

# Oleate Uptake by Cardiac Myocytes Is Carrier Mediated and Involves a 40-kD Plasma Membrane Fatty Acid Binding Protein Similar to That in Liver, Adipose Tissue, and Gut

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## Abstract

Uptake of [ $^3\text{H}$ ]oleate by canine or rat cardiac myocytes is saturable, displays the countertransport phenomenon, and is inhibited by phloretin and trypsin. Cardiac myocytes contain a basic ( $\text{pI} \sim 9.1$ ) 40-kD plasma membrane fatty acid binding protein ( $\text{FABP}_{\text{PM}}$ ) analogous to those recently isolated from liver, adipose tissue, and gut, unrelated to the 12–14-kD cytosolic FABP in these same tissues. An antibody to rat liver  $\text{FABP}_{\text{PM}}$  selectively inhibits specific uptake of [ $^3\text{H}$ ]oleate by rat heart myocytes at 37°C, but has no influence on nonspecific [ $^3\text{H}$ ]oleate uptake at 4°C or on specific uptake of [ $^3\text{H}$ ]glucose. Uptake of long-chain free fatty acids by cardiac muscle cells, liver, and adipose tissue and absorption by gut epithelial cells is a facilitated process mediated by identical or closely related plasma membrane FABPs.

## Introduction

Even though the heart capillary endothelium represents a barrier to free fatty acid (FFA) extraction by the myocyte (1, 2), uptake rates *in vivo* are substantial (1). Indeed, FFA represent the most important substrate for myocardial energy metabolism under basal conditions. With regard to the mechanism of FFA uptake, some have suggested that the transfer of FFA from plasma into the cell is carrier mediated (3, 4) whereas others argue that the process is equilibrative and that the kinetics observed reflect intracellular metabolism rather than membrane transport (5, 6). However, earlier studies (5, 6) of cardiac FFA uptake employed  $\text{Ca}^{2+}$ -intolerant myocytes in which sarcolemma permeability is altered (7). They also failed to consider the contribution of rapid efflux to the shape of the FFA uptake curve. Hence, the nature of the uptake process in cardiac myocytes remains uncertain. By contrast, studies from several laboratories, including our own, have documented that FFA uptake by hepatocytes (8, 9) and adipocytes (10–12) is unequivocally carrier mediated, and that a 40-kD plasma membrane fatty acid binding protein ( $\text{FABP}_{\text{PM}}$ )<sup>1</sup> isolated from

these tissues and from the gut (12–14) plays an essential role in fatty acid uptake (8, 9, 12, 15). The aims of the present study were, therefore (a) to study the uptake of a representative FFA, [ $^3\text{H}$ ]oleate, by well-characterized,  $\text{Ca}^{2+}$ -tolerant dog and rat cardiomyocyte preparations using a technique that allowed accurate determination of initial influx rates; (b) to isolate and characterize a putative cardiac myocyte  $\text{FABP}_{\text{PM}}$ ; and (c) to examine its role in FFA uptake by means of antibody inhibition studies. A preliminary report of our findings has appeared in abstract form (16).

## Methods

**Materials.** D-[1- $^3\text{H}(N)$ ]glucose (15.5 Ci/mmol) and 9,10-[ $^3\text{H}$ ]oleic acid (2.6 Ci/mmol) came from New England Nuclear (Boston, MA); nonradioactive oleate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), phloretin, KCN, and ouabain from Sigma Chemical Co. (St. Louis, MO); bovine serum albumin (BSA, fatty acid free) from Boehringer Mannheim (Indianapolis, IN); and trypsin from ICN (Cleveland, OH).

**Isolation of myocytes.** Cardiac myocytes were isolated from dogs (1–2-yr-old beagles) or rats (55–60-d-old Sprague Dawley) by collagenase perfusion in the presence of  $\text{CaCl}_2$  (7, 17), and resuspended in Hepes-Ringer containing 3% BSA until use. Approximately 85–90% of all cells excluded trypan blue. Of these, up to 80% retained the rod shape. It has been previously shown that, with respect to physiologically relevant parameters, these myocytes are ultrastructurally, pharmacologically, and electrophysiologically indistinguishable from intact heart (7, 17). Data presented below were obtained with canine cardiac myocytes except where the use of rat heart myocytes is specifically indicated.

**Incubation and uptake procedures.** For the uptake studies myocytes were washed three times and resuspended ( $\sim 1 \times 10^6/\text{ml}$ ) in Hanks' buffer containing 10 mM Hepes (HH), pH 7.4, gassed for 5 min with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ . 9,10-[ $^3\text{H}$ ]oleic acid and measured amounts of nonradioactive oleate and BSA were also dissolved in HH at oleate/BSA molar ratios from 0.5:1 to 6:1, producing unbound oleate concentrations of 0.04–21.9  $\mu\text{M}$  (18, 19). Except where specified, final BSA concentration was 208  $\mu\text{M}$ . At zero time 500  $\mu\text{l}$  of the appropriate oleate solution was added to 100  $\mu\text{l}$  of cell suspension in a 15-ml polystyrene tube (Sarstedt, Inc., Princeton, NJ). At specific intervals thereafter uptake was blocked with 5 ml of an ice-cold 200  $\mu\text{M}$  phloretin/0.1% BSA stop solution in HH; the mixture was immediately filtered and the filters counted by liquid scintillation spectrometry (8, 9). Cell-associated radioactivity at zero time was determined by adding stop solution to the cells before adding the [ $^3\text{H}$ ]oleate-BSA. Nonspecific oleate adsorption to the filter was routinely measured and subtracted from experimental values, of which it represented < 15%. In all instances, the cumulative curve of cell associated radioactivity vs. time was linear, with its maximal positive slope, for at least the first 40 s after addition of [ $^3\text{H}$ ]oleate. The initial oleate uptake velocity was estimated as the slope of this initial, linear portion of the cumulative radioactivity curves, as previously described (8, 9, 12).

