme Activities in the Homogenate and the Plasma Membrane Fraction

Enzyme	Homogenate	Plasma membranes	Enrichment
Na^+/K^+-ATPase	0.91 ± 0.38	39.3 ± 5.2	43
K^+-p-nitrophenyl phosphatase	0.14 ± 0.05	7.84 ± 1.06	56
5'-Nucleotidase	0.31 ± 0.06	3.87 ± 0.81	13

(Data are given as mean±SD of five representative preparations. Specific activities are reported as micromoles per milligram.

presence of 1 mM $CaCl_2$ the initial uptake was also inhibited by 32% (1.06 ± 0.12 vs. 1.56 ± 0.21 nmol $\times$ min^{-1} per 10^6 cells in controls, $P > 0.001$).

In additional studies the initial rate of oleate uptake was examined in absence of sodium, by isoosmotic replacement of NaCl with sucrose, LiCl, choline chloride or KCl in the incubation medium. However, after a 20-min preincubation period of cardiomyocytes in Na-free medium, the viability of the cells dropped markedly, as evident by the complete disappearance of the characteristic rod-shaped cell morphology and the observation that < 80% of the cells excluded trypan blue. These effects were seen in presence as well as in absence of 1 mM $CaCl_2$ in the medium. Therefore, the apparent inhibition of [^{3}H]oleate uptake by 62% in the absence of Na compared to the presence of Na was considered as a nonspecific effect due to the marked decrease in cellular viability.

Binding of [^{3}H]oleate to isolated plasma membranes. Since the initial step in any carrier-mediated uptake process represents binding of the ligand to specific membrane binding sites, the interaction of isolated cardiac plasma membranes with [^{3}H]oleate was examined.

Characterization of the isolated sarcolemma fraction. Transmission electron microscopy of the cardiac plasma

membrane fraction revealed a homogeneous preparation of vesicular membrane structures with diameters ranging from 0.06 to 0.15 μm. Contaminating membranes from organelles other than plasma membranes were minimal. Accordingly, sarcolemma marker enzymes were significantly enriched in these preparations (Table I), whereas marker enzyme activities for mitochondria, endoplasmatic reticulum, and lysosomes were not elevated in the plasma membrane fraction compared to the homogenate.

Aliquots of these highly enriched cardiac plasma membrane fractions (100 μg protein) were incubated with 4.54 μM [^{3}H]oleate bound to albumin in various molar ratios and binding was examined as a function of the calculated unbound oleate concentration in the medium. With increasing unbound oleate concentrations incubated binding to the membranes revealed saturation kinetics with a K_D of 42.3 ± 7.8 nM (Fig. 3). Binding was significantly ($P < 0.001$) reduced after heat denaturation of the membranes as well as after pretreatment of the membranes with trypsin (Fig. 4).

Isolation of a fatty acid binding membrane protein. The demonstration of saturable, trypsin inhibitable binding suggested that high affinity binding is due to an intrinsic membrane protein. This suggestion was pursued by application of 300 mg Triton X-100 solubilized proteins from rat myocardial plasma membranes to affinity chromatography over oleate-coupled agarose. SDS-PAGE of the urea eluate of this column revealed a single 40-kD protein (Fig. 5). On gel filtration over an HPLC column this protein comigrated with tracer doses of [^{3}H]oleate, [^{14}C]linoleate, [^{14}C]arachidonate, and [^{3}H]-palmitate but not with other complex lipids such as [^{14}C]-phosphatidylcholine or cholesteryl-[^{14}C]oleate nor with [^{3}H]-glucose, suggesting its specific high affinity for various monomeric long-chain fatty acids.

