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# Differential regulation of cardiac glucose and fatty acid uptake by endosomal pH and actin filaments

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<sup>1</sup>Department of Molecular Genetics, Maastricht University, Maastricht, the Netherlands; <sup>2</sup>German Diabetes Center, Düsseldorf, Germany; <sup>3</sup>Department of Endocrinology, Diabetes Center, VU university Medical Center, Amsterdam, the Netherlands; and <sup>4</sup>Department of Human Health and Nutritional Science, University of Guelph, Guelph, Ontario, Canada

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**Steinbusch LK, Wijnen W, Schwenk RW, Coumans WA, Hoebbers NT, Ouwers DM, Diamant M, Bonen A, Glatz JF, Luiken JJ.** Differential regulation of cardiac glucose and fatty acid uptake by endosomal pH and actin filaments. *Am J Physiol Cell Physiol* 298: C1549–C1559, 2010. First published April 7, 2010; doi:10.1152/ajpcell.00334.2009.—Insulin and contraction stimulate both cardiac glucose and long-chain fatty acid (LCFA) uptake via translocation of the substrate transporters GLUT4 and CD36, respectively, from intracellular compartments to the sarcolemma. Little is known about the role of vesicular trafficking elements in insulin- and contraction-stimulated glucose and LCFA uptake in the heart, especially whether certain trafficking elements are specifically involved in GLUT4 versus CD36 translocation. Therefore, we studied the role of coat proteins, actin- and microtubule-filaments and endosomal pH on glucose and LCFA uptake into primary cardiomyocytes under basal conditions and during stimulation with insulin or oligomycin (contraction-like AMP-activated protein kinase activator). Inhibition of coat protein targeting to Golgi/endosomes decreased insulin/oligomycin-stimulated glucose (–42%/–51%) and LCFA (–39%/–68%) uptake. Actin disruption decreased insulin/oligomycin-stimulated glucose uptake (–41%/–75%), while not affecting LCFA uptake. Microtubule disruption did not affect substrate uptake under any condition. Endosomal alkalinization increased basal sarcolemmal CD36 (2-fold), but not GLUT4, content, and concomitantly decreased basal intracellular membrane GLUT4 and CD36 content (–60% and –62%, respectively), indicating successful CD36 translocation and incomplete GLUT4 translocation. Additionally, endosomal alkalinization elevated basal LCFA uptake (1.4-fold) in a nonadditive manner to insulin/oligomycin, and decreased insulin/oligomycin-stimulated glucose uptake (–32%/–68%). In conclusion, 1) CD36 translocation, just like GLUT4 translocation, is a vesicle-mediated process depending on coat proteins, and 2) GLUT4 and CD36 trafficking are differentially dependent on endosomal pH and actin filaments. The latter conclusion suggests novel strategies to alter cardiac substrate preference as part of metabolic modulation therapy.

cardiac substrate uptake; CD36 translocation; vesicular trafficking; coat proteins

GLUCOSE AND LONG-CHAIN FATTY ACIDS (LCFA) are the major fuels for cardiac contractile activity. Glucose transporter-4 (GLUT4) is the major glucose transporter in the heart, and a number of LCFA transporters have been implicated in cardiac LCFA uptake, such as CD36 (35), fatty acid transport protein (FATP)1 (7), and FATP6 (16). To adequately respond to rapidly varying metabolic demands, the heart is able to acutely

regulate the uptake of both substrates, i.e., via reversible translocation of GLUT4 and CD36 from intracellular membrane compartments (endosomes) to the sarcolemma (35). In contrast to CD36, FATP1 and FATP6 do not traffic between intracellular storage compartments and the sarcolemma in the heart and have only a minor contribution to basal LCFA uptake (18, 19). In addition, FATP1 has been shown to translocate to the cell surface of adipocytes (38), showing that trafficking processes are tissue specific. The most important physiological stimuli to induce cardiac GLUT4 and CD36 translocation are 1) a rise in circulating levels of insulin and 2) an increase in contraction frequency. Hence, the processes of GLUT4 and CD36 translocation bear close resemblance to each other, based on their responsiveness to these same physiological stimuli, and also to various pharmacological stimuli (25). Additionally, the same signaling enzymes are involved in the translocation of both transporters. Specifically, phosphatidylinositol 3-kinase and Akt/protein kinase B are involved in insulin-stimulated translocation of both GLUT4 and CD36, and AMP-activated protein kinase (AMPK) as well as its upstream kinase, the tumor suppressor protein LKB1, are involved in their contraction-stimulated translocation (17). Whether this similarity between GLUT4 and CD36 translocation also extends to the subcellular machineries involved in translocation of each of these transporters remains unexplored.

Because stimulus-induced GLUT4 translocation has been described since the early 1980s, and stimulus-induced CD36 translocation has only recently begun to be examined, there is much more information about the subcellular machinery involved in GLUT4 translocation (4, 32) compared with the CD36 translocation machinery, of which essentially nothing is known (35). Parts of the subcellular GLUT4 translocation machinery are formed by 1) coat proteins, involved in packaging of GLUT4 vesicles during their budding from the endosomal membranes; 2) cytoskeletal filaments along which the subcellular GLUT4 vesicles migrate toward the cell surface; and 3) the recycling endosomes, involved in storage of GLUT4.

Several coat proteins have been proposed to function in GLUT4 recycling: caveolin, clathrin, and  $\beta$ -COP. Caveolin (33, 37) and clathrin (2), predominantly plasmalemmal, have been proposed to play a role in initiation of GLUT4 endocytosis, although this has been disputed by others (31). The fungal metabolite brefeldin-A is known to interfere with the retrograde transport from the Golgi apparatus to the endoplasmic reticulum through inhibition of coat proteins, e.g.,  $\beta$ -COP and Arf1p, involved in formation of transport vesicles resulting

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in impaired sorting from this network (14, 41). Also mistargeting of clathrin/adaptor protein complexes might play a role in this process (23). In addition to the Golgi, the endosomal compartment, presumably involved in storing GLUT4 and CD36, also depends on COP proteins and clathrin for its structural organization, and it will collapse upon brefeldin-A treatment (45). The use of brefeldin-A in studying insulin-stimulated GLUT4 translocation in rat adipocytes also yielded controversial observations: inhibition (22), or no inhibition (21). Hence, the issue as to which coat proteins are necessary for regulation of GLUT4 translocation and what their role is in cardiomyocytes is yet unresolved. To date, brefeldin-A has not yet been used to investigate the regulation of GLUT4 and CD36 translocation in the heart.

It is well established that cortical filamentous actin plays a role in insulin-induced GLUT4 translocation (9, 43). Specifically, insulin-induced GLUT4 translocation depends on continuous cycles of actin polymerization/depolymerization, because both actin stabilizing and destabilizing agents are inhibitory to this process (20). Actin structures support ruffling of the plasma membrane, important for local concentration of t-SNAREs (42). Controversy exists about the role of the microtubules in GLUT4 translocation, because microtubule-disrupting agents have been reported to inhibit insulin-induced GLUT4 translocation (29) and not to affect this process (1, 36). None of these filament disruptors have been used to examine insulin-induced GLUT4 translocation in cardiomyocytes, or contraction-induced GLUT4 translocation or stimulus-induced CD36 translocation in any cell type.

One of the key features of endosomes is their acidic interior, a feature which they share with lysosomes, but not (or, to a lesser extent) with other organelles (15, 40). Therefore, this low endosomal pH is thought to be important for endosomal functioning, as has been established in receptor-mediated endocytosis. Endosomal acidification is achieved by the proton-pumping action of the vesicular-type ATPase (v-ATPase or  $V_1V_0$ -ATPase), a large multisubunit complex specifically localized to endosomes and lysosomes (15). On the basis of pharmacological agents able to disrupt the pH gradient between endosomes and cytoplasm, as investigated in adipocytes, skeletal muscle, and heart, the role of endosomal pH in regulation of subcellular GLUT4 localization is controversial. For example, it has been reported that bafilomycin-A, a specific inhibitor of the v-ATPase, stimulates (6) or inhibits GLUT4 translocation (8, 46). Furthermore, dissipation of the endosomal/cytoplasmic pH gradient with the proton ionophore monensin did not affect insulin-induced GLUT4 translocation (32). However, no studies have yet been undertaken to assess the role of endosomal functioning in contraction-induced GLUT4 translocation in the heart. With respect to CD36, the sole piece of evidence about the role of endosomes in CD36 translocation comes from our earlier work in skeletal muscle, in which we detected an intracellular membrane fraction enriched in CD36 and the transferrin receptor, an established component of the recycling endosomes (3).