Where indicated, cells were preincubated for 15 min at 4°C (phloretin) and 37°C (KCN, CCCP, ouabain, trypsin) under condi-

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1. Abbreviations used in this paper: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FABP, fatty acid binding protein;  $\text{FABP}_{\text{PM}}$ , plasma membrane FABP.

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tions previously described (8, 9) before determining [<sup>3</sup>H]oleate uptake. To evaluate whether Na<sup>+</sup> substitution affects the uptake process, myocytes were resuspended in HH buffer in which Na<sup>+</sup> had been isosmotically replaced by K<sup>+</sup>, Li<sup>+</sup>, choline, or sucrose; the experiment was performed immediately thereafter. The absence of Na<sup>+</sup> or the presence of the inhibitors listed above did not affect cell viability as assessed by trypan blue exclusion and the average number of myocytes which retained the rod shape. These variables were assessed on a separate aliquot of identically treated cells, during the uptake study.

**Isolation and characterization of a sarcolemma protein with high affinity for fatty acids.** Isolated canine cardiac myocytes (~ 50 × 10<sup>6</sup> cells, 1.5 mg of cell protein/10<sup>6</sup> cells) were resuspended (1:10 vol/vol) in 1 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, pH 7.5 and sonicated three times (10 s each in ice). From resulting cell fragments a crude plasma membrane preparation (4 μg of membrane protein/mg cell protein) was obtained by differential centrifugation. Membrane proteins were extracted by gentle sonication for 30 min at 0°C in a 2 M NaCl, 0.05 M phosphate buffer, pH 7.6, and subsequent stirring at 4°C for 60 min. This procedure, which extracted ~ 20% of membrane proteins, has recently been described in detail elsewhere (20). After dilution (1:1) and centrifugation (2,500 g for 30 min at 4°C) the supernatant was passed through an oleate agarose affinity column (14). After washing extensively with 1 M NaCl, pH 8, bound proteins (~ 300 ng) were eluted with 2 vol of 8 M urea, diluted 4:1 and resuspended, after ultrafiltration, in 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.6. When necessary, the FABP<sub>PM</sub> was further purified on a 75 × 4.6-mm TSK phenyl 5PW HPLC-hydrophobic interaction chromatography column (LKB Instruments, Gaithersburg, MD) using a reverse ammonium sulfate gradient (from 1.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 M phosphate, pH 6.7, to 0.1 M phosphate, pH 6.7 in 30 min) at a flow of 1 ml/min; the absorbance was measured at 220 nm. Purity at various stages of the isolation process was monitored with SDS-polyacrylamide gel electrophoresis (SDS-PAGE), analytical isoelectric focusing, and gel permeation HPLC (600 × 7.5-mm TSK 2000 SW column with a 75 × 7.5-mm precolumn) using an eluting solvent consisting of 0.1 M NaCl, 0.01 M phosphate, pH 6.7, and measuring ultraviolet (UV) absorbance at 210 nm.

The affinity of the purified FABP<sub>PM</sub> for oleate was confirmed by co-chromatography. After being concentrated and resuspended in 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.6, the protein was gently sonicated with [<sup>3</sup>H]oleate (23°C for 10 min) and injected into a Superose 12 HPLC-gel permeation column (300 × 10 mm, Pharmacia Fine Chemicals, Piscataway, NJ) using the eluting solvent described above at a flow rate of 0.5 ml/min. The collected fractions were counted and the resulting <sup>3</sup>H peak compared to the 40-kD protein peak as monitored at 210 nm. The Western blot technique (21) was used to assess the immunologic cross-reactivity between the cardiac FABP<sub>PM</sub> and an antibody to the rat liver FABP<sub>PM</sub> produced in rabbits as previously described (14, 20). The assay was performed on samples subjected to SDS-PAGE (20).

**Antibody inhibition studies.** The role of the myocardial FABP<sub>PM</sub> in oleate uptake was examined by antibody inhibition studies employing the antibody to the rat liver FABP<sub>PM</sub>. To avoid possible immunologic and species differences, these studies were conducted with rat heart myocytes; the strong immunologic cross-reactivity between this antibody and rat heart FABP<sub>PM</sub> has been reported previously (20). Isolated rat heart myocytes (1 × 10<sup>6</sup>/ml, 1 ml) were incubated for 1 h at 4°C with 500 μg of the IgG fraction of the antiserum to rat liver FABP<sub>PM</sub> or with 500 μg of the IgG fraction of the corresponding preimmune serum. The cells were then washed and resuspended in HH buffer, and aliquots of treated and control cells were incubated at 37° and 4°C with 208 μM [<sup>3</sup>H]oleate/BSA (1:1). Initial oleate uptake velocity was determined as described above.

To assess the specificity of the antibody effect, [<sup>3</sup>H]glucose uptake was also studied in aliquots of antibody-treated and control cells. The technique employed involved minor modifications of the one described for oleate uptake: cells were resuspended in Krebs-Ringer buffer containing 10 mM Hepes, and to the mixture of cells and glucose solution (100 μl each) was added a stop solution containing 200

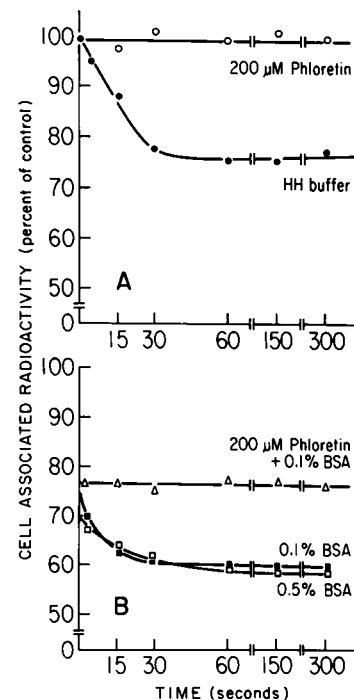
μM phloretin in Krebs-Ringer buffer containing 10 mM Hepes, but no BSA. Initial glucose uptake velocity was calculated from the initial (30-s) linear portion of the cumulative uptake curve.

