Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles

Joost J. F. P. Luiken,* Lorraine P. Turcotte,† and Arend Bonen[1,*]

Department of Kinesiology,* University of Waterloo, Waterloo, Ontario, Canada N2L 3G1, and Department of Exercise Sciences,† University of Southern California, Los Angeles, CA 90089

Abstract **Giant sarcolemmal vesicles were isolated from rat heart and hindlimb muscles for *a*) characterization of long-chain fatty acid transport in the absence of metabolism and *b*) comparison of fatty acid transport protein expression with fatty acid transport. Giant vesicles contained cytosolic fatty acid binding protein. Palmitate uptake was completely divorced from its metabolism. All palmitate taken up was recovered in the intravesicular cytosol as unesterified FA. Palmitate uptake by heart vesicles exhibited a K_m of 9.7 nm, similar to that of muscle (K_m = 9.7 nm). V_{max} (2.7 pmol/mg protein/s) in heart was 8-fold higher than in muscle (0.34 pmol/mg protein/s). Palmitate uptake was inhibited in heart (55–80%) and muscle (31–50%) by trypsin, phloretin, sulfo-*N*-succinimidyloleate (SSO), or a polyclonal antiserum against the 40 kDa plasma membrane fatty acid binding protein (FABPpm). Palmitate uptake by heart and by red and white muscle vesicles correlated well with the expression of fatty acid translocase (FAT/CD36) and fatty acid binding protein FABPpm, which may act in concert. The expression of fatty acid transport protein (FATP), was 10-fold lower in heart vesicles than in white muscle vesicles. It is concluded that long-chain fatty acid uptake by heart and muscle vesicles is largely protein-mediated, involving FAT/CD36 and FABPpm. The role of FATP in muscle and heart remains uncertain.**—Luiken, J. J. F. P., L. P. Turcotte, and A. Bonen. **Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles.** ***J. Lipid Res.*** **1999.** 40: **1007–1016.**

Supplementary key words FAT/CD36 • FABPpm • FATP • uptake kinetics • muscle

Although long-chain fatty acids (LCFA) are important substrates for heart and skeletal muscle, their mechanism of cellular uptake remains controversial. The question is whether LCFA uptake is *a*) due to passive diffusion or *b*) mediated by transport proteins. Investigations with artificial phospholipid bilayers suggested that the rate of FA flip-flop across the bilayer was extremely fast favoring passive diffusion as the likely mechanism (1). On the other hand, investigations with cellular systems yielded a saturable uptake process indicating the involvement of membrane LCFA receptors (c.f. ref. 2). However, saturability could also be due to a passive diffusion mechanism with cellular metabolism being rate limiting (3).

There have been a number of studies that present kinetic evidence for protein-mediated uptake of LCFA in heart and skeletal muscle (4–7). However, in none of these studies has it been possible to examine LCFA uptake in the absence of any oxidation and esterification. Nonetheless, three putative FA transporters have been found in these tissues. The 40–43 kDa plasma membrane fatty acid binding protein (FABPpm) appeared to be a peripheral membrane protein (8) suggesting that it cannot act as a classical transporter. Instead, it could be involved in LCFA trapping or assisting LCFA to surpass the unstirred fluid layer. The 88 kDa highly glycosylated fatty acid translocase (FAT), which was identified as the rat homolog of human glycoprotein IV or CD36 (9), is an integral membrane protein and probably has one or two membrane spanning regions (9, 10), so that it may act as a translocase rather than a LCFA pore. In contrast, fatty acid transport protein (FATP), an integral membrane protein with six putative membrane spanning regions, could function as a more classic transport protein (11).

In a previous study (4), FA uptake by freshly isolated cardiac myocytes was found to be largely protein-mediated (±80%). However, in the same study two lines of evidence indicated that LCFA uptake is tightly coupled to its subsequent metabolism. First, initial FA uptake could be modulated by agents that alter the activity of intracellular LCFA handling enzymes. Second, upon cellular entry LCFA were rapidly esterified. In order to obtain specific information about the mechanism of LCFA uptake, it is important to study this process in the absence of metabolism. Therefore, we chose giant sarcolemmal vesicles as a model of study. These vesicles are isolated by collagenase treatment in a KCl-containing medium. Compared to vesicles obtained with classic procedures, including homogenization

Abbreviations: FAT/CD36, fatty acid translocase; FABPpm, plasma membrane-bound fatty acid binding protein; FATP, fatty acid transport protein; LCFA, long-chain fatty acid.

[1] To whom correspondence should be addressed.

or sonication of tissue, the giant vesicles are at least an order of magnitude larger in diameter which gives them a much larger capacity for harboring substrate, thereby reducing the onset of efflux (12). In addition, these giant vesicles are oriented fully right-side out.

Giant sarcolemmal vesicles have been used to study uptake of glucose and lactate in skeletal muscles (12–14) However, giant vesicles have not been used as a model for investigation of substrate uptake by the heart. Our first aim, therefore, was to modify the procedure for isolation of vesicles from skeletal muscle for obtaining giant membrane vesicles from heart tissue. Second, we wanted to study the kinetics of LCFA uptake in both heart and muscle vesicles, and investigate whether differences in uptake of LCFA, if any, would parallel the well-known differences between the lipid-metabolizing capacities in these tissues. Third, we wished to examine which putative LCFA transporter(s) is (are) involved in LCFA uptake in heart and muscle. This was assessed *a*) by the use of specific inhibitors of LCFA uptake, and *b*) by comparing the differences in LCFA uptake by giant vesicles from heart and skeletal muscle with the expression of FABPpm, FAT/CD36 or FATP in the plasma membranes of these giant vesicles.

EXPERIMENTAL PROCEDURES

Materials

[9,10-^{3}H]palmitate, [^{14}C]mannitol, [^{3}H]d-glucose, [^{3}H]octanoate were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Bovine serum albumin (BSA) (fraction V, fatty acid-free) and trypsin were from Boehringer Mannheim Corp. (Indianapolis, IN). Phloretin and collagenase type VII were from Sigma Chemical Co. (St. Louis, MO). Collagenase type II was from Worthington Biochemical Co. (Lakewood, NJ). Sulfo-N-succinimidyl oleate (SSO) was a gift from Dr. N. A. Abumrad. A polyclonal rabbit antibody raised against rat hepatic membrane fatty acid binding protein (6) was used to detect FABPpm in heart and muscles vesicle membranes (gift from Dr. D. Sorentino). An affinity-purified rabbit polyclonal antibody described by Schaffer and Lodish (11) was used to detect FATP in heart and muscle vesicles (gift from Dr. J. Schaffer). A monoclonal antibody to CD36 (Cedarlane Laboratories, Hornby, Ontario, Canada) was used to detect FAT/CD36. In other studies CD36 has been shown to be the human analog to rat FAT (9). Western blot reagents were from Bio-Rad Laboratories (Hercules, CA), and the enhanced chemiluminescence (ECL) kit and anti-rabbit Ig, horseradish peroxidase were from Amersham Life Sciences (Oakville, Ontario, Canada).