The present study was designed to examine the involvement of components of the vesicular trafficking machinery, i.e., vesicular coat proteins, cytoskeletal filaments, and endosomal pH, in the regulation of LCFA uptake (through CD36 translocation), and evaluate the possible similarities and/or differences with the regulation of glucose uptake (through GLUT4 translocation).

We chose a pharmacological approach to interfere with the functioning of coat proteins (using brefeldin-A), cytoskeletal proteins (using latrunculin-B and colchicine), and endosomal v-ATPase (using bafilomycin-A and monensin) rather than a genetic approach. We chose this approach because 1) brefeldin-A, latrunculin-B, colchicine, bafilomycin-A, and monensin are frequently used established inhibitors; and 2) coat proteins, cytoskeletal proteins, and endosomal v-ATPase are involved in multiple crucial cellular functions, so that their genetic ablation would seriously hamper cell viability. In contrast to a genetic approach, a pharmacological approach to block functioning of selected trafficking proteins can be undertaken in a relative short time span ( $\leq 60$  min) without detrimental effects on cell survival.

The involvement of these trafficking components in the regulation of substrate uptake was studied in regard to insulin as well as contraction-stimulation. To mimic contraction signaling, we used the  $F_1F_0$ -ATPase inhibitor oligomycin, which, just like increased muscular work, induces AMPK activation through an increase of the AMP-to-ATP ratio (25). Given that different signaling routes appear to be involved in contraction-induced versus insulin-induced transporter translocation, we also analyzed whether these differences can be attributed to vesicular coat proteins, cytoskeletal filaments, or the endosomal pH. For this purpose, it was investigated whether agents disrupting  $\beta$ -COP functioning (brefeldin-A), actin filaments (latrunculin B), and microtubules (colchicine) or endosomal pH (monensin and bafilomycin A) were able to alter insulin- and oligomycin-induced glucose and LCFA uptake.

## MATERIALS AND METHODS

**Materials.** Palmitic acid and [ $^3$ H]deoxyglucose were obtained from GE Healthcare (Little Chalfont, UK). [ $^3$ H]chloroquine was from Moravak Biochemicals (Brea, CA). Laminin, insulin, oligomycin, monensin, bafilomycin-A, brefeldin-A, latrunculin-B, and colchicine were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) (fraction V), dependent on the application, was derived from MP Biomedicals (Irvine, CA) (for cell isolation and incubation purposes), or from Sigma (other purposes). Collagenase type II was from Worthington (Freehold, NJ). CD36 was detected with a monoclonal antibody (MO25) directed against human CD36, kindly provided by Dr. N. N. Tandon (Thrombosis and Vascular Biology Laboratory, Otsuka America Pharmaceutical, Rockville, MD). Rabbit anti-mouse immunoglobulin horseradish peroxidase and swine anti-rabbit immunoglobulin horseradish peroxidase were obtained from DAKO (Glostrup, Denmark). Nonfat dry milk and Western blot reagents were from Bio-Rad (Hercules, CA) and the enhanced chemiluminescence kit was from Amersham Pharmacia Biotech (Buckingham, UK). The fluorescently labeled monoclonal antibody against the Golgi matrix 130-kDa protein (GM-130) was from BD Biosciences (San Jose, CA), and the anti- $\alpha$ -tubulin antibody was from Iowa Hybridomabank (clone E7) (Iowa City, IA). Texas red-conjugated phalloidin and DAPI were from Molecular Probes (Eugene, OR), and goat-anti-mouse-TRITC was purchased from DAKO. Sulfo-*N*-succinimidylolate (SSO) is routinely synthesized in our laboratory, as has been previously described (39). Purity of these compounds was confirmed with infrared spectroscopy (kindly performed by Dr. van Genderen, Eindhoven Technical University, Eindhoven, the Netherlands).

**Experimental animals.** Male Lewis rats, 200–250 g, were purchased from Charles River Laboratories. All animal procedures were approved by the Experimental Animal Committee of Maastricht University, Maastricht, the Netherlands.

*Isolation of cardiomyocytes followed by measurement of substrate uptake.* Cardiomyocytes were isolated from male Lewis rats (200–250 g) using a Langendorff perfusion system, as previously described (28). Only when >80% of the cells had a rod-shaped appearance and excluded Trypan blue were they used for subsequent tracer uptake studies. Uptake of [ $^{14}\text{C}$ ]palmitate (in complex with BSA) and [ $^3\text{H}$ ]deoxyglucose into cardiomyocytes incubated in cell incubation medium was measured as previously described (28). Substrate uptake was modulated by insulin and various pharmacological inhibitors (see figures for exact concentrations and incubation times). All these inhibitors were dissolved in DMSO, of which the final concentration in the cell suspensions never exceeded 0.5%. At this concentration, DMSO did not affect cellular substrate utilization. All agents were added 15–45 min before measurement of substrate uptake at the minimal concentration at which they exerted the maximal effect. None of these agents, alone or in combination and including SSO, were found to affect the percentage of cells that 1) were rod-shaped and 2) excluded Trypan blue, as parameters of cellular integrity.

*Isolation of giant vesicles followed by measurement of substrate uptake.* Giant vesicles were prepared from rat heart, as described previously (27). Uptake of [ $^9,^{10}\text{-}^3\text{H}$ ]palmitate (in complex with BSA) and [ $^3\text{H}$ ]D-glucose were measured as described previously (27). All pharmacological inhibitors used were added to giant vesicles 20 min before determination of palmitate or glucose uptake (see figures for exact concentrations and incubation times).

*[ $^3\text{H}$ ]chloroquine uptake assay.* Cardiomyocytes (0.5–1.0 mg wet mass/ml) were preincubated with or without pharmacological agents similarly as in the substrate uptake assay. At the start of the incubation, 4  $\mu\text{l}$  [ $^3\text{H}$ ]chloroquine (250  $\mu\text{Ci/ml}$  diluted 1:125 in Milli-Q) were added. After a 30-min incubation period, 1.5 ml cell suspension was pulse centrifuged. The supernatant was aspirated, and the lower part of the cup, containing the cell pellet, was snipped and vortexed in 5 ml Opti-Fluor (Perkin Elmer, Waltham, MA) until the pellet was completely dissolved. Samples were counted with a Wallace liquid scintillation counter.

*Microscopy.* Cells were seeded on laminin-coated coverslips. After attachment, cells were washed and incubated with the corresponding inhibitors in cell incubation medium. Cells were fixed with (ice-cold) methanol, washed for 15 min with 0.2% Triton X-100 in PBS, and blocked with 2% BSA in PBS. Fluorescent dyes or primary antibodies [GM-130 (1:100), phalloidin-Texas red (1:100) and  $\alpha$ -tubulin (1:50)] were incubated for 1 h at room temperature in PBS with 2% BSA. For the detection of tubulin, the cells were subsequently washed with PBS and incubated for 1 h with goat-anti-mouse-tetramethylrhodamine isothiocyanate (1:100) in PBS with 1% BSA. Cells were washed and mounted with Glycerol DABCO from Sigma. Nuclei were stained with DAPI (1:8,000 in PBS). Cells were evaluated with a Zeiss confocal microscope with a Bio-Rad laser. Image analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

*Surface detection of CD36.* Directly after isolation, cell suspensions of cardiomyocytes (100,000 cells/well) were plated in laminin-coated (20  $\mu\text{g/ml}$ ) 35-mm culture plates and were allowed to attach to the plates for 1 h. Afterward, the cells were incubated with 0.35% DMSO or with the pharmacological agents as described for the substrate uptake assay. As previously described (44), cells were biotinylated with the cell membrane-impermeable reagent sulfo-NHS-LC-biotin in bicarbonate medium at a final concentration of 1 mg/ml for 45 min at 4°C while shaking. The reaction was quenched with ice-cold glycine in bicarbonate medium (100 mM). After a brief wash with ice-cold bicarbonate medium, cells were lysed by scraping in 250  $\mu\text{l}$  lysis buffer. Lysates were incubated at 4°C for 1 h while rotating and were centrifuged for 10 min at 13,000 g at 4°C. Supernatant (15  $\mu\text{l}$ ) was used as total lysate sample, and 200  $\mu\text{l}$  supernatant was incubated overnight with streptavidin beads. Samples were briefly centrifuged, and beads were washed twice with lysis buffer. Biotinylated proteins were eluted by incubation of the streptavidin beads for 5 min at 95°C in sample buffer. Samples were subjected to SDS-

polyacrylamide gel electrophoresis, followed by Western blotting, as previously described (25).