## Results

### Evaluation of methods

**Effectiveness of the stop solution.** To allow accurate estimation of initial oleate influx, a stop solution should block bidirectional membrane transport while removing material surface bound but not yet internalized. As previously reported for hepatocytes (8) and adipocytes (10–12), the combination phloretin/BSA is an effective stop solution for use with cardiac myocytes. After preloading of myocytes with [<sup>3</sup>H]oleate for 30 s, 200 μM phloretin completely blocked the efflux observed in the presence of buffer alone (Fig. 1 A). Concentrations of BSA of 0.1% or higher virtually instantly removed surface-bound material followed by progressive efflux of intracellular radioactivity (Fig. 1 B). The combined 200 μM phloretin/0.1% BSA stop solution removed surface material while blocking further efflux.

**Initial oleate uptake reflects influx rather than dissociation from albumin or intracellular events.** Over a wide range of absolute concentrations of oleate and BSA, the concentration of unbound oleate is precisely determined by the oleate/albumin molar ratio (18, 19). Nevertheless, at low and nonphysiologic oleate/albumin concentrations the velocity of hepatocellular oleate uptake increases with increasing concentrations of substrate, despite a fixed molar ratio (22, 23). Recent experiments in isolated hepatocytes have shown that, for a given molar ratio, there exists a critical concentration of oleate:albumin beyond which further increases in the concentration of substrate complex produce no further increase in oleate uptake velocity (23); this maximal oleate uptake velocity for a given molar ratio was found to be a saturable function of the equilibrium unbound oleate concentrations defined by the various



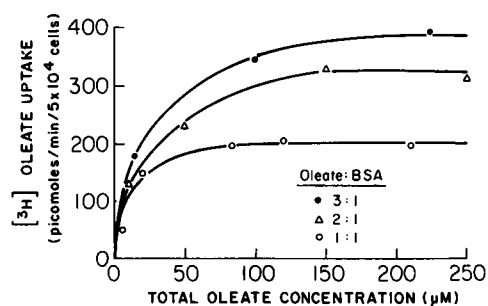
**Figure 1.** Representative experiment demonstrating the effect of various stop solutions on oleate efflux from canine cardiac myocytes. Cells were incubated for 30 s with 104:208 μM [<sup>3</sup>H]oleate/BSA. At time zero, the stop solution was added and the cell associated radioactivity monitored over the next 5 min as described in Methods. Compared to the control buffer (●), phloretin alone (○) blocks oleate efflux (A) while BSA alone (■, □) virtually instantly reduces the amount of surface bound oleate (lower y-axis intercept), while permitting subsequent cellular efflux (B). The combination phloretin/BSA (Δ) blocks oleate efflux while removing surface bound oleate.

molar ratios studied (23). This suggests that at low concentrations of oleate/albumin measured uptake rates may be dissociation limited. However, physiologic concentrations of oleate and albumin exceed the observed critical concentrations of oleate/albumin complexes, indicating that, at physiologic concentration of substrate, hepatocellular oleate uptake is, in fact, driven by the equilibrium unbound oleate concentration (23). Under these conditions measured uptake rates reflect cellular removal mechanisms, rather than limited dissociation of substrate complexes (23, 24). Analogous experiments in isolated adipocytes (D. Sorrentino and P. D. Berk, unpublished observations) and cardiac myocytes (Fig. 2) yielded similar results. Accordingly, except where specific circumstances dictated otherwise, all experiments in the present paper were conducted at a BSA concentration of 208  $\mu\text{M}$ , well in excess of the level at which substrate complex dissociation rates or oleate availability are factors which limit uptake velocity. That under the conditions employed uptake velocity was related to the oleate/BSA ratio and consequent unbound oleate concentration, rather than to the total oleate concentration, was corroborated by the finding that oleate uptake was similar at a given ratio whether the ratio was obtained by keeping the BSA concentration constant at 208  $\mu\text{M}$  and varying the oleate concentration or by using a fixed 208  $\mu\text{M}$  concentration of oleate and varying the BSA concentration (Fig. 3).

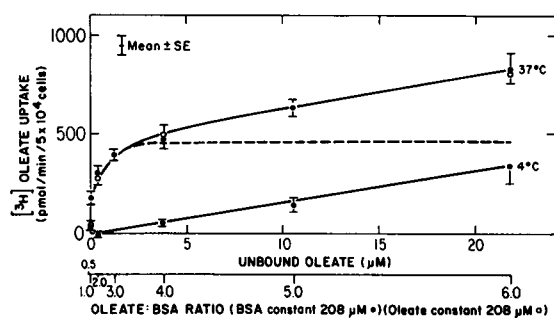
Oleate uptake at 37°C was linear for at least 40 s. This initial slope of the cumulative oleate uptake curve, which was used to calculate initial oleate uptake velocities, mainly reflects influx rather than intracellular metabolism: removal of glucose from the medium, which decreases esterification up to 50% (6), did not affect this slope. Finally, uptake rates did not reflect substrate depletion since the initial influx did not exceed 1–2% of the incubated substrate.