Isolation of giant sarcolemmal vesicles

Giant vesicles from skeletal muscle were essentially isolated as previously described by us (14) and others (12, 13). Briefly, rat hindlimb muscles from both legs were cut into thin layers (1–3 mm thick) and incubated for 1 h at 34°C in 140 mm KCl/10 mm MOPS (pH 7.4), collagenase type VII (150 units/ml) and aprotinin (10 mg/ml). At the end of the incubation, the supernatant was collected and the remaining muscle tissue was washed with KCl/MOPS and 10 mm EDTA which resulted in a second supernatant. Both supernatants were pooled, and Percoll and aprotinin were added to final concentrations of 16% (v/v) and 10 mg/ml, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (w/v) and a 1-ml KCl/MOPS upper layer. This sample was centrifuged at 60*g* for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in KCl/MOPS, and recentrifuged at 900*g* for 10 min. The pellet was resuspended in KCl/MOPS to a protein concentration of 2–3 μg/μl.

For isolation of giant sarcolemmal vesicles from the heart, hearts were collected from 3–6 rats. With respect to the isolation procedure for skeletal muscle, the following modifications were made. The hearts were cut through the middle along the lengthwise axis, and incisions with a scalpel were made following the direction of the fibers. These heart slices were incubated with collagenase type II and 0.8 mm $CaCl_2$ for 90 min at 34°C. The final vesicle pellet was suspended at a protein concentration of 0.4–0.8 μg/μl.

Substrate transport studies

Palmitate uptake studies were performed by addition of 40 μl 0.1% BSA in KCl/MOPS containing radiolabeled 0.3 μCi [^{3}H]palmitate (concentration range, 4–69 μm) and 0.06 μCi [^{14}C]mannitol to 40 μl of vesicle suspension. The incubation was carried out for 15 s, unless otherwise specified. Palmitate uptake was terminated by the addition of 1.4 ml ice-cold KCl/MOPS containing 2.5 mm $HgCl_2$ and 0.1% BSA. The sample was quickly centrifuged in a microfuge at 12,000 rpm for 1 min. The supernatant was discarded, and radioactivity was determined in the tip of the tube. Non-specific uptake was measured by adding the stop solution prior to addition of the radiolabeled palmitate solution.

Glucose uptake studies were carried out by addition of 40 μl 0.1% BSA in KCl/MOPS containing 0.3 μCi [^{3}H]d-glucose (200 μm) and 0.06 μCi [^{14}C]mannitol. Incubations were carried out at room temperature for 1 min. The incubation was ended and radioactivity was determined as described above.

Octanoate uptake studies were performed by addition of 40 μl 0.1% BSA in KCl/MOPS containing 1.5 μCi [^{3}H]octanoate (15 μm) and 0.06 μCi [^{14}C]mannitol. Incubations were carried out at room temperature for 1 min. The incubation was ended and radioactivity was determined as described above except that the vesicle pellet was washed twice with stop solution.

Determination of palmitate esterification

When the stop solution was added at the end of the incubations, followed by the discarding of the supernatant, the inside of the tube was cleaned with cotton tips. Subsequently, the vesicle pellet was resuspended in 100 μl of a hypotonic solution consisting of 10 mm MOPS (pH 7.4) and 0.1% BSA and was frozen in liquid nitrogen. Upon thawing, the sample was centrifuged at 50,000 rpm at 4°C for 1 h using a Beckman Ti70 rotor. Both pellet and supernatant were collected. The pellet was washed with 50 μl hypotonic solution, and both supernatants were pooled. Both pellet and supernatant were subjected to lipid extraction by transferring them to 6 ml chloroform–methanol 2:1 in 10-ml plastic centrifugation tubes. These mixtures were shaken for 2 h at room temperature. 0.5 ml of methanol was added and the mixtures were centrifuged at 3800*g* for 10 min. The supernatant fluids were transferred to another plastic centrifugation tube containing 1.0 ml chloroform. Then, 1.5 ml 50 mm NaCl was added, and the mixtures were thoroughly vortexed and centrifuged at 3800*g* for 10 min. The upper aqueous phase and the interface were carefully aspirated. The chloroform phase was transferred to a glass tube, gently evaporated under a stream of N_2, and redissolved in 50 μl of chloroform–methanol 2:1. This sample was spotted on an oven-dried silica gel plate. Plates were placed in a sealed tank and subjected to thin-layer chromatography according to Gorski and Bonen (15). Parallel with the samples, a known quantity of [^{3}H]palmitate was subjected to lipid extraction and subsequently chromatographed, allowing correction for the loss of radioactivity in the course of this procedure.

Determination of enzyme activities and cytosolic FABP content

Frozen heart samples were homogenized as previously described (16). Heart homogenates and heart vesicle suspensions were subsequently analyzed for 5′ nucleotidase (17), lactate dehydrogenase (18), and citrate synthase activities (19). Heart type fatty acid binding protein (H-FABP) content was determined by a sandwich-type ELISA as previously described (20). Protein concentrations were determined by the bicinchoninic acid assay using BSA as a standard.

Western blotting

Giant vesicles (50 μg protein) were separated on 10% SDS-polyacrylamide gels (150 V for 1 h). Proteins were then transferred to Immobilon polyvinylidene difluoride membranes (100 V for 90 min). These membranes were gently shaken for about 16 h in buffer A (20 mm Tris-base, 137 mm NaCl, 0.1 m HCl, pH 7.5), 0.1% (v/v) Tween-20) and either 10% (w/v) non-fat dry milk (preceding anti-FABPpm antiserum, anti-CD36 antibodies or anti-GLUT4 antiserum) or 10% BSA (w/v) (preceding anti-FATP antibodies) at room temperature. Subsequently, membranes were incubated for 1 h with either a polyclonal antiserum against GLUT4 (1:7000), a polyclonal antiserum against FABPpm (1:2500), a monoclonal antibody to CD36 (1:500), or polyclonal anti-FATP antibodies (1:1500) in buffer A containing 10% non-fat dry milk, followed by 3 washes in buffer A and by incubation for 1 h with either rat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:7000, following anti-GLUT4 antibodies; 1:2500, following anti-FABPpm antiserum; 1:1500 following anti-FATP antibodies) or donkey anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (1:5000) in buffer B. Membranes were washed as before with buffer B. Detection occurred with an enhanced chemiluminescence detection method by exposing the membranes to film (Hyperfilm-ECL) at room temperature according to the instructions of the manufacturer. Film was developed and fixed in GBX fixer/replenisher. Protein band densities were obtained by scanning the films using an Abaton scanner connected to a Macintosh computer with appropriate software.