*Cellular fractionation of rat cardiomyocytes.* Freshly isolated cardiomyocytes were incubated with oligomycin or trafficking inhibitors similarly to the substrate uptake experiments. Subsequently, cell suspensions were processed according to the fractionation procedure as previously described (26).

*Other procedures.* Cellular wet mass was obtained from cell samples taken during the incubation period and determined after centrifugation for 2–3 s at maximal speed in a microcentrifuge and subsequent removal of the supernatant. Protein was quantified with the bicinchoninic acid protein assay (Pierce, Rockford, IL) according to manufacturer's instructions.

*Data presentation and statistical analysis.* All data are presented as means  $\pm$  SE for the indicated number of myocyte preparations. Statistical difference between groups of observations was evaluated by ANOVA or Student's *t*-test.  $P \leq 0.05$  was considered significant.

## RESULTS

*Verification of the disrupting actions of brefeldin-A, latrunculin-B, and colchicine at the ultrastructural level in cardiomyocytes.* One of the reported cellular effects of brefeldin-A treatment is fragmentation of the Golgi apparatus (13). To evaluate the effectiveness of brefeldin-A on Golgi disruption in cardiomyocytes, cells were treated, fixed, and stained for Golgi marker 130, a Golgi-localized protein. The Golgi apparatus was detectable as an elongated structure stretching from the vicinity of the nucleus through the sarcoplasmic reticulum, parallel along the contractile fibers (Fig. 1A). With brefeldin-A, this staining became less intense and more dispersed. The lack of high-intensity staining after brefeldin-A treatment indicates that the Golgi apparatus, and likely also the endosomes, have been fragmented (Fig. 1A).

Latrunculin-B has been used as an inhibitor of actin polymerization and thereby as a destabilizing agent for actin filaments (20). Treatment with latrunculin-B was evaluated by actin staining with phalloidin-Texas red. The striated actin pattern, a characteristic feature of cardiomyocytes, was markedly less pronounced and more dispersed upon treatment with 20  $\mu\text{M}$  latrunculin-B (Fig. 1B). In addition, black holes appeared between the actin striations of latrunculin-B-treated cardiomyocytes, indicating that the actin cytoskeleton has ruptured at several intracellular locations. Finally, cell morphology altered from rod-shaped with rectangular ends to a shorter and rounder appearance (Fig. 1B). Hence, the subcellular actin network was damaged by latrunculin-B treatment.

Colchicine is a disruptor of the microtubule network (1). Optimal concentrations were established using immunofluorescence staining of control and colchicine-treated cardiomyocytes. Incubation of cardiomyocytes for 1 h in the presence of 1  $\mu\text{M}$  colchicine induced structural changes in the microtubule network. The perinuclear staining became less prominent and the overall organization of the microtubules was disrupted, as indicated by a more diffuse staining pattern in treated cells (Fig. 1C). Another well-established colchicine-induced change in cell morphology is the loss of cell shape (34). Clearly, colchicine treatment of cardiomyocytes caused a metamorphosis from the characteristic rod-shaped appearance into a more spread-out, less-defined cell shape.

Taken together, we could verify the effectiveness of brefeldin-A, latrunculin-B, and colchicine in cardiomyocytes.

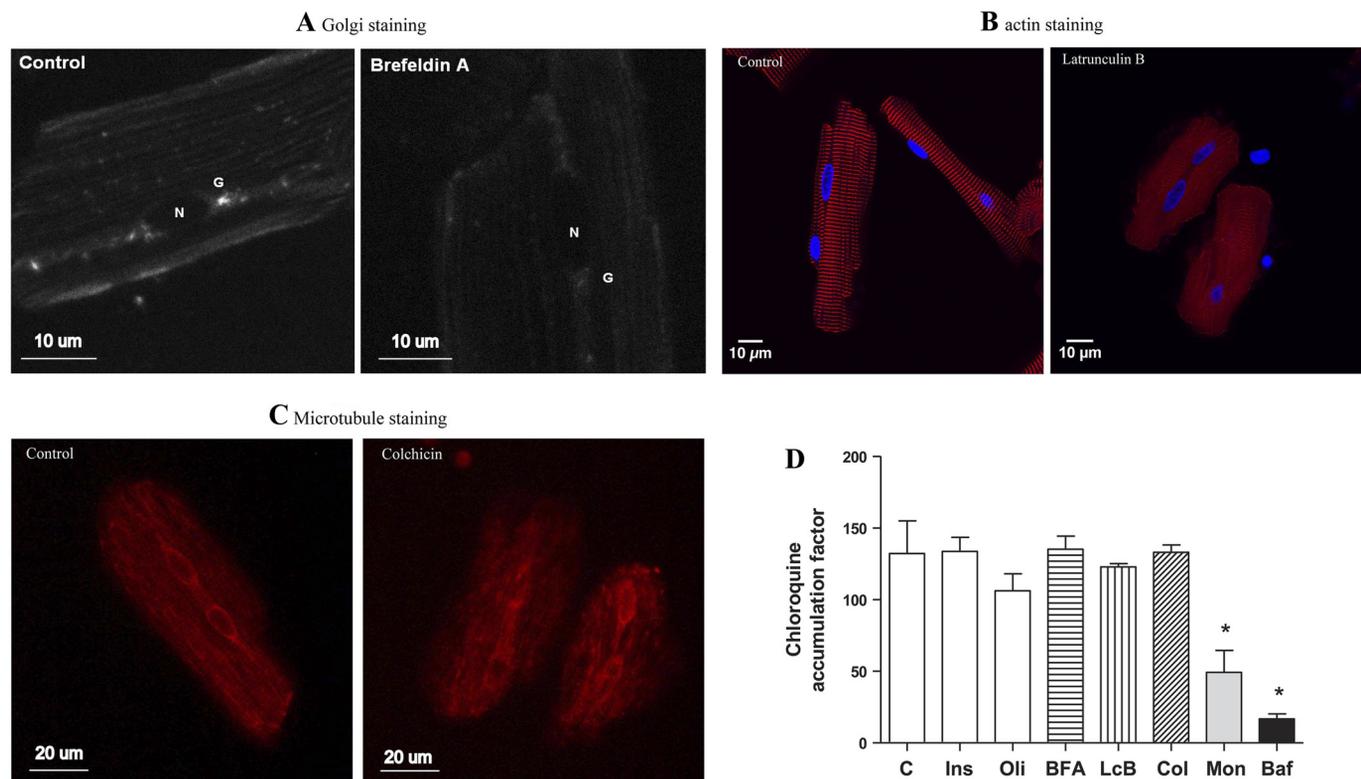


Fig. 1. Effect of trafficking inhibitors on cardiomyocyte morphology and endosomal function. *A*: cardiomyocyte suspensions were incubated without or with 20  $\mu\text{g/ml}$  brefeldin-A (45 min) before fixation and Golgi staining with GM-130. *B*: cardiomyocyte suspensions were incubated without or with 20  $\mu\text{g/ml}$  latrunculin-B (45 min) before fixation and actin staining with phalloidin Texas red. *C*: cardiomyocyte suspensions were incubated without or with 1  $\mu\text{M}$  colchicine (45 min) before fixation and staining of microtubules with  $\alpha$ -tubulin. *D*: cardiomyocyte suspensions were incubated without or with 100 nM insulin (20 min), 5  $\mu\text{M}$  oligomycin (20 min), 20  $\mu\text{g/ml}$  brefeldin-A (45 min), 20  $\mu\text{g/ml}$  latrunculin-B (45 min), 1  $\mu\text{M}$  colchicine (45 min), 100 nM monensin (25 min), or 100 nM bafilomycin-A (25 min) at the time periods indicated before incubation (30 min) with trace amounts of [ $^3\text{H}$ ]chloroquine for assessment of endosomal function. Data are means  $\pm$  SE of 4–6 experiments carried out with different cardiomyocyte preparations. \*Significantly different from cardiomyocytes without additions ( $P < 0.05$ ). C, control; Ins, insulin; Oli, oligomycin; BFA, brefeldin-A; LcB, latrunculin-B; Col, colchicine; Mon, monensin; Baf, bafilomycin-A.

