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Uptake of oleate by isolated rat adipocytes is mediated by a 40-kDa plasma membrane fatty acid binding protein closely related to that in liver and gut

(carrier-mediated transport/fatty acids/membrane proteins)

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ABSTRACT **A portion of the hepatocellular uptake of nonesterified long-chain fatty acids is mediated by a specific 40-kDa plasma membrane fatty acid binding protein, which has also been isolated from the gut. To investigate whether a similar transport process exists in other tissues with high transmembrane fatty acid fluxes, initial rates (V_o) of [^{3}H]-oleate uptake into isolated rat adipocytes were studied as a function of the concentration of unbound [^{3}H]oleate in the medium. V_o reached a maximum as the concentration of unbound oleate was increased (K_m = 0.30 ± 0.03 μM; V_{max} = 2470 ± 90 pmol/min per 5 × 10^4 adipocytes) and was significantly inhibited both by phloretin and by prior incubation of the cells with Pronase. A rabbit antibody to the rat liver plasma membrane fatty acid binding protein inhibited adipocyte fatty acid uptake by up to 63% in dose-dependent fashion. Inhibition was noncompetitive; at an immunoglobulin concentration of 250 μg/ml V_{max} was reduced from 2480 ± 160 to 1870 ± 80 pmol/min per 5 × 10^4 adipocytes, with no change in K_m. A basic (pI ≈ 9.1) 40-kDa adipocyte plasma membrane fatty acid binding protein, isolated from crude adipocyte plasma membrane fractions, reacted strongly in both agar gel diffusion and electrophoretic blots with the antibody raised against the corresponding hepatic plasma membrane protein. These data indicate that the uptake of oleate by rat adipocytes is mediated by a 40-kDa plasma membrane fatty acid binding protein closely related to that in liver and gut.**

Free fatty acids have been widely believed to enter cells passively (1–4). However, the high transmembrane fluxes of long-chain free fatty acids that occur in certain tissues, including the liver, gut, adipose tissue, and myocardium, suggest the existence of more specialized membrane transport processes for free fatty acids, at least in these sites. We recently demonstrated that the hepatocellular uptake of free fatty acids is effected by a specific plasma membrane carrier-mediated transport system (5, 6). Fatty acid uptake via this system is sodium coupled and ouabain inhibitable, energy dependent, and mediated by a 40-kDa plasma membrane fatty acid binding protein (6, 7). Antibodies to this protein selectively inhibit the hepatocellular uptake of free fatty acids while having no effect on cell viability or on the uptake of other classes of organic anions, such as bromosulfophthalein and bilirubin, or conjugated bile acids (6), which enter the hepatocyte via their own specific carrier-mediated transport processes (8–12). An immunologically identical protein has been isolated from the luminal microvillous membranes of jejunal enterocytes (13), where it presumably mediates fatty acid absorption. These plasma membrane fatty acid binding proteins are unrelated to the previously described and lower molecular weight cytosolic fatty acid binding proteins, which occur in similar tissues (14–16).‡

Other studies have shown that the movement of free fatty acids into and out of the adipocyte also has features suggestive of carrier-mediated transport (17, 18). In this report we demonstrate that the adipocyte carrier system involves the same 40-kDa membrane fatty acid binding protein previously isolated from liver (7) and gut (13), and that antibodies raised against the hepatic protein inhibit fatty acid uptake in adipocytes.

MATERIALS AND METHODS

Preparation of Antibodies to h-FABP$_{PM}$. Sinusoidally enriched plasma membranes were prepared from rat livers as previously described (19, 20). h-FABP$_{PM}$ was extracted from these membranes and purified by a modification (21) of our original procedure (7) involving preparative isoelectric focusing, affinity chromatography over oleate-coupled agarose, and hydrophobic interaction high-performance liquid chromatography (HPLC). The purified antigen, which was eluted as a single sharp peak corresponding to a molecular mass of 40 kDa from gel permeation HPLC columns (TSK-2000, Bio-Rad) and migrated as a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE), was used to prepare polyclonal monospecific antisera in rabbits as previously reported (7, 20). Prior to use of the antibodies, albumin was separated from the immunoglobulin fraction by ammonium sulfate precipitation and ion-exchange chromatography over DEAE-cellulose and discarded.