Furthermore, an antibody to this protein was raised in rabbits. In double immunodiffusion studies the antibody to the heart fatty acid binding membrane protein produced a single precipitin line between the purified protein, the mixture of solubilized membrane proteins and whole tissue homoge-
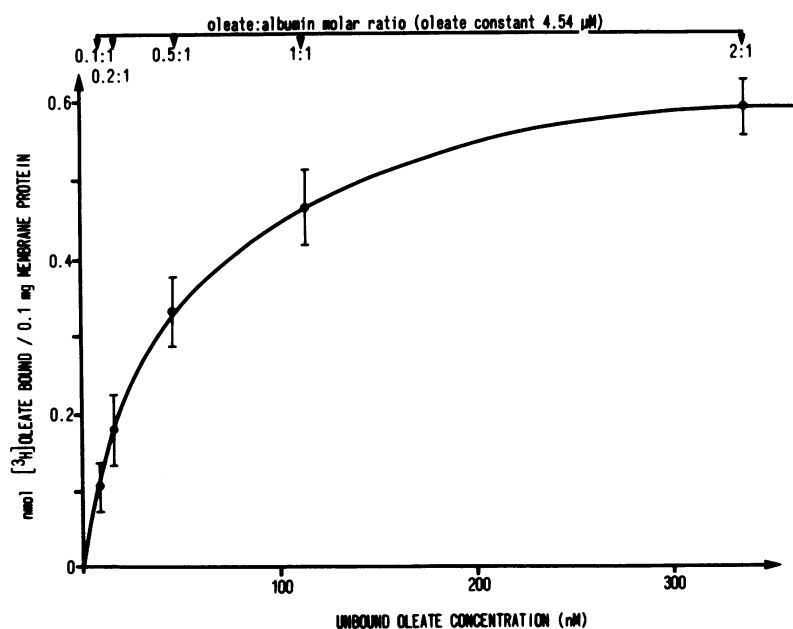
Figure 3. Binding of [^{3}H]oleate to isolated plasma membranes of cardiomyocytes. Aliquots of cardiac plasma membrane fractions (100 μg protein) were incubated at room temperature in a final volume of 400 μl with 4.54 μM [^{3}H]oleate bound to albumin in various molar ratios. The unbound oleate concentration was calculated as described in Methods. After incubation for 10 min equilibrium binding to the plasma membranes was assessed by a vacuum filtration assay. Values are means±SD of three replicate experiments. The binding parameters were generated from a weighted least-squares fit of the individual data points from each experiment to a rectangular hyperbola (K_D 42.3 ± 7.8 nM; maximal binding capacity 0.682 ± 0.104 nmol/0.1 mg membrane protein).
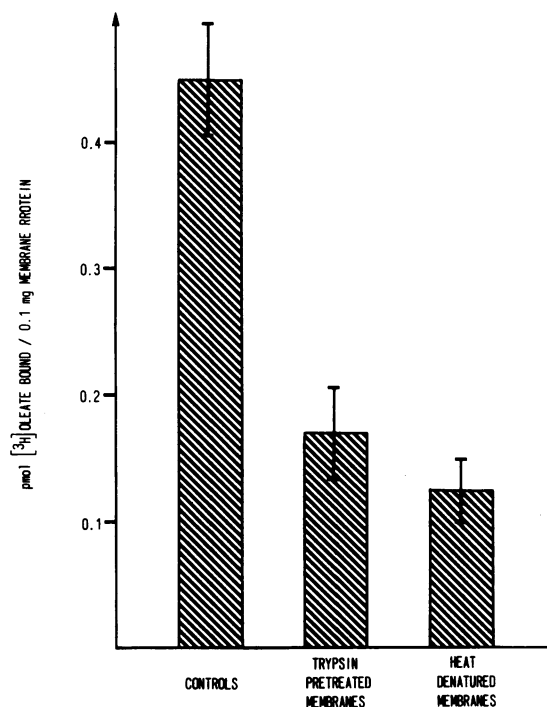
Figure 4. Effect of trypsin pretreatment and heat denaturation on binding of [^{3}H]oleate to plasma membranes of cardiomyocytes. 4.54 μM [^{3}H]oleate/albumin (1:1) was incubated with 100 μg membrane protein aliquots of native, trypsin-pretreated, or heat-denatured plasma membranes of cardiomyocytes (prepared as described in Methods). After incubation for 10 min, binding was assessed by a vacuum filtration assay. Values are means±SD of three replicate experiments.