*Verification of alkalinizing action of monensin and bafilomycin-A in cardiomyocytes.* Intracellular accumulation of divalent weak bases, such as chloroquine, is a sensitive pH indicator of the interior of acidic organelles (12). In nontreated cardiomyocytes, the chloroquine accumulation factor was  $133 \pm 22$ -fold, which was not influenced by addition of insulin, oligomycin, brefeldin-A, latrunculin-B, or colchicine (Fig. 1D). In contrast, elevation of the endosomal pH by monensin or bafilomycin-A markedly decreased chloroquine accumulation (Fig. 1D). Hence, monensin and bafilomycin-A effectively interfere with endosomal functioning via alkalization of the acidic lumen.

*Excluding direct effects of trafficking-inhibiting agents on GLUT4 and CD36.* The possibility exists that the used trafficking inhibitors would affect glucose or LCFA uptake into cardiomyocytes through direct interaction with GLUT4 or CD36 at the plasma membrane. We investigated this option by preparation of giant vesicles which solely consist of sarcolemmal membranes encapsulating soluble cytoplasm. Intracellular organelles such as the endosomal recycling compartment are absent (27), so that translocation of transporters cannot occur. Hence, during incubation of giant vesicles with selected stimuli, the amount of GLUT4 and CD36 present at the vesicular membrane remains fixed, and therefore alterations in transport rates can only be due to changes in the activities of these substrate transporters. We observed that none of the used

trafficking inhibitors altered the uptake rates of glucose and LCFA into giant vesicles (Fig. 2), indicating that any inhibitory effect of these agents on regulation of substrate uptake into cardiomyocytes cannot be ascribed to a direct inhibition of sarcolemmal GLUT4 or CD36.

*Excluding nonspecific effects of trafficking-inhibiting agents on insulin and AMPK signaling.* To demonstrate that the cytoskeleton disruptors, brefeldin-A and latrunculin-B, did not interfere with oligomycin-induced AMPK activation, we evaluated Ser79 phosphorylation of the AMPK substrate acetyl-CoA carboxylase (ACC) by Western blotting (Fig. 3A). Symmetrically, we determined insulin-induced Akt-Ser473 phosphorylation (Fig. 3B). Insulin and oligomycin enhanced phosphorylation of Akt and ACC, respectively, by  $>10$ -fold (Fig. 3), in agreement with earlier observations in cardiomyocytes (23, 28). However, none of the used trafficking inhibitors were observed to alter the phosphorylation states of these markers of insulin and AMPK signaling (Fig. 3). Hence, any effects of these agents on regulation of substrate uptake into cardiomyocytes cannot be attributed to nonspecific effects of these compounds on insulin and AMPK signaling.

*Effects of trafficking-inhibiting agents on substrate uptake into cardiomyocytes.* Insulin and oligomycin were used to stimulate cardiac substrate uptake. As displayed in Fig. 4, insulin and oligomycin treatment increased glucose uptake by 5- to 12-fold and 1.7- to 2.5-fold, respectively, and LCFA uptake by 1.3- to 1.5-fold and 1.7- to 2.0-fold, respectively, in

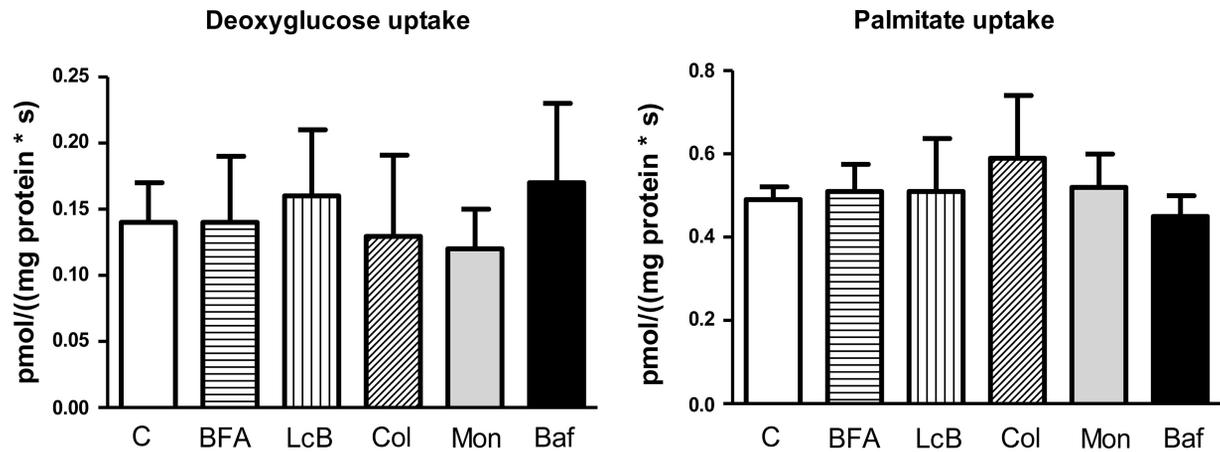


Fig. 2. Effects of trafficking inhibitors on intrinsic activity of substrate transporters in heart-derived giant vesicles. Giant vesicle suspensions were incubated without or with 20  $\mu\text{g/ml}$  brefeldin-A, 20  $\mu\text{g/ml}$  latrunculin-B, 1  $\mu\text{M}$  colchicine, 100 nM monensin, or 100 nM bafilomycin-A before execution of glucose uptake studies (2 min) or palmitate uptake studies (15 s). Data are means  $\pm$  SE of 3–4 experiments carried out with different cardiomyocyte preparations.

agreement with our earlier observations (25, 26). Brefeldin-A treatment did not alter basal glucose and LCFA uptake but markedly inhibited insulin-stimulated glucose uptake ( $-42\%$ ), oligomycin-stimulated glucose uptake ( $-51\%$ ), and oligomycin-stimulated LCFA uptake ( $-39\%$ ) and largely inhibited insulin-stimulated LCFA uptake ( $-68\%$ ; Fig. 4A). Latrunculin-B treatment did not alter basal glucose uptake and also did not alter LCFA uptake, neither under basal nor upon treatment of cardiomyocytes with insulin or oligomycin (Fig. 4B). In contrast, this actin filament-disrupting agent markedly inhibited insulin-stimulated glucose uptake ( $-41\%$ ) and largely inhibited oligomycin-stimulated glucose uptake ( $-75\%$ ; Fig. 4B). Colchicine treatment neither influenced basal glucose and LCFA uptake nor insulin- or oligomycin-stimulated glucose and LCFA uptake (Fig. 4C). Monensin and bafilomycin-A exerted very similar effects on substrate uptake into cardiomyocytes, bolstering the notion that these agents act via the same mechanism, i.e., disruption of low endosomal pH. Specifically, monensin or bafilomycin-A did not alter basal glucose uptake, while simultaneously increasing basal LCFA uptake by 1.3- and 1.4-fold, respectively. Furthermore, monensin and bafilomycin-A markedly inhibited insulin-stimulated glucose uptake ( $-32\%$  and  $-52\%$ , respectively) and largely inhibited oligo-

mycin-stimulated glucose uptake ( $-49\%$  and  $-68\%$ , respectively), but these agents had no effect on insulin- or oligomycin-stimulated LCFA uptake (Fig. 4D).

When combining the results obtained with all applied trafficking inhibitors, we conclude that insulin/oligomycin-stimulated glucose and LCFA uptake resemble each other in their similar dependence on coat protein-mediated vesicle formation and their independence of microtubules. In contrast, the endosomal pH is differentially involved in basal and insulin/oligomycin-stimulated glucose and LCFA uptake. Moreover, insulin/oligomycin-stimulated glucose uptake, but not LCFA uptake, is dependent on actin filaments.