Preparation of Adipocytes. Male Sprague–Dawley rats, 200–220 g, obtained from Charles River Breeding Laboratories, were sacrificed with pentobarbital, 50 mg/kg i.p., followed by rapid exsanguination. The epididymal fat pads were removed immediately and adipocytes were prepared from the fat pads by digestion for 45 min at 37°C with purified collagenase (Boehringer Mannheim), 1 mg/ml, as previously described (22, 23). Cells were filtered through nylon mesh, washed four times with Krebs–Ringer Hepes buffer (KRH), pH 7.4, containing 2% bovine serum albumin (hereafter referred to as albumin) and 1 mM glucose and resuspended at 1 × 10^6 cells per ml in KRH containing 0.2%

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‡To avoid confusion, we suggest that fatty acid binding proteins (FABPs) be designated with prefixes h-, g-, a-, my-, or sm-, to designate origin from hepatic, gut, adipose, myocardial, or skeletal muscle sites, as already proposed by others (16), and a subscript C or PM, to indicate cytosolic or plasma membrane subcellular localization. Thus, h-FABP$_{PM}$ denotes the liver fatty acid binding protein from plasma membrane.

albumin and 2 mM glucose (incubation buffer) at room temperature until needed. The adipocyte preparations were virtually free of contaminating stromal/vascular elements as readily assessed by light microscopy (23). Viability was evaluated by using a conventional insulin-stimulated lipogenesis assay (24) employing 1-[^{14}C]glucose as substrate. By this technique, there was no loss of viability between freshly isolated cells and those incubated for up to 2 hr.

Assay of Fatty Acid Uptake. Fifty microliters of cell suspension was added at zero time to 950 μl of incubation buffer containing 180 μM albumin and various quantities of [^{3}H]oleate at pH 7.4, in 15-ml conical polystyrene tubes (Sarstedt, Princeton, NJ). The final concentration of albumin was 171 μM; concentrations of oleate varied such that the oleate-to-albumin molar ratio ranged from 0.25:1 to 6:1, providing unbound oleate concentrations of 0.018–21.9 μM (6). Incubations were conducted at 37°C and 4°C. At various times ranging from 5 sec to 3 min the uptake process was stopped by rapid addition of 5 ml of a stop solution consisting of ice-cold 400 μM phloretin/0.1% albumin, and the quantity of [^{3}H]oleate taken into the cells was determined by a rapid filtration method (5, 6). The "uptake" at zero time and appropriate filter blanks were determined and applied as corrections to the experimental measurements as previously described (5); the latter averaged 0.1% of total incubated radioactive material and ≤10% of the experimental uptake values. In a limited series of incubations the concentration of [^{3}H]oleate was fixed at 171 μM, and the albumin concentration varied such that the oleate-to-albumin molar ratio ranged from 0.5:1 to 2:1. Initial oleate uptake velocities in these studies were compared with those determined in the studies described above, in which identical oleate-to-albumin molar ratios [and hence, unbound oleate concentrations (6)] were achieved with a constant albumin concentration of 171 μM and appropriately varied concentrations of oleate.

Immunofluorescence Studies. Isolated rat adipocytes, which had been incubated with rabbit anti-h-FABP$_{PM}$, and control rat adipocytes, which had been incubated with corresponding preimmune rabbit serum, were fixed in an aqueous solution of buffered 2% (wt/vol) paraformaldehyde for 1 hr (25), during which time the specimens were rotated at room temperature. The cell suspensions were then spun at 15,600 × *g* in an Eppendorf 5414 microcentrifuge for 5 min, following which the supernatant cell-rich layer was harvested. The cells were washed three times by resuspending them in excess phosphate-buffered saline (PBS; 0.075 M NaCl/0.015 M NaH_2PO_4/0.06 M Na_2HPO_4) followed by recentrifugation as above. They were then incubated for 1 hr at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Meloy Laboratories, Springfield, VA) diluted 1:50 in PBS, during which time the specimens were again rotated. The cells were again washed three times in PBS, after which suspensions were mounted on glass slides, cover-slipped, and viewed with a Nikon Optiphot epifluorescence microscope with a halogen lamp and B-mode excitation, using a 515 W absorption filter and a 469 auxiliary filter (25). All photographs were taken, developed, and printed under equal conditions.