### Kinetic characteristics

**Saturability.** With canine cardiac myocytes, increasing concentrations of unbound oleate produced saturation of initial



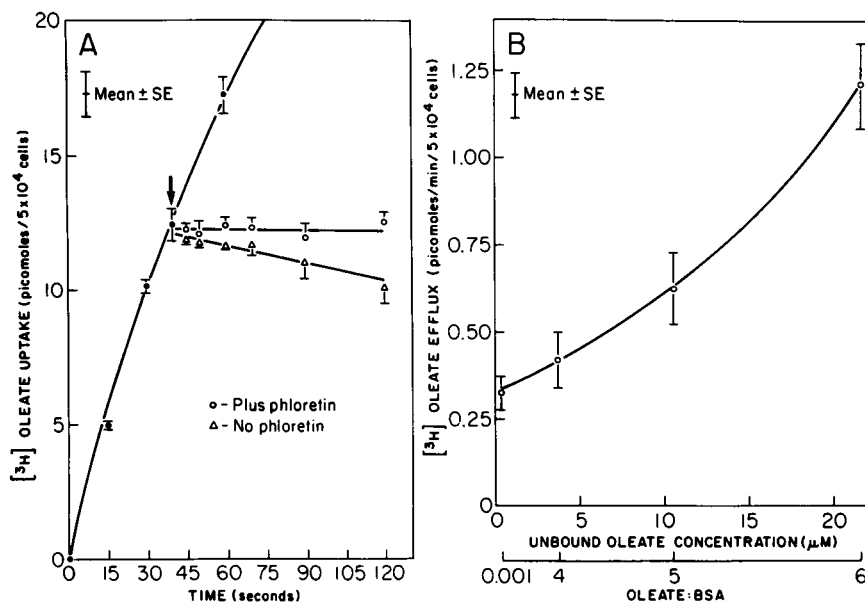
**Figure 2.** Oleate uptake by canine cardiac myocytes as a function of total oleate concentrations at three representative oleate/BSA molar ratios. Although the equilibrium unbound oleate concentration is constant for a given molar ratio, irrespective of the absolute concentrations of oleate and BSA (18, 19) each curve appears to indicate saturation of uptake. However, experiments conducted in isolated hepatocytes and adipocytes have shown that at each oleate/BSA molar ratio the apparent saturation of uptake with increasing total oleate concentrations reflects dissociation limited availability of unbound oleate at low oleate/BSA concentrations and cellular removal at higher oleate/BSA concentrations (23). Accordingly, in subsequent experiments with cardiac myocytes, oleate/BSA concentrations > 150  $\mu\text{M}$  were used, at which concentrations measured uptake velocities reflect cellular removal of unbound oleate.



**Figure 3.** Initial oleate uptake velocities as measured at 37°C and 4°C from incubation media containing oleate and BSA at oleate/BSA molar ratios of 0.5:1 to 6:1. Abscissa also indicates resulting concentrations of unbound oleate, calculated from these molar ratios by the stepwise equilibrium method (18, 19). Dashed line, representing specific uptake curve, is the difference between observations at 37°C and 4°C, and was fitted by computer directly to the Michaelis-Menten Equation (see text). Error bars indicate standard errors of four to five replicates. Most studies were conducted with 208  $\mu\text{M}$  BSA, and varied concentrations of oleate. However, over the range employed in these experiments, uptake velocities for a given molar ratio were the same whether the ratio was achieved by keeping BSA constant and varying the oleate concentrations (●) or by keeping oleate constant and varying the BSA concentration (○).

uptake velocity when measured at 37°C (Fig. 3). An additional, linear component, believed to represent nonspecific uptake, was apparent at unbound oleate concentrations > 4  $\mu\text{M}$ . It is the only component observed at 4°C. Although quantitatively important at oleate/BSA ratios  $\geq$  3:1, this linear component is probably insignificant *in vivo*, where FFA/BSA ratios typically fluctuate between 0.5:1 and 2:1 (25). When this linear component was subtracted from the uptake curve observed at 37°C, a single, saturable process was demonstrated. Computer analysis, conducted as previously described for hepatocytes (26), indicated a  $V_{\text{max}}$  of  $475 \pm 9$  (SE) pmol/min per 50,000 cells and a  $K_m$  of  $186 \pm 10$  nM. Similar results were obtained with rat heart myocytes, in which the specific, saturable component had a  $V_{\text{max}}$  of  $585 \pm 18$  pmol/min per 50,000 cells and a  $K_m$  of  $125 \pm 11$  nM.

**Countertransport and trans-stimulation.** To demonstrate that myocardial oleate uptake exhibits these additional features of carrier-mediated transport (27, 28) 50- $\mu\text{l}$  aliquots of canine cardiac myocytes ( $1 \times 10^6/\text{ml}$ ) were incubated at 37°C with 50  $\mu\text{l}$  of [ $^3\text{H}$ ]oleate/BSA at a molar ratio of 0.1:1 (final concentrations 5:50  $\mu\text{M}$ ), and cell associated radioactivity determined at 10–15-s intervals as described in Methods. After an incubation period of 40 s, 2.5 ml of a solution containing 250  $\mu\text{M}$  BSA and 0, 1,000, 1,250, or 1,500  $\mu\text{M}$  unlabeled oleate, with or without 200  $\mu\text{M}$  phloretin, was added, and cell associated radioactivity monitored over the next 80 s. A representative set of experiments, employing 1,500  $\mu\text{M}$  oleate:250  $\mu\text{M}$  BSA, is illustrated in Fig. 4 A. In contrast to control cells, which continue to accumulate [ $^3\text{H}$ ]oleate, addition of unlabeled oleate/BSA in the presence of phloretin blocks subsequent influx and efflux of [ $^3\text{H}$ ]oleate, resulting in a constant level of cell associated radioactivity over the subsequent 80 s. By contrast, addition of the same unlabeled oleate/albumin solution in the absence of phloretin results in progressive efflux of [ $^3\text{H}$ ]oleate at a rate of  $1.21 \pm 0.14$  pmol/min per  $5 \times 10^4$  myocytes. From the cell-associated radioactivity at 40 s, the