*Involvement of CD36 in alterations seen in cardiomyocytes treated with alkalinizing agents.* We observed an increase in basal LCFA uptake in cardiomyocytes treated with alkalinizing agents. To establish whether CD36 was involved in this process, we applied the specific CD36 inhibitor SSO (11, 18). In agreement with earlier studies (26), SSO pretreatment partially inhibited LCFA uptake into nonstimulated cardiomyocytes (control;  $-30\%$ ) (Fig. 5A). Furthermore, SSO completely reduced the increase in basal LCFA uptake in monensin- and bafilomycin-A-treated cells, i.e., to the same residual level as observed for nonstimulated cardiomyocytes (Fig. 5A). This indicates that

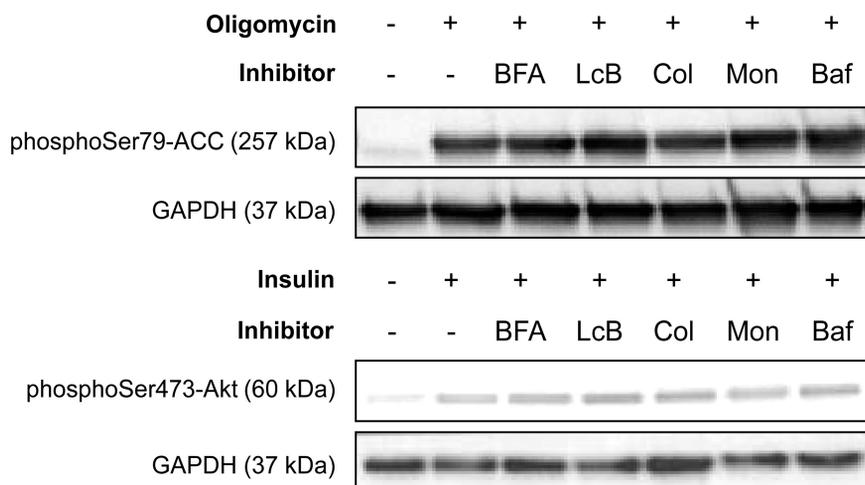


Fig. 3. Effect of trafficking inhibitors on phosphorylation of signaling proteins in insulin and AMP-activated protein kinase (contraction) signaling. Cardiomyocyte suspensions were preincubated without or with 20  $\mu\text{g/ml}$  brefeldin-A (45 min), 20  $\mu\text{g/ml}$  latrunculin-B (45 min), 1  $\mu\text{M}$  colchicine (45 min), 100 nM monensin (25 min), or 100 nM bafilomycin-A (25 min) at the time periods indicated and were subsequently incubated with or without 100 nM insulin or 5  $\mu\text{M}$  oligomycin for 15 min. Subsequently, samples were processed for detection of phospho-Ser79-acetyl-CoA carboxylase (ACC) and phospho-Ser473-Akt by Western blotting.

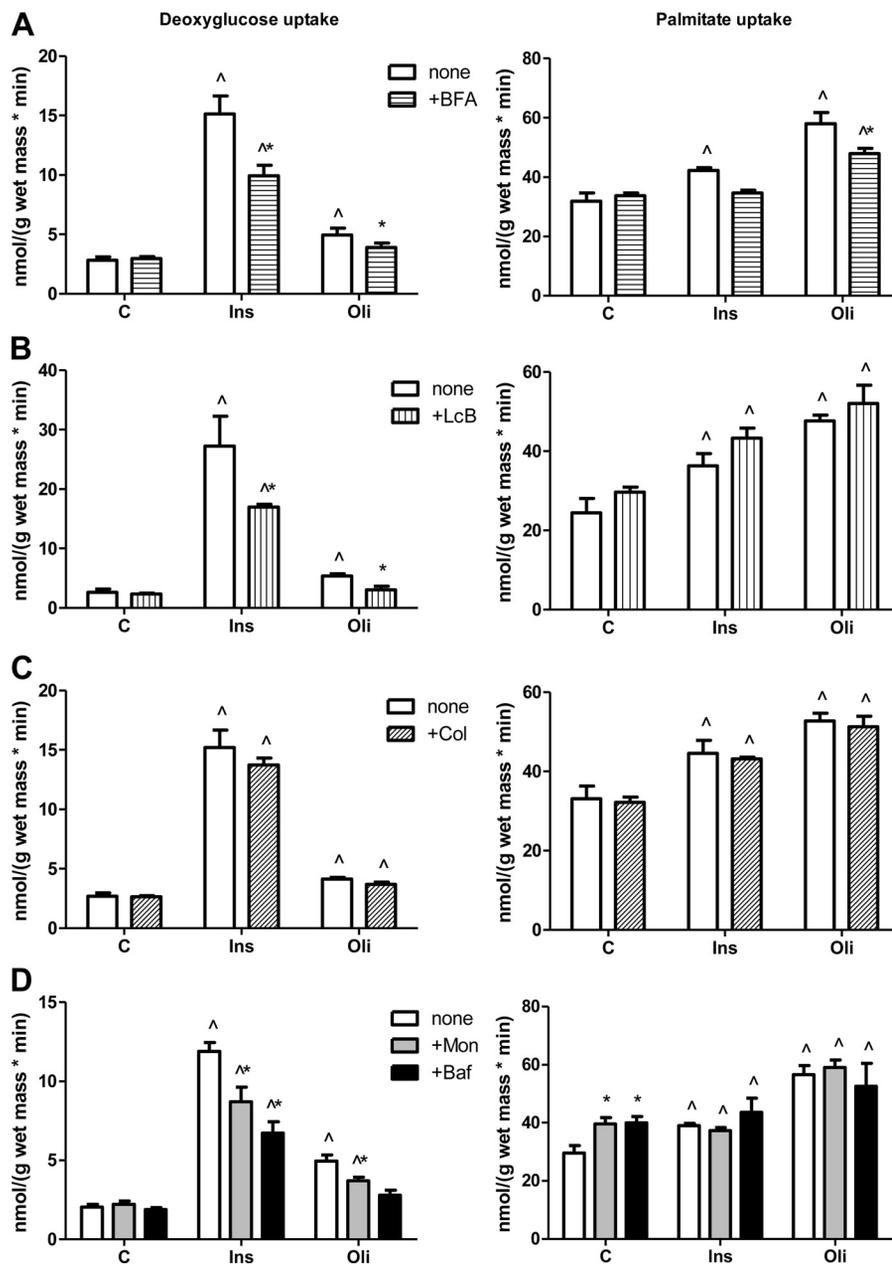


Fig. 4. Effects of trafficking inhibitors on glucose and light-chain fatty acid (LCFA) uptake into cardiomyocytes. Cardiomyocyte suspensions were pre-incubated without or with 20  $\mu$ g/ml brefeldin-A (45 min) (A), 20  $\mu$ g/ml latrunculin-B (45 min) (B), 1  $\mu$ M colchicine (45 min) (C), or 100 nM monensin (25 min) or 100 nM bafilomycin-A (25 min) (D) at the time periods indicated and were subsequently incubated with or without 100 nM insulin or 5  $\mu$ M oligomycin for 15 min before execution of palmitate uptake studies (3 min) or deoxyglucose uptake studies (3 min). Data are means  $\pm$  SE of 4–12 experiments carried out with different cardiomyocyte preparations. \*Significantly different from cardiomyocytes stimulated with insulin or oligomycin in the absence of trafficking inhibitors ( $P < 0.05$ ); <sup>^</sup>significantly different from cardiomyocytes without trafficking inhibitor ( $P < 0.05$ ).

CD36 is necessary for the action of these endosomal pH-elevating agents on LCFA uptake into cardiomyocytes. SSO treatment did not affect glucose uptake into cardiomyocytes, confirming the specificity of this compound for CD36 (Fig. 5A). Additionally, none of the trafficking inhibitors decreased cardiomyocyte glucose uptake in either the absence or the presence of SSO (Fig. 5A).