Data are represented as mean ± SEM. Statistical significance was calculated with Student's *t*-test.

RESULTS

Characterization of giant vesicles

In order to separate palmitate uptake by heart and skeletal muscle from subsequent metabolism, we chose to use giant sarcolemmal vesicles, derived from these tissue, as a model. Giant vesicles are an established model for substrate uptake studies in muscle, as substrate metabolism is absent and the vesicles are oriented right side out (12–14). However, no such model has been described for studies of the heart. Applying the established procedure for isolation of giant vesicles from hindlimb muscle but using a different type of collagenase, we succeeded in harvesting giant vesicular structures from heart tissue. These vesicles had a diameter of 15.2 ± 5.4 μm (n = 400), which makes them of similar size compared to giant vesicles from muscular origin (13.8 ± 6.2 μm; n = 100, in agreement with our earlier measurements (14)). Compared with heart homogenates, these vesicles showed a loss in citrate synthase (**Fig. 1**). The presence of both lactate dehydrogenase and cytoplasmic H-FABP (Fig. 1), demonstrated that the intravesicular space was of cytoplasmic origin. This is expected, because in the formation of vesicles, as proposed by Ploug et al. (12), cytoplasmic material will be trapped. The intravesicular concentration of H-FABP, serving as a sink for palmitate after its uptake, was measured to be 1.92 nmol/mg protein (muscle vesicles also contain H-FABP, 0.10 nmol/mg protein). The sarcolemmal nature of the vesicular membranes was indicated by the 28.5-fold enrichment in 5′-nucleotidase (Fig. 1). This corresponds well to the enrichment reported previously for skeletal muscles giant vesicles (14). Microscopic observations demonstrated that these vesicles from heart (and from muscle) were completely exclusive for trypan blue (not shown), indicating that they represent intact structures. Thus, based on morphological observations and marker protein analysis, heart giant vesicles bear striking similarities to giant vesicles of muscular origin.

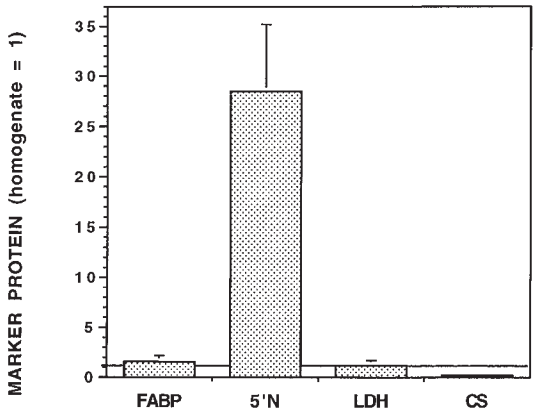

Fig. 1. Marker protein enrichment in heart sarcolemmal vesicles respective to heart tissue. Heart tissue was homogenized as described in Experimental Procedures, prior to enzyme determinations. Enzyme activity (5′ nucleotidase, 5′N; lactate dehydrogenase, LDH; citrate synthase, CS) or protein content (cytosolic H-FABP, FABP) in both homogenized tissue and vesicle suspensions were calculated as activity/mg protein or μg protein/mg protein. Subsequently, enzyme enrichment in vesicles was calculated by dividing obtained vesicle values by obtained tissue values, whereby the heart values were arbitrarily set at 1 (see horizontal line). Data are means ± SEM of 3 independent experiments.

Palmitate uptake kinetics by heart and muscle vesicles

Palmitate uptake was measured after 15 s of incubation, as this was well within the linear range for palmitate uptake in both heart and muscle vesicles. Studying palmitate uptake as function of the exogenous palmitate concentration can provide insight in the mechanism of permeation of palmitate across the sarcolemmal membranes of giant vesicles. After adjusting the data for the diffusive component of palmitate uptake (using procedures reported elsewhere (21, 22)), it is evident that both in the heart and in the muscle vesicles, palmitate uptake was saturable with increasing unbound palmitate concentrations (**Fig. 2**; the unbound palmitate concentrations were calculated according to Richieri, Anel, and Kleinfeld (23)). The K_m in

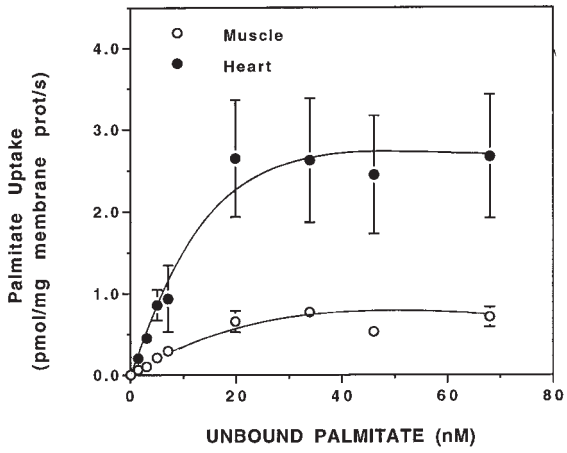

Fig. 2. Palmitate uptake by heart and muscle giant sarcolemmal vesicles as function of the exogenous palmitate concentration. Uptake of palmitate was expressed against the exogenous non-protein bound palmitate concentrations as calculated from the ratio between the added palmitate concentration and the concentration of BSA according to Richieri et al. (24), muscle ○; heart ●. Data are the means ± SEM of 3 experiments carried out with different vesicle preparations.

heart was estimated to be 9.7 nm, which is similar to that of mixed-muscle giant vesicles (K_m = 9.7 nm). In contrast, the V_{max} was 8-fold higher in heart giant vesicles, amounting to 2.7 pmol/mg protein per s compared to 0.34 mol/mg protein per s in skeletal muscle.

Fate of sequestered palmitate in giant vesicles

Clearly, giant heart and muscle vesicles are capable of sequestering palmitate. To investigate whether the radiolabeled palmitate is translocated across the sarcolemma and/or is partitioned in the plasma membrane, vesicles were fractionated into their cytosolic and plasma membrane compartments, after which these were analyzed for palmitate and its esterification products using thin-layer chromatography. In both heart and muscle vesicles (**Table 1**) radioactivity was completely recovered as unesterified palmitate in the intravesicular cytosolic fraction. In addition, there was no detectable production of $^{14}CO_2$, even up to 30 min after palmitate addition (data not shown). This indicates that all of the palmitate sequestered by the vesicles is translocated while none is metabolized. Similar results were obtained in giant vesicles obtained from skeletal muscle (data not shown).