**Figure 4.** Countertransport and trans-stimulation of [<sup>3</sup>H]oleate efflux in isolated canine cardiomyocytes. (A) 50 μl of [<sup>3</sup>H]oleate/BSA (5:50 μM) was added to 50 μl of cells and cell-associated radioactivity determined at various times as described in Methods. Control cells were incubated for 120 s (●). In the experimental incubations 2.5 ml of unlabeled oleate/BSA (1,500:250 μM) with (○) or without (△) 200 μM phloretin, was added to the cells after 40-s incubation with the isotope (arrow), and the cell associated radioactivity monitored over the next 80 s. The figure shows that efflux of [<sup>3</sup>H]oleate occurs against the prevailing concentration gradient (see text) while phloretin virtually abolishes the phenomenon. ( $\bar{x} \pm SE$ ,  $n = 3$ ). (B) [<sup>3</sup>H]-oleate efflux rates measured under the conditions described for A, are plotted versus the extracellular unlabeled oleate/BSA molar ratios and consequent unbound oleate concentrations following addition of unlabeled oleate:albumin solutions containing no phloretin. Under these conditions [<sup>3</sup>H]oleate efflux increases with increasing extracellular unbound oleate. In the presence of phloretin efflux was minimal at all unbound oleate concentrations studied (see text).

packed cell volume in the incubation mixture (10 μl) and an assumed cellular water space of 50%, the average intracellular [<sup>3</sup>H]oleate concentration at 40-s incubation is estimated at ~ 3.0 μM. By contrast, the extracellular free unlabeled oleate concentration after addition of the 1,500 μM oleate:250 μM BSA is 21.9 μM (18, 19). Hence, this experiment illustrates phloretin-inhibitable efflux of [<sup>3</sup>H]oleate from cardiac myocytes against a sevenfold concentration gradient. Even if intracellular oleate is compartmentalized at concentrations in excess of that calculated above, it is unlikely that the concentrations would be sufficient to completely abolish the inwardly directed oleate concentration gradient established by the experimental manipulations. Aside from establishing an inwardly directed oleate concentration gradient, the relative volumes and concentrations of the labeled and unlabeled oleate solutions employed abruptly reduce the specific activity of extracellular oleate, minimizing further uptake of [<sup>3</sup>H]oleate and therefore the possible effects on cell associated radioactivity of displacement of labeled substrate from its albumin carrier.

As illustrated in Fig. 4 B, in the absence of phloretin, [<sup>3</sup>H]-oleate efflux rates were found to increase with increasing oleate/albumin ratios, and hence, with increasing unbound oleate concentrations, in the unlabeled oleate:albumin solutions added at 40 s. This indicates trans-stimulation of [<sup>3</sup>H]-oleate efflux by extracellular oleate. The efflux rates in the absence of phloretin exceeded in all cases those observed in the presence of phloretin which averaged  $0.15 \pm 0.10$  pmol/min per  $5 \times 10^4$  cells, and did not differ as a function of the oleate/albumin solution employed (data not shown).

#### Oleate uptake in the presence of various inhibitors and ions.

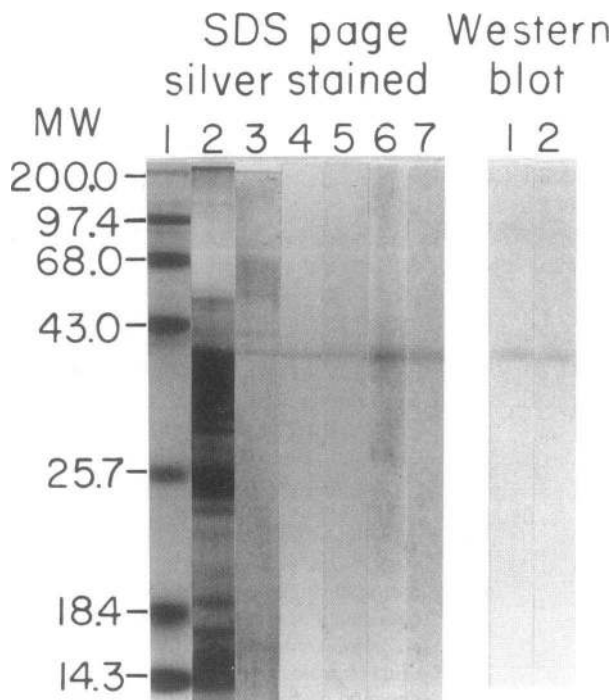
In canine cardiac myocytes, as in hepatocytes (8, 9), phloretin (400 μM) and trypsin (0.3 mg/ml) significantly inhibited oleate uptake to  $27 \pm 3$  and  $64 \pm 3\%$ , (both  $P < 0.005$ ) respectively, of control. KCN (1 mM), CCCP (100 μM), and ouabain (1 mM),

which all decrease hepatocellular uptake of oleate (9), had no effect in myocytes under the conditions employed. Total or partial substitution of NaCl in the medium with LiCl, choline Cl, KCl or sucrose, which decreases fatty acid uptake in hepatocytes (9), stimulated uptake in myocytes by 15–55% (significant,  $P < 0.01$ , for LiCl and choline Cl).