The involvement of CD36 in the increased basal LCFA uptake in monensin- and bafilomycin-A-treated cardiomyocytes suggests that both endosomal inhibitors induce CD36 translocation to the sarcolemma. To verify this, surface protein biotinylation was performed, allowing quantification of CD36-translocation to the cell surface. We found the amount of CD36 in the biotin-labeled fraction to be increased in insulin-treated (3.7-fold) or oligomycin-treated (4.0-fold) cardiomyocytes compared with nonstimulated cells (Fig. 5, B and D). This

confirmed earlier observations using this procedure in insulin- and oligomycin-stimulated Chinese hamster ovary cells (44), and the suitability of this procedure to quantify the cell surface presence of recycling proteins. An increase in CD36 cell surface content was also observed upon treatment of cardiomyocytes with monensin (2.5-fold) or bafilomycin-A (2.0-fold), whereas no alterations were seen in GLUT4 surface presence (Fig. 5, C and D). To assess whether the increase in sarcolemmal CD36 in monensin or bafilomycin-A treated cardiomyocytes was due to translocation from intracellular membrane compartments, subcellular fractionation was performed. First of all, this method confirmed the biotinylation experiments; the presence of CD36 in the sarcolemmal fraction was found to be increased upon cardiomyocyte treatment with monensin (1.3-fold) or bafilomycin-A (1.2-fold), but not upon brefeldin-A, latrunculin-B, or colchicine treatment (Fig. 6).

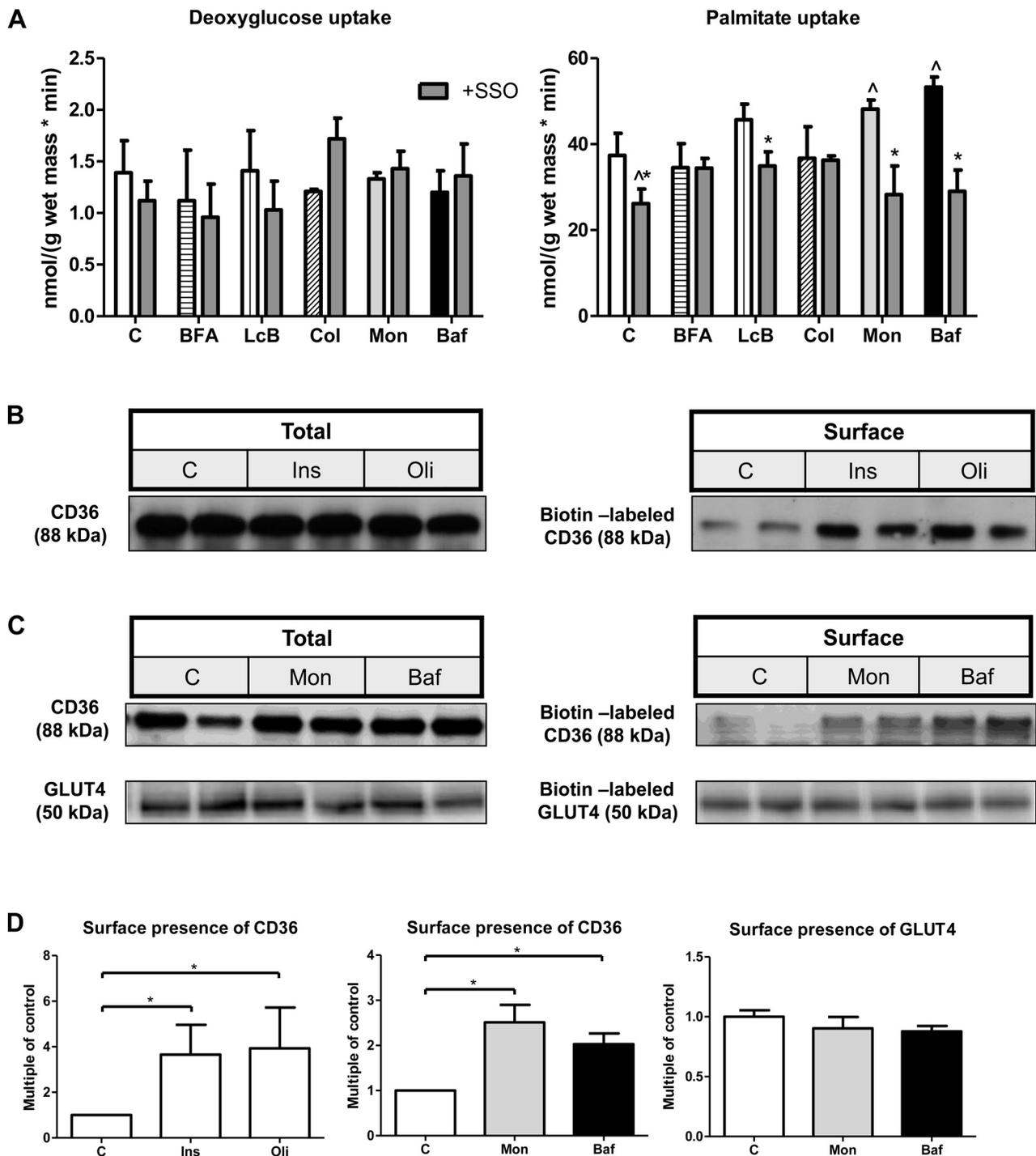


Fig. 5. Involvement of CD36 in increased basal LCFA uptake into cardiomyocytes after monensin/bafilomycin-A treatment. **A**: modulation of the stimulatory effects of trafficking inhibitors on LCFA uptake by sulfo-*N*-succinimidylleate (SSO). Cell suspensions were preincubated with DMSO (basal) or with 500  $\mu$ M SSO (dissolved in DMSO), after which the cells were washed twice, as described in MATERIALS AND METHODS. Subsequently, cells were incubated with or without 100 nM monensin (25 min), 100 nM bafilomycin-A (25 min), 20  $\mu$ g/ml brefeldin-A (45 min), 20  $\mu$ g/ml latrunculin-B (45 min), or 1  $\mu$ M colchicines (45 min) before execution of palmitate uptake studies (3 min) or deoxyglucose uptake studies (3 min). Data are means  $\pm$  SE of 3–4 experiments carried out with different cardiomyocyte preparations. \*Significantly different from cardiomyocytes without SSO ( $P < 0.05$ ); <sup>^</sup>significantly different from cardiomyocytes without inhibitor ( $P < 0.05$ ). **B–D**: effect of trafficking inhibitors on CD36 surface presence: seeded cells were incubated with DMSO (control), 100 nM insulin (20 min), 5  $\mu$ M oligomycin (20 min), 100 nM monensin (25 min), or 100 nM bafilomycin-A (25 min) before biotinylation and cell lysis. CD36 and glucose transporter-4 (GLUT4) were detected by Western blotting before biotinylation (total) and after biotinylation (surface). Values are relative to control presence of CD36 at the sarcolemma. Data are means  $\pm$  SE of 3–4 experiments carried out with different cardiomyocyte preparations. Representative Western blots are displayed. \*Significantly different from myocytes without additions ( $P < 0.05$ ).