Inhibition of palmitate uptake in heart and muscle vesicles by vesicular disruption, competitive substrates, or protein-modifying agents

When either muscle or heart vesicles were subjected to a cycle of freeze-thawing, palmitate uptake was reduced by more than 80% (**Fig. 3**). However, this observation does not allow any discrimination between passive diffusion or carrier-mediated transport as the likely mechanism, but it does suggest that palmitate transport depends on vesicular integrity.

Inhibition of substrate uptake by competitive substrates is generally considered to be evidence for carrier-mediated uptake. Competition experiments were carried out at a low palmitate/BSA ratio (0.27) and at high inhibitor concentrations, as described elsewhere (24). Addition of an excess of cold palmitate was able to compete with the radiolabeled palmitate for vesicular uptake (inhibition 51% in muscle vesicles and 54% in heart vesicles). Also, oleate, another FA, inhibited palmitate uptake substantially (inhibition 64% in muscle vesicles and 65% in heart vesicles). However, there was no significant effect of high concentrations of octanoate or glucose on palmitate uptake. The effects of competing substrates on palmitate uptake were similar in muscle and heart vesicles (**Fig. 4**). Apparently, palmitate is taken up by muscle tissues via an LCFA transport system which is specific for LCFAs, but which is unable to transport medium-chain FA and other physiologically important substrates.

In order to obtain additional evidence for the involvement of a protein component in this process, we tested

TABLE 1. Palmitate uptake into giant sarcolemmal vesicles and recovery of palmitate in lipid fractions in the plasma membrane and the intravesicular cytosolic fraction of heart and skeletal muscle giant sarcolemmal vesicles

Tissue	Palmitate Uptake into Vesicles	Palmitate Recovery in Lipid Fractions					
		Compartment	PL	MG	DG	FA	TG
	pmol/mg prot/15s						
Heart	24.9 ± 3.7	PM	ND	ND	ND	ND	ND
		IC	ND	ND	0.5 ± 0.3	25.9 ± 4.7	ND
Muscle	8.1 ± 0.5	PM	ND	ND	ND	ND	ND
		IC	ND	ND	ND	8.0 ± 0.3	ND

Palmitate uptake (15 s) was determined in giant sarcolemmal vesicles obtained from heart and muscle. In parallel experiments, palmitate recovery was determined by TLC after fractionating the vesicles into a plasma membrane (PM) compartment and an intravesicular cytosolic (IC) compartment as described in Experimental Procedures. Palmitate uptake and recovery data are expressed as pmol/mg protein/15 s. Recovery of radiolabeled palmitate, determined as the sum of radioactivity in all the collected lipid fractions, amounted to 106% using heart vesicles and 99% using muscle vesicles. Data are means ± SEM of 3 experiments carried out with different vesicle preparations. PL, phospholipids; MG, monoacylglycerol; DG, diacylglycerol, FA, unesterified fatty acid; TG, triacylglycerol; ND, not detectable.

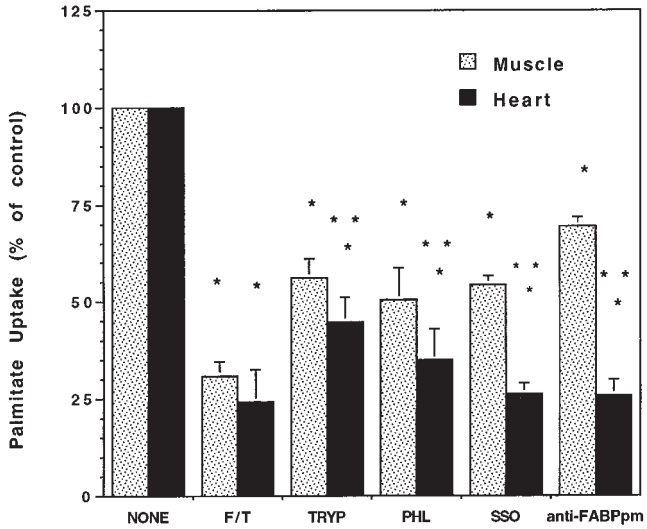

Fig. 3. Effect of physical and chemical modulations on palmitate uptake by heart and muscle vesicles. None, no additions; F/T, cycle of freeze-thawing; Tryp, 0.5% trypsin; Phl, 0.2 mm phloretin; SSO, 50 μm SSO; FABPpm, 8.1 μg/μl polyclonal anti-FABPpm antiserum. Data are the means ± SEM of 3–5 experiments carried out with different vesicle preparations. *Significantly different from control ($P < 0.05$). **Heart value significantly different from muscle value ($P < 0.05$).

the possible influence of a variety of compounds with the ability to interfere with the function of putative fatty acid transporters through different mechanisms of action. For this purpose, the following agents were used: *1*) trypsin, which hydrolytically removes peptide chains at the medium-facing leaflet of the sarcolemma; *2*) phloretin, a non-selective inhibitor of carrier-mediated membrane transport processes (25, 26); *3*) sulfo-N-succinimidyloleate (SSO), a fatty acid derivative with a highly reactive sulfo-N-succinimidyl moiety, which covalently modifies FA binding proteins (27); *4*) a polyclonal antiserum against FABPpm.

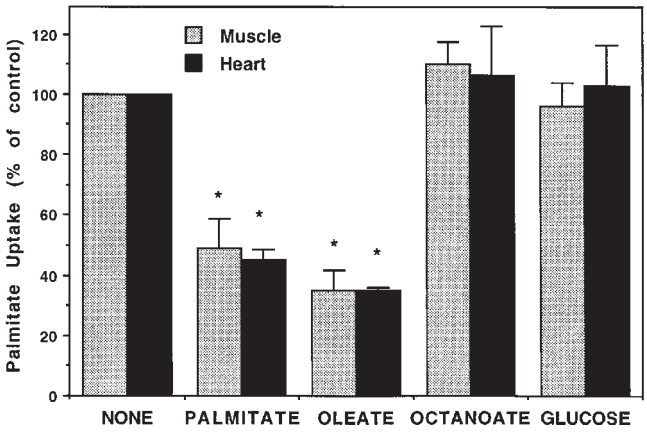

Fig. 4. Competition for uptake between radiolabeled palmitate uptake and unlabeled substrates into heart and muscle vesicles. None, no additions. Additions are 100 μm palmitate, 100 μm oleate, 100 μm octanoate, or 1.0 mm glucose. Data are the means ± SEM of 3 experiments carried out with different vesicle preparations.