nates of the heart and liver. No reactivity was observed between this antibody and rat albumin, whole rat serum, and concentrated rat heart and liver cytosol. Immunoblot analysis of this antibody in dilutions of 1:1,000 to 1:50 with solubilized plasma membrane proteins and whole tissue homogenates of
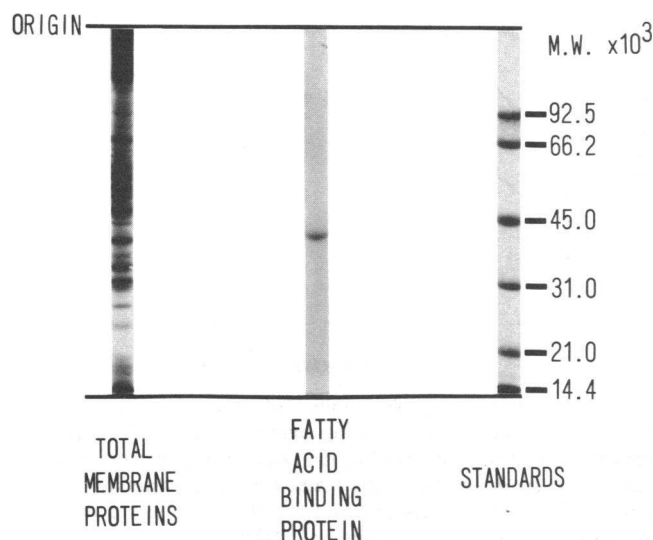

Figure 5. SDS-polyacrylamide gel patterns of the total solubilized cardiac plasma membrane proteins and a single 40-kD fatty acid binding membrane protein isolated by affinity chromatography of cardiac plasma membranes over oleate-coupled agarose.

heart and liver identified a single 40-kD protein in all preparations. Identical results were obtained when the antibody to the liver fatty acid binding membrane protein (19) was employed. It indicates that both 40-kD fatty acid binding membrane proteins isolated from liver (19) and heart share antigenic determinants.

By immunofluorescence studies with this monospecific antibody to the myocardial fatty acid binding membrane protein a predominant staining of the plasma membranes of cardiomyocytes, particularly of the intercalated disks was evident (Fig. 6). Absorption of the antibody with purified antigen or incubation with the preimmune serum abolished the membrane staining pattern.

Functional significance of the fatty acid binding membrane protein. To evaluate the functional significance of the membrane fatty acid binding protein, the effect of the monospecific antibody to this protein on fatty acid binding to myocardial plasma membranes was examined. In presence of 10–200 μg of the IgG fraction of the antiserum, membrane binding of fatty acids progressively decreased with maximal inhibition of binding at 100 μg of the IgG fraction per 100 μg membrane protein. Addition of > 100 μg of the IgG fraction did not further increase inhibition of binding. Studies of equilibrium binding as a function of the incubated unbound oleate concentration revealed a significant reduction of the maximal binding capacity by the antibody (0.148±0.039 nmol/0.1 mg membrane protein) compared to control incubations in presence of the IgG-fraction of the preimmune serum (0.632±0.097 nmol/0.1 mg membrane protein, $P < 0.001$) (Fig. 7). No statistical difference of the K_D values was evident (39.5±4.1 nM vs. 42.4±3.3 nM in controls, $P > 0.05$), indicating that the antibody acts predominantly as noncompetitive inhibitor.

The suggestion that this membrane fatty acid binding protein might also be responsible for the translocation of fatty acids across the plasma membrane into the intracellular space was substantiated, when the effect of the monospecific antibody to this protein on cellular influx kinetics was analyzed. When oleate influx by cardiomyocytes pretreated with the IgG fraction of the antiserum was compared with that of a control preparation pretreated with the IgG fraction of the preimmune serum, a significant inhibition of initial uptake velocity by the antibody was demonstrated. This inhibition was dependent on the concentration of the antibody, reaching a maximum at 100 μg IgG/incubation. Studies of influx as a function of the incubated unbound oleate concentration revealed a predominant noncompetitive inhibition of uptake by the anti-fatty acid binding protein (Fig. 8). This was evident by a marked decrease of V_{max} (1.183±0.107 nmol $\times$ min^{-1} per 10^6 cells in antibody-pretreated cells vs. 2.021±0.236 nmol $\times$ min^{-1} per 10^6 cells in controls, $P < 0.001$), but no statistical difference in the obtained K_m values (76±5 nM vs. 75±8 nM in controls, $P > 0.05$).