Antibody Inhibition of Oleate Uptake. [^{3}H]Oleate uptake was examined in suspensions of isolated adipocytes treated either with the immunoglobulin fraction of the rabbit antiserum to h-FABP$_{PM}$, prepared as described above, or with the corresponding fraction of preimmune serum, which served as a control. Adipocytes (1 ml, 1 × 10^6/ml) were incubated for 60 min on ice with 200–2000 μg of the appropriate immunoglobulin fraction in 1 ml of PBS. Cells were washed twice in incubation buffer and resuspended at 1 × 10^6 cells per ml in the same medium. Subsequently, 50 μl of cell suspension was added to 950 μl of incubation buffer containing 180 μM bovine serum albumin and [^{3}H]oleate at various concentrations, producing oleate-to-albumin molar ratios of 1:1 to 6:1. [^{3}H]Oleate uptake curves for each oleate-to-albumin molar ratio were determined in both antibody-treated and control cells at both 37°C and 4°C, as described above.

Isolation and Characterization of a-FABP$_{PM}$. Crude plasma membrane fractions were prepared from isolated adipocytes by loose Dounce homogenization and differential centrifugation, the proteins were extracted by gentle sonication (30 min on ice) in 2 M NaCl followed by stirring at 4°C for 60 min, and a-FABP$_{PM}$ was isolated by preparative isoelectric focusing, oleate-agarose affinity chromatography (13), and hydrophobic interaction HPLC. The isolated protein was characterized by NaDodSO_4/PAGE, gel-permeation HPLC, and analytical isoelectric focusing. Its relation to h-FABP$_{PM}$ was examined by agar gel diffusion (Ouchterlony method) and electrophoretic blotting, employing in both cases the immunoglobulin fraction of the rabbit anti-rat h-FABP$_{PM}$. Details of the isolation and characterization of the a-FABP$_{PM}$, as well as the analogous protein isolated from cardiac myocyte plasma membranes (my-FABP$_{PM}$) will be published elsewhere (21).

RESULTS

Evaluation of Methods. In detailed studies analogous to those conducted in hepatocytes (5, 6), we established the following.

(*i*) The use of a cold 400 μM phloretin/0.1% albumin stop solution effectively removed surface-bound [^{3}H]oleate while blocking efflux from the cells of material already internalized, permitting accurate quantitation of cellular oleate uptake. In contrast to earlier reports (17, 18), we found appreciable quantities of surface-bound [^{3}H]oleate, which were rapidly removed by the addition of 0.1% albumin to the stop solution (26).

(*ii*) The linear portion of the cumulative uptake curve, observed over at least the first 20 sec of incubation, reflects almost entirely cellular influx. Hence, as suggested by Abumrad *et al.* (17, 18), the slope of this initial portion of the curve affords an accurate quantitative measure of uptake velocity.

(*iii*) The volume of incubation medium relative to cell suspension and the concentration of oleate·albumin had to be sufficiently high that the initial oleate uptake velocity, V_o, did not exceed 2.5% of the total incubated oleate per min. Otherwise, calculated parameters of oleate uptake kinetics were influenced by substrate depletion, the limited dissociation rate of the oleate·BSA complex (27), or both, and did not accurately reflect membrane transport. The experiments supporting these assertions will be reported in detail elsewhere (D.S., W.S., L. Patel, C.-L.K., and P.D.B., unpublished data).