Pretreatment of vesicles with 0.25% (w/v) trypsin or incubation of vesicles in the presence of 0.2 mm phloretin inhibited palmitate uptake into muscle vesicles by 45–50% and in heart vesicles by 55–65%. Pretreatment of vesicles with 50 μm SSO inhibited palmitate uptake in muscle vesicles by 50% and in heart vesicles by 70%. The polyclonal anti-FABPpm antiserum at a concentration at 4.0 μg protein/μl inhibited palmitate uptake into muscle vesicles by 35% and into heart vesicles by 75% (vesicles that were incubated with the same protein concentration of preimmune serum, served as reference point). With all inhibitors tested, the magnitude of the exerted effects was significantly greater in heart than in muscle vesicles.

In all cases, the concentrations of the inhibitors used were the minimal doses required for the maximal inhibitory effects. At the concentrations used, these inhibitors do not interfere with vesicle integrity, as was confirmed by the trypan blue exclusion test. Hydrophobic compounds such as phloretin and SSO were added together with their solvent dimethylsulfoxide (DMSO). This solvent did not affect palmitate uptake by heart giant vesicles at the concentrations used, which never exceeded 1.0% (v/v) (data not shown).

A remarkable feature of all these inhibitors is that they inhibited palmitate uptake substantially. While phloretin and trypsin are general transport inhibitors, SSO and anti-FABPpm supposedly restrict their action to LCFA transport. The mechanism of the inhibitory action of SSO is expected to be specific for FAT (27). This was confirmed in studies with giant vesicles obtained from liver (a tissue that does not express FAT (9)), because in these hepatic giant vesicles SSO did not inhibit palmitate uptake (**Fig. 5**). This also indicated that SSO did not inhibit FABPpm or FATP, both of which are present in liver (11, 28). Thus, given that SSO and FABPm antibodies selectively inhibit FAT and FABPpm, respectively, it was of interest to investigate whether or not the effect of SSO was additive to that of anti-FABPpm. Additivity was first tested at inhibitor concentrations exerting the maximal effect, both in heart and muscle giant vesicles. Palmitate uptake in heart vesicles was 3-fold higher than in skeletal muscle vesicles. In heart vesicles, the maximal inhibition by both compounds individually amounted to 70–80% (Fig. 5), while in muscle vesicles the maximal inhibitory effect was smaller, and amounted to 40–50%. Both in muscle and in heart vesicles, addition of both compounds simultaneously did not inhibit palmitate uptake further than their individual action. Interestingly, the non-inhibitable component of palmitate uptake in muscle vesicles was similar to that observed in heart vesicles. As each inhibitor was used at its own independent maximal inhibitory concentration, it could be argued that failure to observe an additive inhibitory effect of the two inhibitors could be due to the maximal inhibition of only one of the inhibitors. Therefore, we also investigated the combined action of both inhibitors using concentrations at which they independently exerted a partial inhibition on palmitate uptake. This again demonstrated that the effects of both inhibitors were not additive (Fig. 5).

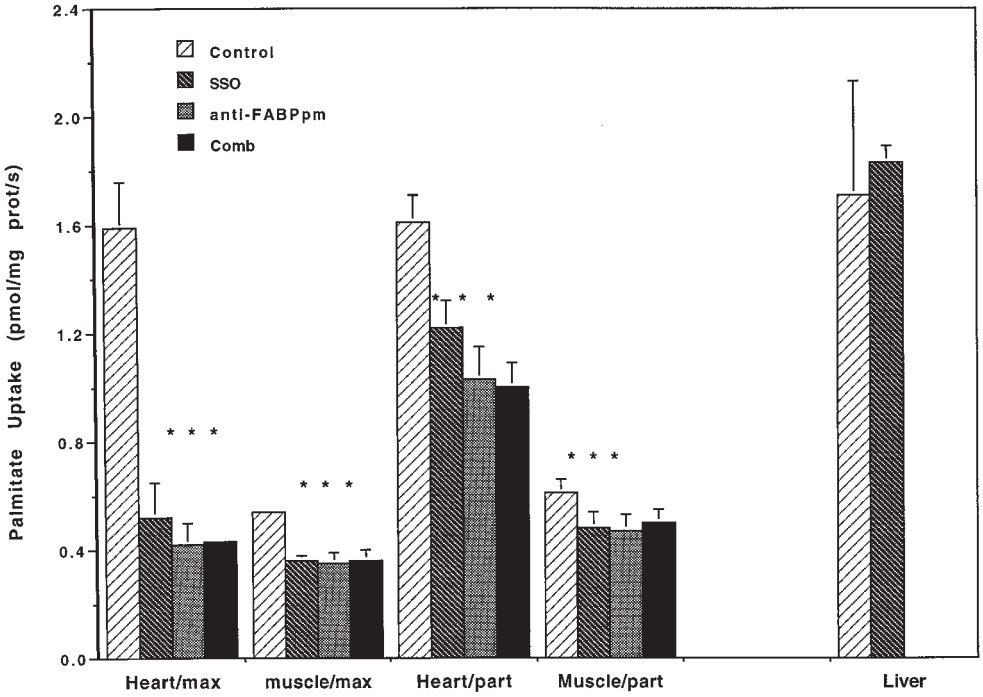

Fig. 5. Investigation of additivity of the effects of SSO and anti-FABPpm on palmitate uptake by heart and muscle giant vesicles. Heart/max and muscle/max: addition of inhibitors at maximally inhibitory doses (50 μm SSO 50 μm; 8.1 μg/μl anti-FABPpm antiserum) to heart and muscle vesicle. Heart/part and muscle/part: addition of inhibitors at partially inhibitory doses (12 μm SSO; 1.3 μg/μl anti-FABPpm antiserum) to heart and muscle vesicles. Comb: the combined addition of SSO and anti-FABPpm antiserum, either at maximal inhibitory concentrations or submaximal inhibitory concentrations to heart and muscle vesicles. The lack of effect of SSO on palmitate uptake in liver giant vesicles is also shown (liver does not express FAT/CD36). Data are the means ± SEM of 3–4 experiments carried out with different vesicle preparations. *Significantly different from control ($P < 0.05$).

Uptake of glucose and octanoate by heart and muscle vesicles

In order to test the specificity of the effects exerted by the applied inhibitors on cellular uptake of LCFA, their effects on uptake of glucose and octanoate were monitored under identical incubation conditions. Glucose uptake by muscle vesicles amounted to 53 pmol/min per mg protein, and is thus of the same order of magnitude as measured by Pilegaard, Juel, and Wibrand (29). SSO and anti-FABPpm had no effect on glucose uptake by both heart and muscle giant vesicles, while the non-selective inhibitor phloretin was able to inhibit more than 90% of the glucose uptake (**Fig. 6**).