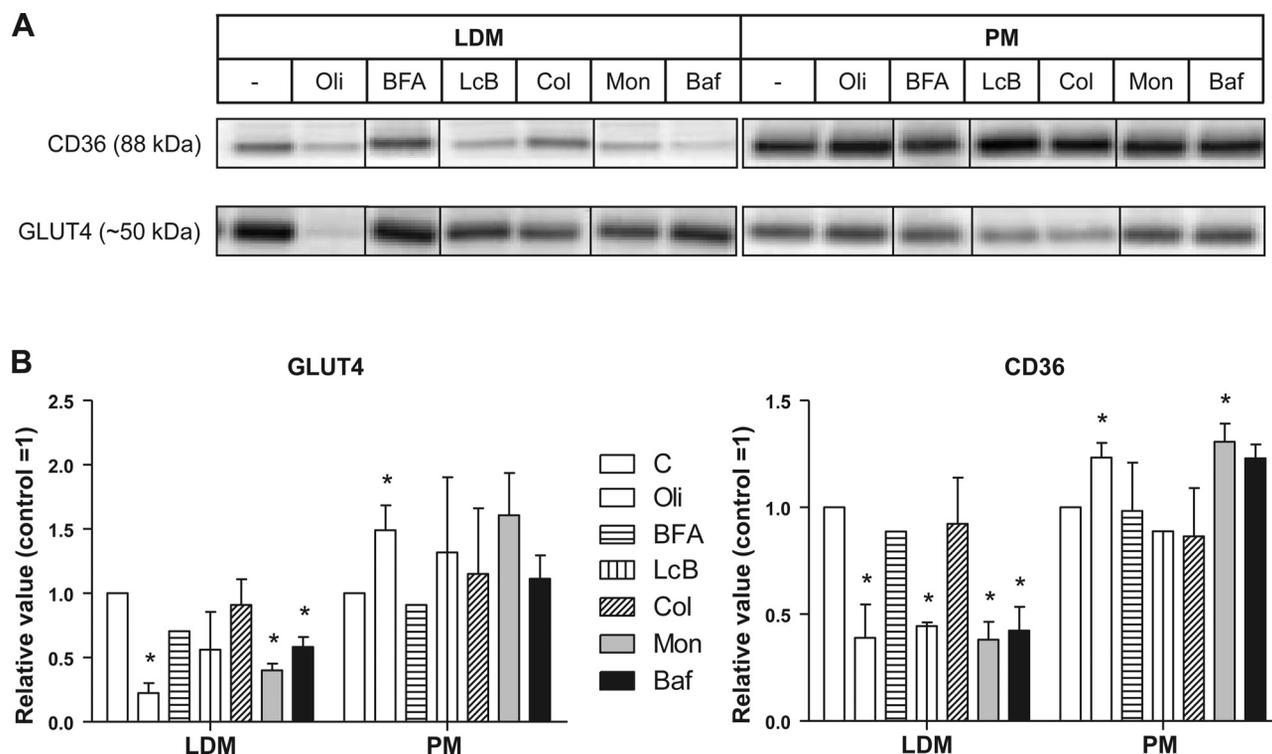


Fig. 6. Effect of trafficking inhibitors on subcellular localization of GLUT4 and CD36. A: cardiomyocyte suspensions were incubated without or with 5  $\mu$ M oligomycin (15 min), 20  $\mu$ g/ml brefeldin-A (45 min), 20  $\mu$ g/ml latrunculin-B (45 min), 1  $\mu$ M colchicine (45 min), 100 nM monensin (25 min), or 100 nM bafilomycin-A (25 min). Cells were processed for fractionation and later detection of GLUT4 and CD36 by Western blotting. Data are means  $\pm$  SE of 3–4 experiments carried out with different cardiomyocyte preparations. Representative Western blots are displayed. Lines indicate that samples were loaded on different blots. B: graphs show quantification and analysis of 3–4 Western blots. \*Significantly different from myocytes without additions ( $P < 0.05$ ). LDM, low-density microsomal fraction; PM, plasmalemmal fraction.

Importantly, the intracellular membrane content of CD36 was found to be decreased by monensin (–62%), bafilomycin-A (–58%) or latrunculin-B (–55%) treatment, but not by brefeldin-A or colchicine (Fig. 6). This indicates that dissipation of endosomal pH, but not disruption of assembly of coat proteins, actin filaments or microtubule, causes CD36 translocation from intracellular stores to the sarcolemma. In these same subcellular fractions we measured the content of the major cardiac glucose transporter GLUT4, which was not altered by any of the applied trafficking inhibitors, in agreement with the lack of an effect of monensin or bafilomycin-A on cell surface GLUT4 content, and in agreement with the lack of an effect of these inhibitors on basal glucose uptake. However, we found monensin (–60%) or bafilomycin-A (–42%) to reduce the GLUT4 content in the intracellular membrane fraction, i.e., whereas brefeldin-A, latrunculin-B, or colchicines were unsuccessful in this respect. Hence, it seems that monensin and bafilomycin induced an incomplete GLUT4 translocation event. In these subcellular fractionation experiments, oligomycin was used as positive control for CD36 and GLUT4 translocation. Oligomycin treatment was observed to increase sarcolemmal CD36 (1.2-fold) and GLUT4 (1.5-fold) content and to decrease intracellular CD36 (–61%) and GLUT4 (–77%) content, confirming earlier data (25) and indicating the suitability of the subcellular fractionation method.

## DISCUSSION

Translocation of both GLUT4 and CD36 are pivotal processes by which the heart regulates its substrate uptake. To

modulate cardiac substrate preference, e.g., to favor glucose over LCFA utilization, it would be of interest to identify subcellular components specifically involved in the translocation of either GLUT4 or of CD36. However, the signal transduction cascades, as far as these have been investigated, appear not to discriminate between translocation of GLUT4 and that of CD36 in cardiomyocytes (see Introduction and also Ref. 35).

In the present study, we have extended the comparison between the translocation of GLUT4 and that of CD36 in cardiomyocytes by investigating the possible similarities and differences in the reliance on trafficking structures. We applied compounds that disrupt selected components of the subcellular trafficking machinery and have come to the following conclusions. 1) Glucose uptake and LCFA uptake, stimulated by either insulin or oligomycin, show similar dependence on coat proteins. 2) Actin filaments do not play a role in LCFA uptake but are involved in glucose uptake stimulated by insulin or oligomycin, whereas microtubules are neither involved in insulin/oligomycin-stimulated glucose uptake nor in insulin/oligomycin-stimulated LCFA uptake. 3) Proper endosomal proton pumping is necessary for insulin/oligomycin-stimulated glucose uptake as well as for maintaining low basal LCFA uptake rates and intracellular retention of CD36.

Taken together, we have identified trafficking elements that are 1) similarly involved in the regulation of glucose and LCFA uptake (coat proteins), 2) not involved in the regulation of uptake of both substrates (microtubules), and 3) differen-

tially involved in the regulation of glucose uptake versus LCFA uptake (actin filaments and endosomal pH).

*Involvement of coat proteins in regulation of substrate uptake into cardiomyocytes.* This is the first study that has used brefeldin-A to investigate the role of coat proteins in the regulation of cardiac substrate uptake. Under our experimental conditions, brefeldin-A treatment resulted in the disruption of Golgi structures, confirming that this fungal inhibitor indeed affected coat protein-mediated processes in cardiomyocytes. Brefeldin-A did not alter basal glucose or LCFA uptake in cardiomyocytes, nor did it alter the subcellular localization of GLUT4 and CD36. This indicates that coat proteins are not involved in the maintenance of a low surface content of GLUT4 and CD36 in nonstimulated cardiomyocytes.

In contrast, brefeldin-A inhibited, at least partially, both insulin- and oligomycin-stimulated glucose uptake and insulin- and oligomycin-stimulated LCFA uptake. Hence, coat proteins are involved in stimulation of glucose and LCFA uptake upon insulin or oligomycin treatment of cardiomyocytes. Because brefeldin-A inhibits both COP and clathrin targeting to Golgi/endosomal membranes, it cannot be discriminated whether malfunctioning of  $\beta$ -COP versus clathrin is responsible for brefeldin-A-induced inhibition of insulin/oligomycin-stimulated glucose and LCFA uptake. Notwithstanding, the inhibition of stimulus-induced LCFA uptake by brefeldin-A provides the first evidence that CD36 translocation is a vesicle-mediated process dependent on coat proteins, just like GLUT4 translocation.

*Involvement of cytoskeletal elements in regulation of substrate uptake into cardiomyocytes.* Like brefeldin-A, inhibitors of actin filaments such as cytochalasin-D or latrunculin-B, have not been used in the context of cardiac substrate uptake. We were able to confirm that latrunculin-B effectively disrupts the actin network in cardiomyocytes, indicating that this drug is suitable to study the involvement of the actin filaments in subcellular processes. Latrunculin-B did not affect basal glucose or LCFA uptake, nor did it alter basal presence of CD36 and GLUT4 at the sarcolemma, and the basal presence of GLUT4 in the intracellular compartments. Surprisingly, latrunculin-B decreased the basal presence of CD36 in the intracellular compartments, indicating that disruption of actin polymerization dynamics results in an initiation of CD36 translocation but not in completion of this process. The functional relevance of this failing CD36 translocation is presently in completely understood.