Initial Uptake Velocity of [^{3}H]Oleate as a Function of the Unbound Oleate Concentration in the Medium. At 37°C, for all oleate-to-albumin molar ratios studied, the plot of cumulative [^{3}H]oleate uptake vs. time was linear for at least the first 20 sec, during which time the net rate of oleate accumulation by the cells was maximal. V_o was estimated as the slope of this linear portion of the cumulative uptake curve and is expressed as pmol/min per 5 × 10^4 adipocytes. When V_o, as determined at 37°C, was plotted as a function of the calculated initial concentration of unbound oleate in the medium (6, 28), apparent saturation of uptake with increasing concentrations of unbound oleate was observed (Fig. 1). By contrast, when [^{3}H]oleate uptake was measured at 4°C, it was substantially slower than was observed at 37°C; moreover, V_o at 4°C increased linearly with concentration. When the uptake component observed at 4°C, and believed to

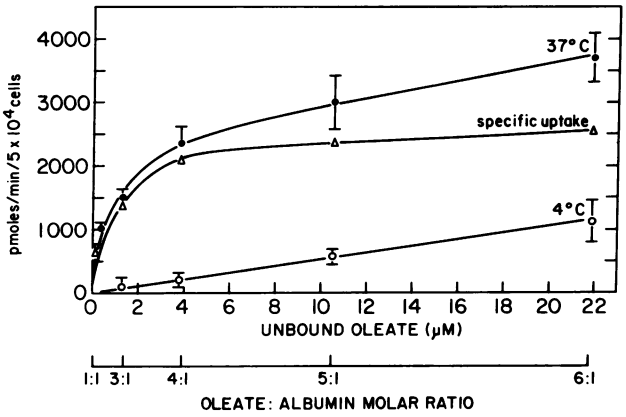

FIG. 1. Initial rates of uptake of [^{3}H]oleate by isolated rat adipocytes plotted as a function of the concentration of unbound oleate in the incubation medium. Uptake rates were determined at 37°C and 4°C; the latter were taken as measures of nonspecific uptake. The curve denoting specific uptake was obtained by subtracting data obtained at 4°C from that obtained at 37°C. Values are mean ± SEM of three to five replicate studies.

represent nonspecific uptake, was subtracted from values obtained at 37°C, a curve representing specific uptake was derived, which indicated unequivocal saturation (Fig. 1). When the specific uptake vs. concentration curve was fitted directly to the Michaelis–Menten equation, using the simulation, analysis, and modeling program (SAAM) of Berman and Weiss (29) on the IBM mainframe computer system of the City University of New York, the calculated V_{max} (±SD) was 2470 ± 90 pmol/min/5 × 10^4 adipocytes, and the K_m was 0.30 ± 0.03 μM. The K_m is thus substantially above reported plasma concentrations of unbound oleate *in vivo* (6, 28, 30, 31). Over the range of oleate-to-albumin molar ratios of 0.5:1 to 2:1, initial oleate uptake velocities determined in the presence of a fixed albumin concentration and variable oleate concentration differed by only ±5% from uptake velocities observed, for the same molar ratios, in studies in which the oleate concentration was fixed and the albumin concentration was varied. Hence, possible diffusional gradients due to variations in albumin concentration had no significant effect on [^{3}H]oleate uptake velocity under the conditions studied.

Phloretin inhibits oleate uptake in both hepatocytes (5, 6) and adipocytes. By contrast, complete replacement of sodium in the incubation medium with 140 mM KCl, 140 mM LiCl, or 240 mM sucrose had virtually no effect on oleate uptake in the adipocyte, which was also unaffected by 1 mM ouabain. As reported previously (6), both ouabain and sodium substitution significantly inhibit oleate uptake in the hepatocyte. A variety of metabolic inhibitors known to inhibit free fatty acid uptake in hepatocytes (5, 6), including KCN and carbonylcyanide *m*-chlorophenylhydrazone (CCCP), were without appreciable inhibitory effect on [^{3}H]oleate uptake in adipocytes. Pretreatment of adipocytes with trypsin, 1 mg/ml, for 30 min at 37°C produced no significant inhibition of [^{3}H]oleate uptake; use of higher trypsin concentrations or longer incubation times resulted in cell lysis. By contrast, prior incubation with Pronase, 1 mg/ml, inhibited [^{3}H]oleate uptake by 42% compared to nontreated controls, when the cells were subsequently incubated with 171 μM [^{3}H]oleate-albumin. These data suggest the participation of a membrane protein in the saturable process by which adipocytes sequester oleate.