Uptake of octanoate, a medium-chain FA, was much slower than that of palmitate: in muscle vesicles octanoate uptake was 2–3% of that of palmitate, while in heart vesicles this was less than 1%. No significant difference in octanoate uptake was measured between heart and muscle vesicles. Neither SSO nor anti-FABPpm had an effect on octanoate uptake by heart vesicles (**Fig. 7**).

Presence of putative LCFA transporters in heart and muscle vesicles

The data above strongly suggest that palmitate uptake is protein-mediated in heart and muscle vesicles. We subsequently determined which putative LCFA transporters are present in the plasma membrane of the vesicles obtained from heart and from red and white skeletal muscles. With the use of a polyclonal antiserum, which was also used in the inhibition studies, FABPpm was observed at 43 kDa (**Fig. 8**). It is present in heart vesicles as well as in muscle vesicles. In heart vesicle membranes the amount of FABPpm is higher than in red muscle or white muscle vesicle membranes (Fig. 8).

Incubating blots with human anti-CD36 antibodies resulted in labeling of a band with a molecular mass of 88 kDa (Fig. 8). This is the highly glycosylated fatty acid translocase (FAT) (9), which was identified as the rat homolog of human glycoprotein IV or CD36, and is referred to as FAT/CD36 (9). The presence of this putative transporter in heart vesicle membranes is also greater than in red and white muscle vesicle membranes (Fig. 8).

We observed that FATP, in contrast to the two other fatty acid transport proteins, is most prominently present in the white muscle vesicles. Heart vesicle membranes contained 10-fold less FATP than white muscle vesicle membranes (Fig. 8).

Because palmitate uptake can be measured in vesicles from heart and from red and white skeletal muscles, it was possible to compare the relative expression of fatty acid transport proteins with the rates of palmitate transport (**Fig. 9**). This demonstrated that vesicle FABPpm and

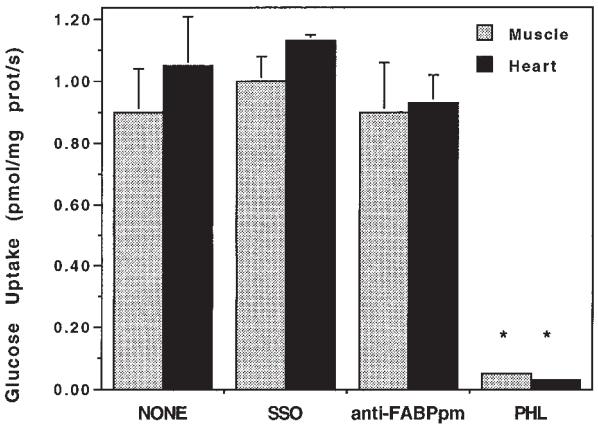

Fig. 6. Glucose uptake by heart and muscle giant vesicles: effect of inhibitors. None, no additions; SSO, 50 μm SSO; FABPpm, 8.1 μg/μl anti-FABPpm antiserum; 0.2 mm phloretin. Glucose uptake in heart vesicles was 15% higher than in muscle vesicles. This is consistent with differences in their plasma membrane GLUT4 content in the vesicles as was determined with Western blotting (data not shown). In contrast to vesicles, total GLUT-4 in heart is much greater than in muscle (data not shown). Data are the means ± SEM of 3 experiments carried out with different vesicle preparations *Significantly different from control ($P < 0.05$).

FAT/CD36 were positively related to the rates of palmitate uptake by the giant vesicles. In contrast, FATP was negatively correlated with the rate of FA transport into the vesicles (Fig. 9).

DISCUSSION

Validation of the methods

It has been suggested that formation of giant vesicles from the plasma membrane is the result of a disequilibrium between cellular exocytosis and endocytosis, caused by high extracellular KCl concentrations (12). The action of collagenase on the extracellular collagen matrix is then probably important for creating space for the excision of vesicles from the plasma membrane. As the extracellular matrix in the heart is made of a firmer constitution than that of the skeletal muscle, a different type of collagenase is required than the one that is used in the procedure for obtaining giant vesicles from skeletal muscle. Collagenase type II, commonly used in the isolation of cardiac myocytes from the heart provides a much higher yield of giant vesicles than does type VII. These cardiac giant vesicles could serve as a new model for studies on plasma membrane-related processes in heart research. The tremendous advantage of giant vesicles over vesicles obtained with classical procedures (i.e., disruption of tissue by homogenization or sonication) is that these giant vesicles are fully right side out (data not shown, see also ref. 12), and that the limiting vesicle membrane is of sarcolemmal origin.

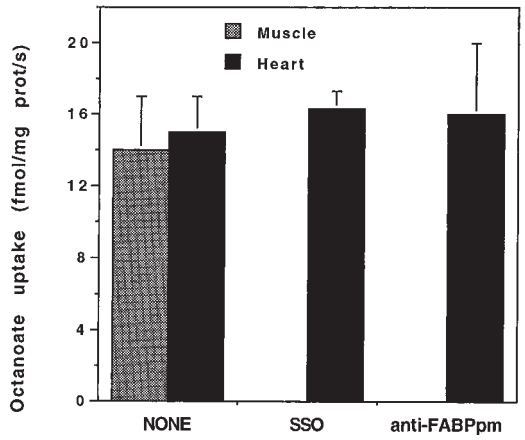

Fig. 7. Octanoate uptake by heart and muscle giant vesicles: effect of inhibitors. None, no additions; SSO, 50 μm SSO; FABPpm, 8.1 μg/μl anti-FABPpm antiserum. Data are the means ± SEM of 3 experiments carried out with different vesicle preparations; *significantly different from control ($P < 0.05$).