#### Isolation of a FABP<sub>PM</sub>

Preliminary efforts to isolate a canine cardiac myocyte FABP<sub>PM</sub> in appreciable quantities directly from crude heart homogenate, utilizing the combination of preparative isoelectric focusing and affinity chromatography successfully employed for FABP<sub>PM</sub> from other tissues (20), were complicated by the presence of relatively large quantities of contaminating proteins in such homogenates. Furthermore, due to the large proportion of endothelial cells, the possibility exists that plasma membranes from homogenates may not entirely represent sarcolemma preparations. Accordingly the small scale procedure starting from isolated myocytes described in Methods was employed instead. Although the resulting quantities of membrane proteins were too small to employ preparative isoelectric focusing, a combination of affinity chromatography and HPLC procedures including hydrophobic interaction chromatography, successfully purified the protein of interest to homogeneity.

When applied to plasma membranes from isolated canine cardiac myocytes, the procedures for extraction and oleate agarose affinity chromatography of FABP<sub>PM</sub> yielded a major band on SDS-polyacrylamide gel electrophoresis having the same molecular mass as the FABP<sub>PM</sub> isolated from rat liver, adipose tissue, jejunum, and myocardium (20) (Fig. 5). In contrast to the latter proteins, which are readily purified to homogeneity (20), the canine protein was frequently accompanied by a contaminant of either slightly greater (Fig. 5) or lesser molecular



**Figure 5.** (Left) SDS-PAGE of membrane proteins. Standards (1), total canine myocardial membrane proteins (2) and proteins purified by oleate agarose affinity chromatography from dog heart (3) and rat liver (4), adipose tissue (5), jejunum (6), and heart (7). MW, molecular weight (kD). (Right) Western blot of rat hepatocyte and canine myocyte FABP<sub>PM</sub> against the antibody to the rat hepatocyte FABP<sub>PM</sub>. The myocyte FABP<sub>PM</sub>, isolated by affinity chromatography, had been further purified by hydrophobic interaction HPLC.

weight, which appeared as a shoulder on the gel permeation HPLC elution peak (Fig. 6 A). When further purified, as described, by hydrophobic interaction HPLC, the FABP<sub>PM</sub> eluted from the gel permeation column as a single, symmetrical peak at an elution time of 32.5 min. After incubation with [<sup>3</sup>H]oleate, rechromatography of the eluted canine FABP<sub>PM</sub> through a 300 × 10-mm Superose-12 HPLC gel permeation column again yielded a symmetric peak which co-chromatographed with the labeled fatty acid; its retention time corresponded to a molecular weight of 40 kD (Fig. 6 B). In addition to a molecular weight (40 kD) and isoelectric point (pI ~ 9.1) similar to those of the other FABP<sub>PM</sub> previously reported, the canine cardiac myocyte FABP<sub>PM</sub> gave a positive Western blot with a rabbit antiserum raised against the rat liver FABP<sub>PM</sub> (Fig. 5).

#### Antibody inhibition studies

At 37°C the antibody to rat liver FABP<sub>PM</sub> significantly inhibited uptake of [<sup>3</sup>H]oleate into isolated rat heart myocytes, compared to cells treated with preimmune serum (216 ± 2 vs. 358 ± 34 pmol/min per 50,000 cells, *P* < 0.02) (Fig. 7). The degree of inhibition at 37°C (40%) was similar to that in adipocytes treated with corresponding antibody concentrations (12). By contrast, the antibody had no effect compared to preimmune serum on nonspecific oleate uptake at 4°C, (2.7 ± 0.10 vs. 2.9 ± 0.9 pmol/min per 50,000 cells, *P* > 0.5). Antibody-treated cells retained their predominantly rod shape and viability. Specificity of the antibody effect for oleate trans-

port was indicated by the glucose uptake studies: uptake of 5 μM glucose in treated and control cells was identical (15.3 ± 2.4 vs. 16.0 ± 0.6 pmol/5 min per 10<sup>6</sup> cells, *P* > 0.5) (Fig. 7).