Immunofluorescence Studies. Isolated rat adipocytes demonstrated an intense plasma membrane pattern of specific immunofluorescence (Fig. 2), similar to that observed with hepatocytes (7). By contrast, control adipocytes showed only a minimal quantity of background immunofluorescence. Pretreatment of adipocytes with Pronase as described above reduced specific immunofluorescence virtually to background levels (data not shown). No specific immunofluorescence was observed in gastric and colonic mucosal epithelial cells, erythrocytes, or Kupffer cells.

Antibody Inhibition of Oleate Uptake. When adipocytes were preincubated with the rabbit antibodies to rat h-$FABP_{PM}$, [^{3}H]oleate V_o, as measured at 37°C, was significantly inhibited at all oleate-to-albumin molar ratios studied, as compared with control cells preincubated with preimmune rabbit serum. By contrast, the antibody had no effect on the nonspecific uptake component observed at 4°C (Fig. 3). The effect of the antibody on specific oleate uptake was established by computer comparisons of the specific uptake versus unbound oleate concentration curves observed in the presence and absence of antibody, using SAAM (29). The antibody inhibited oleate uptake in dose-dependent fashion, achieving a maximum of 63% inhibition at an immunoglobulin concentration of 1000 μg/ml, in studies employing oleate albumin 1:1 (Fig. 4). This is comparable to the degree of inhibition reported in hepatocytes (6). In the particular study illustrated in Fig. 3, V_{max} for [^{3}H]oleate uptake was reduced by 25% after antibody treatment, from 2480 ± 160

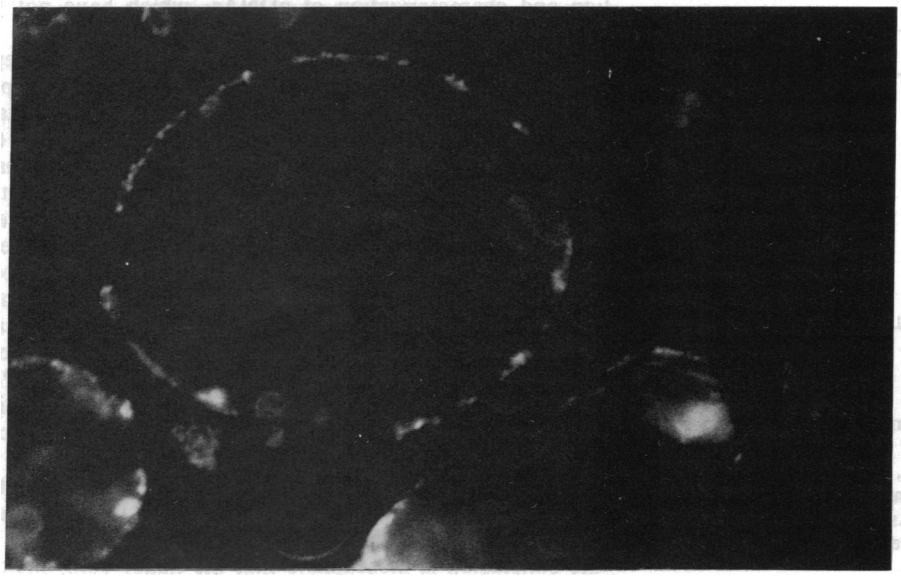

FIG. 2. Immunofluorescence. Isolated adipocytes incubated first with rabbit antiserum to rat h-$FABP_{PM}$ and then with goat anti-rabbit immunoglobulin show an intense membrane pattern of immunofluorescence. (×400.) Substitution of preimmune rabbit serum for anti-h-$FABP_{PM}$ resulted in only minimal background immunofluorescence and no membrane staining (data not shown).