The heart giant vesicles, obtained in our studies, are luminescent spherical structures surrounded by membranes. In this respect they have the same microscopic appearance as muscle giant vesicles. In addition, they possess the same diameter, so that differences in substrate uptake cannot be explained due to size differences. Their intactness is apparent from their ability to exclude trypan blue. Heart vesicles showed a loss in citrate synthase, but were enriched in 5′-nucleotidase. In addition, levels of lactate dehydrogenase and H-FABP are retained. The loss in citrate synthase indicates that mitochondria, and thus the β-oxidizing machinery, are absent. The enrichment factor of the plasma membrane marker 5′-nucleotidase (28.5 fold) is similar to that reported for skeletal muscle vesicles (14, 28). The slight enrichment of both cytoplasmic proteins is likely due to the absence of contractile proteins (13), as the contractile apparatus accounts for a considerable portion of the protein mass in cardiac myocytes. The retention of FABP is also evidence that these giant membrane vesicles are derived from cardiac myocytes and not from endothelial cells or fibroblasts, as these cell types lack H-FABP (20). The intravesicular concentration of cytoplasmic H-FABP was measured to be 1.92 nmol/mg protein. Because we established that all of the palmitate sequestered by the vesicles is taken up but not metabolized, it appears that H-FABP serves as a sink for LCFAs. We calculated that at the K_m of palmitate uptake only about 2% of the H-FABP was occupied and at maximal rates of palmitate uptake only 10% of the H-FABP was occupied. Thus, it is evident that under all conditions, the amount of cytosolic FABP is large enough to accommodate all of the sequestered palmitate, and, therefore, is unlikely to be responsible for the observed saturability of transport. Similar calculations in muscle vesicles indicated that cytosolic FABP can also accommodate all of the sequestered palmitate (i.e., maximal H-FABP occupancy ⩽20%).

Evidence for protein-mediated transport

As already mentioned, giant membrane vesicles offer a unique possibility as opposed to cellular systems to study FA uptake in the absence of metabolism. Using these vesi-

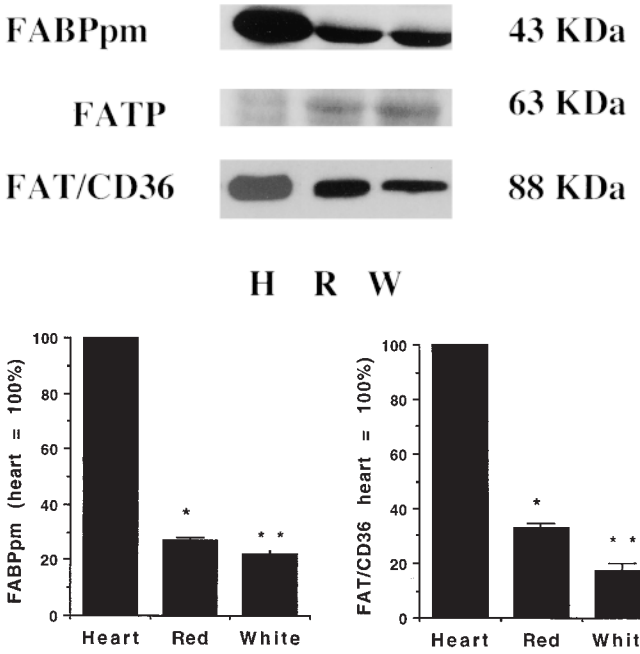

Fig. 8. FABPpm, FAT/CD36, and FATP proteins in heart and in red and white muscle vesicles. Western blots were based on 30 μg vesicle protein per lane. Representative blots are shown (top). For quantification of transporters, data are the means ± SEM of 4–8 experiments carried out with different vesicle preparations. The heart data for FABPpm and FAT/CD36 (bottom) were set to 100% and the data from the red and white vesicles were set relative to this; *heart vs. red muscle ($P < 0.05$); **red muscle vs. white muscle ($P < 0.05$).

cles, evidence was obtained for an important role for a protein component in the bulk uptake of LCFA. This evidence was based on our observations that palmitate uptake was saturable, inhibitable by general carrier-mediated membrane transport inhibitors as well as by anti-FABPpm antiserum and SSO, and that other LCFA, but not medium-chain FA, were able to compete for uptake.

Interestingly, the V_{max} of heart vesicles for palmitate transport was 8-fold greater than that of muscle vesicles, while the K_m for palmitate uptake in heart and muscle vesi-

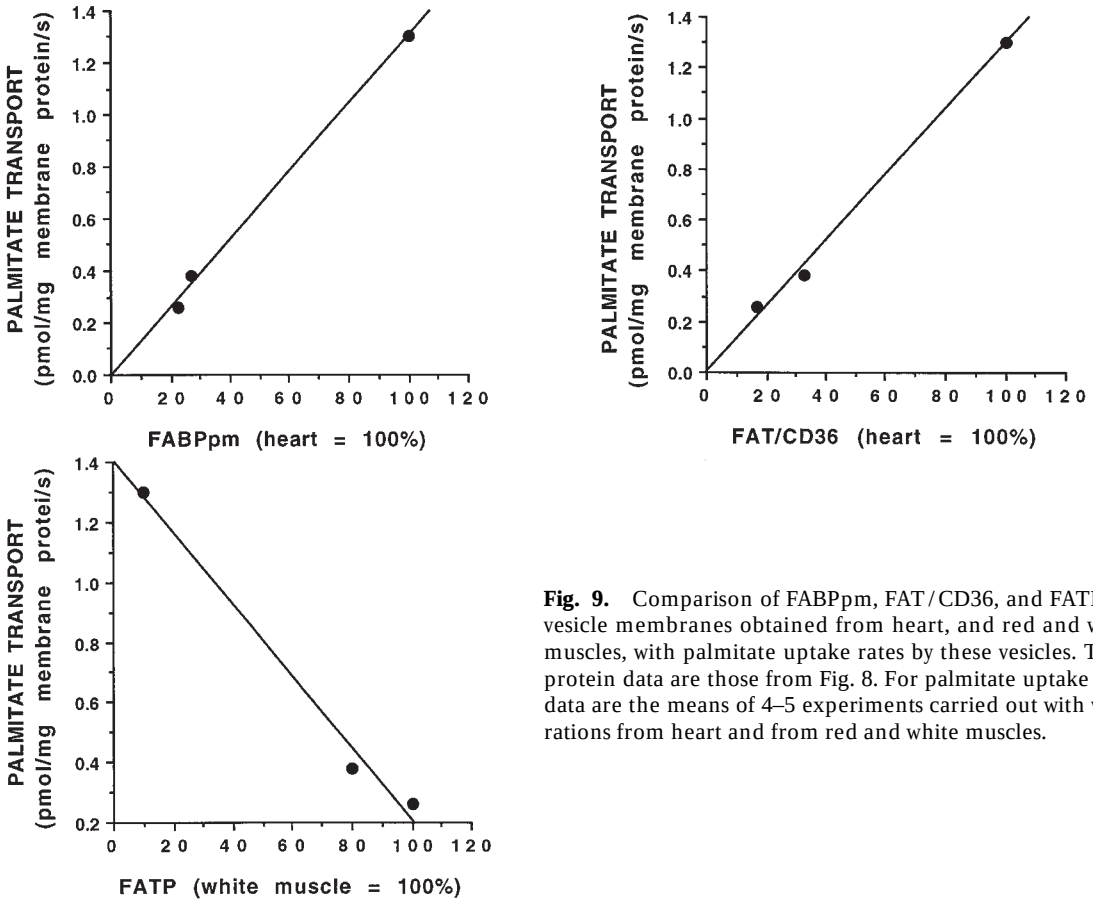

Fig. 9. Comparison of FABPpm, FAT/CD36, and FATP proteins, in vesicle membranes obtained from heart, and red and white skeletal muscles, with palmitate uptake rates by these vesicles. The transport protein data are those from Fig. 8. For palmitate uptake experiments, data are the means of 4–5 experiments carried out with vesicle preparations from heart and from red and white muscles.