More interestingly, latrunculin-B markedly inhibited insulin-stimulated glucose uptake and completely inhibited oligomycin-stimulated glucose uptake. The inhibition of insulin-stimulated glucose uptake is regarded as a classical latrunculin-B action in adipocytes as a result of actin filament disruption (24) and confirms the effectiveness of this drug in cardiomyocytes. In contrast, the inhibition of AMPK-mediated glucose uptake by latrunculin-B and the lack of an effect of latrunculin-B on basal or insulin/oligomycin-stimulated LCFA uptake are novel observations. This suggests that actin filaments are specifically involved in stimulus-induced GLUT4 translocation and not in CD36 translocation.

Conversely, we found that colchicine, while successfully disrupting the microtubule network in cardiomyocytes, had no effect on subcellular GLUT4 and CD36 distribution and on glucose and LCFA uptake into cardiomyocytes under any

condition. This indicates that, at least in the heart, this filamentous network does not provide the scaffolding system along which the intracellular GLUT4 and CD36 vesicles recycle between the endosomal compartment and the cell surface.

In conclusion, this is the first study to identify a subcellular component, i.e., actin filaments, involved in selective translocation of one of the transporters, and to provide evidence that GLUT4 translocation and CD36 translocation are separate events. Combined with the brefeldin-A data, it appears that the processes of GLUT4 translocation and CD36 translocation depend on mutually shared trafficking structures, as well as on structures specifically dedicated to the translocation of either one of these substrate transporters.

*Involvement of endosomal functioning in regulation of substrate uptake into cardiomyocytes.* The role of the endosomal pH in translocation processes such as that of GLUT4, as investigated in adipocytes and skeletal muscle, is controversial (see Introduction). We used two structurally unrelated agents with different action modes to compare the role of endosomal pH in the regulation of glucose and LCFA uptake in cardiomyocytes. Monensin and bafilomycin-A both blocked intracellular accumulation of the divalent weak base chloroquine in cardiomyocytes, which confirms their dissipating effect on endosomal proton trapping. The other trafficking inhibitors used had no effect on chloroquine accumulation, indicating that there are no nonspecific effects of these compounds on the endosomal pH.

Our observations on the effects of both endosomal inhibitors on substrate uptake into cardiac myocytes revealed that endosomal pH plays an entirely different role in subcellular distribution of GLUT4 than of CD36. Namely, we observed that both inhibitors markedly increased basal LCFA uptake without altering basal glucose uptake. In agreement with this, two independent techniques for measurement of cell surface content of proteins show that both endosomal inhibitors increase the sarcolemmal content of CD36, but not that of GLUT4 (Fig. 5, C and D, and Fig. 6). These results suggest that monensin/bafilomycin-A-induced LCFA uptake is due to CD36 translocation, and that the lack of an effect of monensin and bafilomycin-A is due to a lack of an effect on GLUT4 translocation. Definite evidence that the selective action of monensin/bafilomycin-A on LCFA uptake is mediated via CD36 translocation from intracellular stores to the sarcolemma was provided by the sensitivity of monensin/bafilomycin-enhanced LCFA uptake to inhibition by SSO. Surprisingly, monensin and bafilomycin-A reduced the GLUT4 content within the intracellular membrane fraction, suggesting that both alkalinizing agents similarly induced the budding of GLUT4 and of CD36 vesicles from the endosomal compartment. However, only the CD36-containing vesicles are able to reach the cell surface upon endosomal alkalization.

A possible explanation for the role of endosomal alkalization in budding of GLUT4 and CD36 vesicles from the endosomal membranes could include that one of the endosomal proteins regulating this budding process is repressed by low pH, and becomes deinhibited by a dissipation of the pH gradient. Furthermore, our results suggest that CD36-containing vesicles would travel directly to the sarcolemma upon budding from endosomal membranes, whereas GLUT4-containing vesicles might travel to the sarcolemma via a hypothetical endosomal GLUT4 transit compartment, in which GLUT4

would be unpacked and subsequently reloaded into different transport vesicles for further transport to the sarcolemma. However, in this intermediary station the proteins that regulate vesicle budding might be repressed by endosomal alkalization, instead of activated in case of those in the original endosomal GLUT4 stores. Then, GLUT4 arriving in this compartment upon cardiomyocyte treatment with endosomal alkalizing agents would get stuck here.

Remarkably, this subcellular distribution of both substrate transporters, with CD36 being at the cell surface and GLUT4 intracellular during endosomal alkalization, resembles that of the early insulin-resistant heart, as observed in young adult obese Zucker rats (10) and in rats fed a high-fat diet for 10 wk (30). This suggests that endosomal alkalization in the early insulin-resistant heart could result in selective cell surface accumulation of CD36 and hence might contribute to the development of insulin resistance through increased intracellular LCFA accumulation. The loss of the endosomal to cytoplasmic pH gradient might possibly be due to a reduced energy reserve associated with mitochondrial dysfunction during insulin resistance (5). The remaining ATP produced would then only be used for the most necessary cellular processes, and less ATP would be made available for proton pumping into the endosomal compartment.

Another striking difference about the role of the endosomal pH in the regulation of glucose versus LCFA uptake is that insulin/oligomycin-stimulated glucose uptake was partially blocked by monensin/bafilomycin-A treatment, while these compounds had no effect on insulin/oligomycin-stimulated LCFA uptake. The lack of an effect of monensin/bafilomycin-A on insulin/oligomycin-stimulated LCFA uptake must be appreciated in view of their stimulatory action on basal LCFA uptake: namely, the effects of monensin/bafilomycin-A on one hand and insulin/oligomycin on the other were nonadditive. Importantly, this nonadditivity is regarded as kinetic evidence that all these factors use a common molecular mechanism to increase LCFA uptake. Because insulin and oligomycin themselves do not or only slightly alkalize the endosomes (Fig. 1D), the effects of insulin and oligomycin on translocation must be downstream of the alkalization process. Hence, insulin/oligomycin treatment might activate the same budding protein as endosomal alkalization does, but then via phosphorylation.

The blockade of insulin/oligomycin-stimulated glucose uptake treatment of cardiomyocytes with endosomal alkalizing agents is in agreement with earlier studies on insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes (8) and cardiomyocytes (46). Perhaps, the hypothetical GLUT4 transit compartment with budding proteins inhibited by a rise in pH might offer an explanation not only for the incomplete GLUT4 translocation, but also for the inhibition of insulin/oligomycin-induced GLUT4 translocation upon endosomal alkalization. Namely, GLUT4 induced to translocate from the endosomal GLUT4 stores upon insulin/oligomycin treatment would not be further processed in this transit compartment when the pH gradient between this compartment and the cytoplasm has been dissipated by alkalizing agents.

Nonetheless and irrespective of the provided possible explanations, it is clear that the endosomal pH plays a differential role in the regulation of cardiac glucose and LCFA uptake.

**Concluding remarks.** The ability of brefeldin-A to inhibit insulin- or oligomycin-stimulated LCFA uptake provides the first evidence for the vesicle-dependent nature of CD36 translocation upon stimulation of cardiomyocytes with hormonal and pharmacological stimuli. This demonstrates that the similarity in the regulation of translocation of GLUT4 and CD36 extends beyond the signaling pathways (as reviewed in Ref. 35) to the vesicular trafficking machinery. However, actin filaments are solely involved in GLUT4 recycling, and the endosomal proton pump has differential roles in GLUT4 and CD36 recycling. With the actin filaments being specifically involved in GLUT4 recycling, it might be speculated that there is also a filamentous network entirely dedicated to CD36 recycling. However, such a candidate filament network is not provided by the microtubules.

Finally, the differential involvement of the endosomal proton pump and actin filaments in the regulation of GLUT4 and CD36 distribution in cardiomyocytes provides novel therapeutic strategies to alter substrate preference in metabolic disease. For instance, pharmacological inhibitors that are specifically targeted to the heart to disturb vesicle traffic along actin filaments (for inhibition of glucose uptake) could theoretically be used as therapeutic agents to correct substrate preference in the hypertrophied heart, which is characterized by excess glucose utilization. Conversely, in metabolic diseases such as type-2 diabetes, cardiospecific pharmacological agents that stimulate v-ATPase activity would lower LCFA uptake in the lipid-overloaded heart and may thereby restore insulin-stimulated glucose uptake and prevent or reverse the development of metabolic cardiomyopathy.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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