To evaluate whether the inhibition of oleate uptake by the antibody marks a specific effect on the fatty acid uptake system or represents a more general interference with membrane transport processes, the effect of this antibody on cellular influx of 3-O-[^{14}C-methyl]-D-glucose was analyzed. The uptake parameters observed with cardiomyocytes pretreated with the IgG-fraction of the antiserum to the fatty acid binding membrane protein (K_m 4.0±0.5 mM; V_{max} 154±14 nmol $\times$ min^{-1} per 10^6 cells) or with the IgG-fraction of the preimmune serum

Figure 6. Indirect immunofluorescence staining of plasma membranes of cardiomyocytes in normal rat heart sections after incubation with a rabbit antiserum to the membrane fatty acid binding protein.

(K_m 4.4±0.3 mM; V_{max} 135±21 nmol × min^{-1} per 10^6 cells) did not significantly differ ($P > 0.05$). This indicates that the anti-membrane fatty acid binding protein has no effect on glucose transport, and that the transport competence for glucose is not altered by the IgG pretreatment procedure of the cells.

Discussion

The observation that influx of oleate into isolated cardiomyocytes demonstrates saturation kinetics and temperature dependency is compatible with the hypothesis of a membrane

carrier–mediated uptake mechanism. A saturable transport component of myocardial fatty acid uptake was already proposed by earlier studies of Samuel et al. (36) and Paris et al. (37). On the other hand, De Grella and Light (38, 39) interpreted this saturable uptake phenomenon as a result of intracellular fatty acid metabolism. In fact, in those earlier studies net accumulation of fatty acids within the cells was determined and it was not differentiated between the fatty acid metabolism and cellular influx component. Therefore, in the present study the initial rates of fatty acid uptake were determined. They were calculated from the slopes of the cumulative uptake
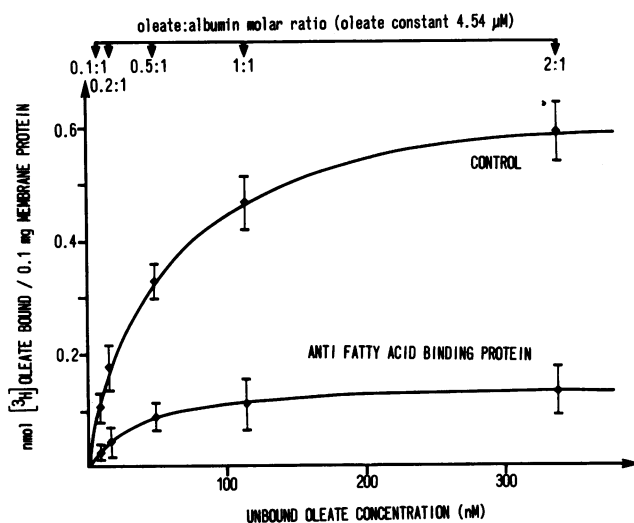