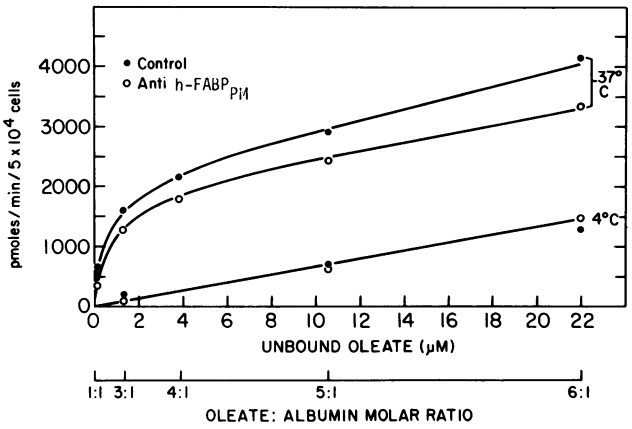

FIG. 3. Inhibition of [^{3}H]oleate uptake into isolated adipocytes by the immunoglobulin fraction of anti-h-FABP$_{PM}$ serum. Cells treated in a final volume of 2 ml with 500 μg of the specific immune immunoglobulin fraction (○) or with corresponding quantities of control immunoglobulin (●) were incubated at 37°C or 4°C with 171 μM albumin and [^{3}H]oleate at various concentrations, producing the oleate-to-albumin molar ratios and unbound oleate concentrations indicated. The specific immune immunoglobulin fraction had no effect on nonspecific oleate uptake as measured at 4°C, but it inhibited uptake at 37°C at all oleate concentrations studied. Values are means of two separate studies.

to 1870 ± 80 pmol/min per 5 × 10^4 cells ($P < 0.025$), while K_m was unaltered (0.32 ± 0.07 vs. 0.32 ± 0.02 μM in controls), suggesting that the antibody acted principally as a noncompetitive inhibitor of oleate uptake. These data are consistent with prior observations that uptake into rat hepatocytes of both oleate (6) and bromosulfophthalein (11) was inhibited noncompetitively by antibodies against the corresponding liver plasma membrane oleate or bromosulfophthalein/bilirubin binding proteins. These studies further suggest that specific oleate uptake by adipocytes is mediated by a protein identical to, or at least immunologically closely related to, the one that mediates fatty acid uptake in hepatocytes.

Identification, Isolation, and Characterization of a-FABP$_{PM}$. When the extraction and purification approach described in *Materials and Methods* was used, adipocyte plasma membrane fractions were found to contain a basic protein (pI = 9.05 ± 0.05) that migrated as a single band of apparent molecular mass 40 kDa on NaDodSO$_4$/PAGE. The protein also eluted from TSK-2000 gel permeation HPLC columns as a single sharp peak of apparent molecular mass

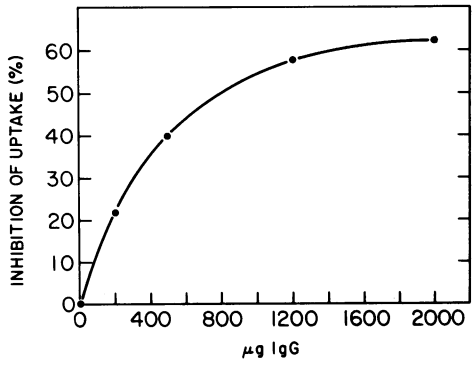

FIG. 4. Effect of anti-h-FABP$_{PM}$ on [^{3}H]oleate uptake by isolated rat adipocytes. Incubations in 171 μM oleate-albumin (1:1) were carried out as described in the text, with either preimmune immunoglobulin or specific immune immunoglobulin at amounts up to 2000 μg per 2-ml incubation mixture. As compared with control incubations, anti-h-FABP$_{PM}$ inhibited oleate uptake velocity in dose-dependent fashion.

40 kDa. In agar gel diffusion studies employing rabbit antibodies to rat h-FABP$_{PM}$, the a-FABP$_{PM}$ gave a line of immunologic identity with h-FABP$_{PM}$. Electrophoresis and immunoblotting using the same antibody showed that adipocyte plasma membranes contain a single strongly crossreactive 40-kDa protein. Further details of the isolation and characterization of a-FABP$_{PM}$ will be reported elsewhere (21).