cles was identical (9.7 nm). As heart and muscle vesicles are similar in size, morphological appearance, and enzyme enrichment pattern, the observed difference in palmitate uptake kinetics could not be explained by differences in any of these parameters. Furthermore, from a mechanistic point of view it is very unlikely that the 8-fold greater uptake of palmitate by heart compared to muscle vesicles could be attributed to passive diffusion. Several observations in this study provide support for this notion: *a*) the non-inhibitable component in the inhibition experiments, which is likely due to passive diffusion, was similar in heart and muscle vesicles, and *b*) the uptake of octanoate, which is known to enter adipocytes (24), fibroblasts (30), and enterocytes (31) via passive diffusion, occurs at a similar rate in heart and muscle vesicles. The similar K_m for palmitate uptake in heart and muscle suggests that the same transport protein or proteins must be operable in both tissues. Combined with the observed V_{max} values, one could conclude that 8-fold greater quantities of the same transporter(s) is (are) present in heart compared to muscle.

Identification of functionally important transporters

We used SSO, an inhibitor of FAT/CD36, and anti-FABPpm to examine palmitate uptake. The specificity of SSO for blocking FAT/CD36 was established in separate experiments using giant vesicles from liver, a tissue that does not express FAT/CD36 (9). In liver vesicles SSO did not inhibit fatty acid uptake. In muscle and heart, neither SSO nor anti-FABPpm inhibited glucose or octanoate uptake by vesicles, while both inhibited palmitate uptake. This suggests that both FAT/CD36 and FABPpm are functionally important in LCFA uptake in the respective tissues. When both agents were administered simultaneously, the observed inhibition was not greater than the inhibitory action of these agents individually. A possible interpretation of this lack of additivity could be that FAT/CD36 and FABPpm cooperate in a concerted fashion to translocate LCFA across the sarcolemma. Whether this means that they could be regarded as two protein-components of a single LCFA transport system in the sarcolemma of heart and skeletal muscle is not known. This awaits more definitive experimental verification.

The greater content of FABPpm in heart vesicles compared to red and white muscle vesicles is congruent with the differences in the rates of palmitate uptake into vesicles obtained from heart and different types of muscles. A similar relationship was present between FAT/CD36 and palmitate uptake in heart and muscle vesicles. This suggests that both FABPpm and FAT/CD36 are physiologically important fatty acid transport proteins in heart and muscle. The greater quantities of these proteins in heart compared to muscle is in agreement with the differences in the capacities for fatty acid metabolism in these respective tissues (heart > red muscle > white muscle). A parallelism between oxidative capacity and expression of putative LCFA transporters in different types of muscle has previously been reported with respect for FAT/CD36 and FABPpm (20, 32, 33).

Surprisingly, our studies have revealed that another putative LCFA transport protein, FATP, was inversely correlated with the palmitate transport rates into heart and into red and white muscle vesicles. FATP content was 10-fold lower in heart vesicle membranes than in muscle vesicle membranes, despite an 8-fold greater maximal palmitate transport rate by heart vesicles. This strongly suggests that FATP is not involved in the bulk uptake of LCFA by heart and muscle, although this does not implicate that FATP is not a LCFA transporter. Perhaps it has an as yet undefined role in muscle and heart, possibly being activated upon a change in phosphorylation state, since rat heart FATP contains a number of potential phosphorylation sites (34).

Conclusion

During the last decade, several plasma membrane proteins were identified as putative LCFA transporters (for review, see ref. 2). Among these, FABPpm (30), FAT/CD36 (35), and FATP (11) were overexpressed in cell lines. In all cases, overexpression resulted in an increase in cellular LCFA uptake, indicating that each of these proteins acts as an LCFA transporter. However, in none of these studies has it yet been possible to discern which of these putative transport proteins is physiologically important. In this regard the present studies, using giant sarcolemmal vesicles, suggest that FABPpm and FAT/CD36 are physiologically relevant transport proteins for LCFA in heart and skeletal muscle.

Comparisons between cardiac myocytes (4) and cardiac sarcolemmal vesicles (present study), where unidirectional palmitate influx is studied in the presence or absence of metabolism, respectively, demonstrate that both systems display identical uptake kinetics (myocyte K_m: 7.5 nm versus vesicle K_m: 9.7 nm). Both systems also display similar inhibitory effects of trypsin, phloretin, and SSO on palmitate uptake (inhibition range from 50 to 80%). This suggests that palmitate translocation across the sarcolemma is the rate-limiting step in palmitate uptake and metabolism by the heart.

Although some studies have shown that the expression of only one of the putative fatty acid transport proteins can increase fatty acid transport (e.g., FATP (11), FABPpm (30), or FAT (35)), another recent study has shown that transfecting a heart muscle cell line (H9c2) with FAT did not increase fatty acid uptake (36). It was suggested that these cells may lack a protein or set of proteins that act as an obligatory partner with FAT in transporting LCFAs. We speculate that this obligatory partner may be FABPpm, which could be absent in H9c2 cells. Based on the inhibition of LCFA uptake when FABPpm and FAT/CD36 are blocked, and based on the known structural properties of these transporters, with FABPpm being a peripheral membrane protein at the extracellular surface (8) and FAT/CD36 being an integral membrane protein having one or two membrane spanning region (9, 10), we want to propose the following model of concerted action of both transporters in sarcolemmal LCFA translocation. FABPpm extracts LCFA from the medium, and subsequently delivers them to FAT/CD36, which in turn translocates the LCFA across the sarcolemma. Whether FABPpm and FAT are actually in physical contact at some stage during this translocation event, is not known. This proposed model requires experimental verification.

In conclusion, we have established that palmitate uptake in giant heart and muscle sarcolemmal vesicles is largely protein-mediated, and that the 8-fold higher uptake in heart vesicles compared to muscle vesicles is due to the greater abundance of FABPpm and FAT/CD36, which may act as components of a single transport system in the translocation of LCFA across the sarcolemma. Based on the inverse relationship between FATP and palmitate transport rates in our studies, we feel that this transport protein may not be important for the uptake of LCFAs in these tissues.

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