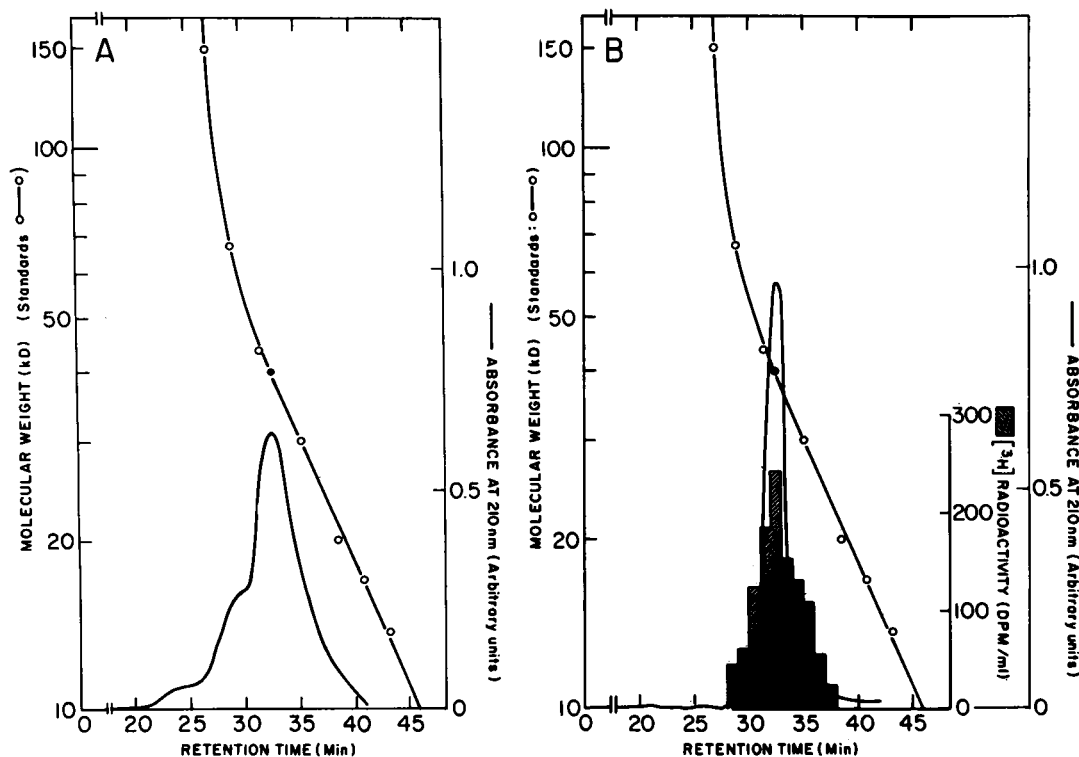
#### Discussion

Fatty acid uptake by cardiac myocytes as well as other cell types was long considered by many (5, 6, 29–31) although not all investigators (3, 4) to occur by diffusion rather than by a specific facilitated process. Unfortunately, these earlier experiments with cardiac myocytes could not satisfactorily quantify FFA influx, since the techniques employed did not block efflux during sample preparation and the cells were prepared and used without calcium, which has been shown to alter sarcolemma integrity and permeability (7).

More recently, we and others have shown that FFA uptake has the kinetic characteristics of a carrier-mediated process in hepatocytes (8, 9) and adipocytes (10–12), and have isolated and characterized basic, 40-kD FABP<sub>PM</sub> from these and other tissues with high transmembrane fluxes of fatty acids (13, 14, 20). That these proteins are similar and mediate FFA uptake has been clearly demonstrated. By immunofluorescence, an antibody raised in rabbits against the rat liver FABP<sub>PM</sub> bound specifically to the plasma membranes of cardiac myocytes, adipocytes, and jejunal enterocytes (12–14). This antibody also inhibited oleate uptake, noncompetitively and in a dose-dependent fashion, in rat hepatocytes (9), adipocytes (12) and, in preliminary studies, in the gut (15). As these data collectively suggested that FFA might be taken up by the heart by mechanisms similar to those of liver, adipose tissue and gut, the current study, initiated in 1986 (16), focused on cardiac myocytes.

The cells employed, isolated from dog and rat, have been shown to resemble intact myocardium, functionally and morphologically, and to conform to the definition of “Ca<sup>2+</sup>-tolerant” (7, 17). We employed a well-validated rapid filtration technique and a 200 μM phloretin:0.1% BSA stop solution, which permits accurate quantitation of cellular influx by blocking bidirectional membrane transport of oleate (8–12) while removing material surface bound but not yet internalized (8, 9, 12). That removal of glucose from the medium, which decreases esterification in these cells by up to 50% (6), did not affect initial uptake velocity suggests that this parameter reflects principally the activity of membrane transport processes rather than an intracellular process such as esterification. Experiments analogous to those performed in other cell types (23) confirmed that myocyte oleate uptake is driven by the equilibrium unbound oleate concentration, rather than by the total oleate concentration.

Initial oleate uptake velocity was saturable with increasing concentrations of unbound oleate; after subtraction of a non-specific linear component it conformed to Michaelis-Menten kinetics with a *V*<sub>max</sub> of 585 pmol/min per 50,000 cells and a *K*<sub>m</sub> of 125 nM for rat cardiomyocytes. In canine cardiomyocytes, isolated from an animal with a lower overall metabolic and heart rate, *V*<sub>max</sub> was not surprisingly somewhat lower at 475 pmol/min per 50,000 cells. In vivo experiments performed with the indicator dilution technique in the dog by Rose and Goresky (1) offer the opportunity to compare FFA influx rates in the working heart with those in the isolated, resting cells. Based on an average heart interstitial space of 13–14% (2) and

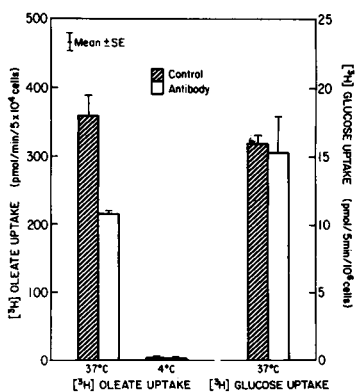


**Figure 6.** HPLC gel permeation elution profile of the canine myocardial FABP<sub>PM</sub> isolated by affinity chromatography before (A) and after (B) further purification by hydrophobic interaction chromatography (for details see text). B also shows co-chromatography of the protein with [<sup>3</sup>H]-oleate at a retention time corresponding to a molecular weight of 40 kD.

on an average cell number of  $40\text{--}50 \times 10^6/\text{g}$  heart (7, 32), it can be calculated that in vivo, at FFA/BSA molar ratios of  $\sim 0.5:1$  to  $1:1$ , FFA influx approximates  $1\text{--}1.5$  nmol/min per 50,000 cells, a value three to five times larger than we observed in isolated cardiomyocytes. While other interpretations are possible, this discrepancy may indicate that FFA influx is dependent on the working load, and as a consequence, that the cardiomyocyte FFA requirements may be modulated at the membrane level. This conclusion is corroborated by the finding that, at low ventricular load, palmitate uptake rates in perfused rat heart are comparable to the data obtained in rat myocytes in the present study (33).