Figure 7. Inhibition of the binding of [^{3}H]oleate to isolated cardiac plasma membranes by the monospecific rabbit antibody to the rat heart fatty acid binding membrane protein. Aliquots of cardiac plasma membrane fractions (100 μg protein) were incubated in the presence of 100 μg of the IgG fraction of the antiserum to the membrane fatty acid binding protein or of the preimmune serum as controls. Binding to the membranes was plotted as a function of the unbound oleate concentration in the medium, which was modulated by incubation of 4.54 μM [^{3}H]oleate bound to albumin in various molar ratios. Values are means±SD of three replicate experiments.
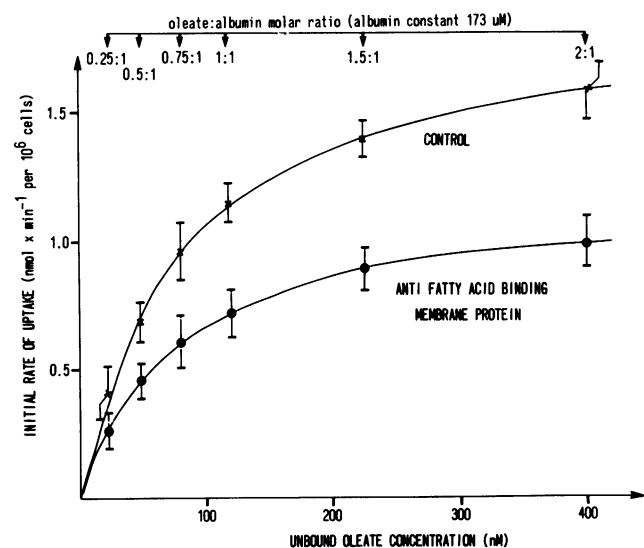
Figure 8. Inhibition of [^{3}H]oleate influx into isolated cardiomyocytes by the anti-cardiac fatty acid binding membrane protein. Cells pretreated with 100 μg of the IgG-fraction of the antiserum were compared to a control preparation pretreated with the preimmune serum. 125 μl of the cell suspensions (2 × 10^6 cells/ml) were incubated with increasing concentrations of [^{3}H]oleate (43–346 μM) bound to a fixed concentration of albumin at 37°C. Illustrated are the initial uptake rates as a function of the calculated unbound oleate concentrations in the incubation media. Values are means±SD of three replicate experiments.

curves, which were maximal and linear over the initial 30-s incubation period. This initial uptake phase predominantly represents the unidirectional cellular influx rate, which is independent of intracellular fatty acid metabolism, since more than 80% of the cell associated fatty acids were still recovered in the unesterified form after 30 s. This cellular fatty acid influx component was temperature dependent and revealed saturation kinetics with a K_m of 78 nM and a V_{max} of 1.9 nmol $\times$ min^{-1} per 10^6 cardiomyocytes, which are similar to the kinetic parameters obtained in hepatocytes (2, 3). The K_m appears to be well above the physiologic concentration of unbound fatty acids in the plasma (10, 11). Based on estimations of cellular fatty acid metabolism in isolated cardiomyocytes (39) and the perfused heart (40), the V_{max} value determined here provides a sufficient reserve capacity for fatty acid influx to meet the energy requirement of cardiomyocytes, even under excercise conditions.

The suggestion of a membrane carrier-mediated uptake system was further substantiated by identification of high affinity membrane binding sites for fatty acids (K_D 42 nM). Moreover, a single specific membrane protein with high affinity to various long chain fatty acids was isolated. This 40-kD protein shares antigenic determinants with the liver fatty acid binding protein (19). However, whether these are in fact identical proteins or just related, similar to the different cytosolic fatty acid binding proteins isolated from liver and heart (41, 42), remains to be established. The physiologic significance of this membrane protein as carrier in the myocardial uptake process of fatty acids is evident, since influx of [^{3}H]oleate was significantly inhibited by a monospecific antibody to this protein, whereas uptake of glucose remained unaltered. These data indicate that fatty acid uptake by cardiomyocytes is at least in part mediated by a specific 40-kD membrane protein. It seems attractive to speculate whether cellular influx of this major class of energy-yielding substrates may be regulated by such a receptor protein.

The driving forces mediating the cellular entry of fatty acids in cardiomyocytes are still unclear. Similar to hepatocellular fatty acid uptake it could be assumed that fatty acid transport across myocardial plasma membranes might also be driven by an active, Na^+-dependent, potential-sensitive translocation process, which was established in studies with basolateral rat liver plasma membrane vesicles (18). In fact, pretreatment of cardiomyocytes with 2 mM ouabain, which is known to inhibit the Na^+/K^+-ATPase, reduced cellular influx of oleate by 38%, suggesting that uptake might be linked to the activity of this enzyme maintaining the physiologic transmembrane Na^+/K^+-gradient. However, in the experimental system of isolated cardiomyocytes a more direct evaluation of the effect of Na depletion on the initial rate of cellular fatty acid uptake was unsuccessful due to a significant drop in cell viability after exposure to a Na-free incubation medium. Therefore, uptake studies with isolated plasma membrane vesicles of cardiomyocytes are planned.

Acknowledgments

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