DISCUSSION

Although it has long been considered that the movement of long-chain fatty acids across cell membranes is invariably passive (1–4), previous studies had already suggested that fatty acids might be taken up in at least some cell types by one or more saturable membrane-associated processes (17, 18, 32–35). Unfortunately, none of these earlier studies was conclusive. Indeed, DeGrella and Light (36, 37) concluded that fatty acids penetrate adult rat cardiac myocytes by passive diffusion and that the apparent saturation of uptake seen in prior studies in fact reflected saturation of an intracellular metabolic step. More recently, Noy *et al.* (4) have argued that the entry of fatty acids into hepatocytes reflects their passive partitioning into the lipid component of the cell membrane.

After demonstrating that the binding of fatty acids to isolated, sinusoidal-enriched rat liver plasma membranes was saturable and sensitive to both trypsin and heat denaturation of the membranes (38), we isolated in 1985 the basic (pI = 9.1) 40-kDa protein, h-FABP$_{PM}$, responsible for the specific component of fatty acid binding by these membranes (7). An immunologically identical FABP was also isolated from the microvillous membranes of jejunal enterocytes (13) and was shown by immunofluorescence to be present in cardiac muscle and in the plasma membranes of intestinal submucosal macrophages (7, 13). An antibody against the rat h-FABP$_{PM}$ was also shown to crossreact with a similar 40-kDa protein in the plasma membranes of mouse 3T3-L1 adipocytes (D. Bernlohr, personal communication). In work presented above and elsewhere (21), we have isolated the membrane FABPs from rat adipocytes and cardiac myocytes and shown them to be immunologically closely related, if not identical, to the protein already isolated from liver and gut. A similar 40-kDa basic protein has also been isolated from canine cardiac myocytes and partially characterized (39). Despite the immunologic studies cited, ultimate proof of the relationships of these various proteins must await amino acid sequencing or the construction and characterization of cDNAs, which have not yet been accomplished.

In parallel studies of fatty acid uptake by isolated hepatocytes, unequivocal evidence for carrier-mediated transport was obtained (5, 6). A role for h-FABP$_{PM}$ in the uptake process was established by antibody inhibition studies (6). The same strategies previously applied to hepatocytes have now been employed to study the uptake of a representative long-chain free fatty acid, [^{3}H]oleate, by isolated rat epididymal fat pad adipocytes. The data confirm that oleate uptake by adipocytes is saturable and inhibited by both phloretin and a protease, in this instance Pronase. These studies also indicate that, over the entire physiologically realizable range of unbound oleate concentrations, oleate uptake is inhibited by an antibody raised against h-FABP$_{PM}$. Although a nonspecific uptake component was not inhibited by antibody, this component is quantitatively significant only at unbound oleate concentrations unlikely to be observed *in vivo*. When this observation is taken in conjunction with earlier studies indicating that such uptake is also independent of intracellular binding or metabolism (17, 18), the conclusion is inescapable that the major component of

fatty acid uptake into adipocytes is effected by a specific membrane transport process closely related to that in the liver cell.

The precise structure of these transport systems is not yet known, but differences in their requirement for external sodium and in their response to a variety of metabolic inhibitors suggest that the hepatocyte and adipocyte fatty acid transport systems are not identical. Although the nature of the differences is not yet clear, the recent finding (21) that the plasma membrane fatty acid binding proteins from hepatocytes, adipocytes, and cardiac myocytes could be extracted with sonication at a high salt concentration and did not require total disruption of the membranes with detergent, as initially reported (7, 13), suggests that it is a superficial membrane protein, rather than an integral protein traversing the entire thickness of the lipid bilayer. If this is the case, then this protein may serve as a common fatty acid acceptor in each of the tissues thus far examined, while actual translocation of fatty acids across the plasma membrane is accomplished by other mechanisms. These might include passive partitioning into the lipid bilayer (4) but are more likely to involve additional transport mechanisms, which may be cell specific, and with different energy sources. This hypothesis is consistent with current knowledge of the well-studied fatty acid transport system in *Escherichia coli*, in which a superficial membrane fatty acid binding protein of 43 kDa is only one component of a more complex fatty acid transport apparatus (40, 41). This model would also explain how antibodies to a common fatty acid binding protein could inhibit fatty acid transport by several membrane transport systems in different tissues, which differ in energy requirements and their responses to hormonal signals.

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