Shortly after our initial characterization of a rat myocardial FABP<sub>PM</sub> (20) Stremmel reported that an antibody to this protein inhibited saturable oleate uptake in isolated rat heart myocytes (34). However, his reported maximal oleate uptake

velocity,  $95$  pmol/min per 50,000 cells, is surprisingly low for an animal with the metabolic rate of the rat and does not correspond to earlier studies of fatty acid removal rates in this species (33). Moreover, it is increasingly apparent that saturable uptake is a necessary but not sufficient criterion to establish that a transport process is carrier mediated (24). In the current report we demonstrate that myocyte oleate uptake also displays countertransport and trans-stimulation, two other important features of carrier-mediated transport (27, 28). Countertransport is typically established by demonstrating cellular efflux of labeled compound against the prevailing concentration gradient upon addition of unlabeled compound to the incubation mixture (27). Demonstration of countertransport, however, is rendered difficult if the substance under study, like oleate, is tightly albumin bound because the added unlabeled species may displace the label from its protein binding sites in the incubation mixture, thereby increasing its free concentration and promoting its uptake. This phenomenon, described by Goresky et al. (35) for the hepatic uptake of palmitate, may in turn result in increased influx of the labeled species rather than demonstrating its predicted efflux from the cells (35–37). We reasoned that if the amount of unlabeled oleate added sufficiently exceeded the amount of extracellular [<sup>3</sup>H]oleate present, the resulting isotopic dilution of extracellular label would insure that subsequent influx of [<sup>3</sup>H]oleate would be negligible. Under these circumstances it would be possible to follow the efflux of the internalized label from the cell by measurements of cellular radioactivity. These predictions were confirmed by the experiment illustrated in Fig. 4 A, which also shows that phloretin, which blocks membrane transport of oleate (Fig. 1), virtually abolished the efflux of the labeled



**Figure 7.** Influence of a rabbit antibody to rat liver FABP<sub>PM</sub> on uptake by isolated rat heart myocytes of [<sup>3</sup>H]oleate and [<sup>3</sup>H]glucose. The antibody inhibited carrier-mediated oleate uptake (37°C), but had no effect on nonspecific oleate uptake (4°C) or on glucose uptake ( $\bar{x} \pm \text{SE}$ ,  $n = 3$ ).

species. Furthermore, as expected (27), [<sup>3</sup>H]oleate efflux rates under these conditions were trans-stimulated by increasing extracellular concentrations of unbound, unlabeled oleate (Fig. 4 B).

By analogy with hepatocyte (8, 9) and adipocyte (10–12) studies, [<sup>3</sup>H]oleate uptake was inhibited by phloretin and a protease, trypsin, suggesting that the carrier is a protein. A basic 40-kD protein which co-chromatographs with oleate was subsequently isolated from cardiac myocyte plasma membranes by procedures including oleate-agarose affinity chromatography, a technique previously employed to isolate analogous FABP<sub>PM</sub> from hepatocytes (14), adipocytes (12, 20), and jejunal enterocytes (13). These proteins are unrelated structurally or immunologically to the family of cytosolic fatty acid binding proteins previously described in these tissues (38). The FABP<sub>PM</sub> of rat liver, gut, adipose tissue, and myocardium are closely related, if not identical (20), and, in antibody inhibition studies, have now all been shown to play a crucial role in specific fatty acid uptake in their respective tissues (9, 12, 15). In the present study, an antibody against rat liver FABP<sub>PM</sub> gave a positive Western blot with the canine cardiac FABP<sub>PM</sub> and significantly inhibited specific [<sup>3</sup>H]oleate uptake by rat myocytes at 37°C, while having no effect on nonspecific oleate uptake as measured at 4°C. Moreover, the antibody did not inhibit myocyte uptake of glucose, which has also been shown under similar conditions to be a specific carrier-mediated process (39), thus demonstrating the selectivity of the antibody effect.

Despite these similarities, the transport processes do not appear fully identical. Fatty acid uptake in liver and gut has been reported to be potential sensitive (40), sodium coupled, and ouabain inhibitable (9, 15). Recent, preliminary studies in the perfused liver suggest that oleate uptake is actively driven by both the electrical and sodium chemical gradients across the plasma membrane (41). However, neither removal of external sodium nor ouabain nor metabolic inhibitors appear to inhibit significantly oleate uptake in adipocytes (10–12); the previously mentioned study by Stremmel (34) failed to demonstrate sodium dependence of myocyte oleate uptake; in the present study, substitution of external Na<sup>+</sup> with Li<sup>+</sup>, K<sup>+</sup>, choline, or sucrose in fact stimulated oleate uptake in cardiac myocytes. Previous observations in cultured chick embryo cardiac myocytes also indicated that fatty acid uptake occurs by a facilitation process without energy dependence (4). The basis for these differences remains to be determined. Preliminary studies conducted in our laboratory suggest (42) that the Na<sup>+</sup> requirement of oleate uptake may be dependent on particular experimental conditions. Another possible explanation is that, as with the well-characterized 43-kD FABP<sub>PM</sub> of *Escherichia coli* (43, 44), the FABP<sub>PM</sub> isolated thus far from mammalian tissues act as acceptors in more complex transport systems involving additional protein components. These additional transport components, and the consequent energy and ion requirements, could be tissue specific. These and other hypotheses are currently being explored.

Despite these questions, the preponderance of evidence from several laboratories now argues against the hypothesis that myocardial fatty acid uptake occurs by diffusion, and strongly suggests that the process is a facilitated one, mediated by a specific plasma membrane protein with high affinity for long-chain FFA, analogous to those isolated from liver, gut, and adipose tissue. Hence, both the absorption and the dispo-

sition of FFA may be subject to regulation at the level of the plasma membrane.

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