THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 11, Issue of March 14, pp. 7085–7092, 1997

Printed in U.S.A.

Insulin-induced Recruitment of Glucose Transporter 4 (GLUT4) and GLUT1 in Isolated Rat Cardiac Myocytes

EVIDENCE OF THE EXISTENCE OF DIFFERENT INTRACELLULAR GLUT4 VESICLE POPULATIONS*

(Received for publication, September 27, 1996, and in revised form, December 13, 1996)

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Using isolated rat cardiomyocytes we have examined: 1) the effect of insulin on the cellular distribution of glucose transporter 4 (GLUT4) and GLUT1, 2) the total amount of these transporters, and 3) the co-localization of GLUT4, GLUT1, and secretory carrier membrane proteins (SCAMPs) in intracellular membranes. Insulin induced 5.7- and 2.7-fold increases in GLUT4 and GLUT1 at the cell surface, respectively, as determined by the nonpermeant photoaffinity label [^{3}H]2-*N*-[4(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine. The total amount of GLUT1, as determined by quantitative Western blot analysis of cell homogenates, was found to represent a substantial fraction (~30%) of the total glucose transporter content. Intracellular GLUT4-containing vesicles were immunoisolated from low density microsomes by using monoclonal anti-GLUT4 (1F8) or anti-SCAMP antibodies (3F8) coupled to either agarose or acrylamide. With these different immunoisolation conditions two GLUT4 membrane pools were found in nonstimulated cells: one pool with a high proportion of GLUT4 and a low content in GLUT1 and SCAMP 39 (pool 1) and a second GLUT4 pool with a high content of GLUT1 and SCAMP 39 (pool 2). The existence of pool 1 was confirmed by immunotitration of intracellular GLUT4 membranes with 1F8-acrylamide. Acute insulin treatment caused the depletion of GLUT4 in both pools and of GLUT1 and SCAMP 39 in pool 2. In conclusion: 1) GLUT4 is the major glucose transporter to be recruited to the surface of cardiomyocytes in response to insulin; 2) these cells express a high level of GLUT1; and 3) intracellular GLUT4-containing vesicles consist of at least two populations, which is compatible with recently proposed models of GLUT4 trafficking in adipocytes.

In mammalian cells, the facilitative uptake of glucose is mediated by a group of specialized glucose transporters (for reviews, see Refs. 1–4). Peripheral insulin-sensitive tissues, such as fat, skeletal muscles, and heart, express a unique transporter isoform (GLUT4),[1] which is largely confined to an intracellular storage site in the basal, nonstimulated state and becomes recruited to the cell surface under the influence of insulin (5, 6) but also other stimuli, such as contraction (7–9) and hypoxia or anoxia (10, 11). This recruitment process is likely to account for a large part of the increase in the rate of glucose uptake observed on stimulation with these agents (12–16).

It has been recently reported that mice expressing a defective GLUT4 gene show cardiac hypertrophy (17), which supports the view that GLUT4 is important for normal function and properties of cardiac myocytes. This fact emphasizes the necessity of a thorough understanding of the mechanisms involved in the control and function of this protein in heart tissue. An important issue is the delineation of the GLUT4 trafficking pathway(s) in cardiomyocytes. In this respect, immunoelectron microscopy studies performed in the rat heart have observed that GLUT4 is localized in cardiac myocytes, under nonstimulated conditions, in small tubulovesicular elements adjacent to the sarcolemma and the transverse tubular system and in the trans-Golgi region (18), and that insulin stimulates the recruitment of intracellular GLUT4 carriers to the sarcolemma and to the T-tubular system. However, there is no information on biochemical grounds regarding the characteristics of the intracellular GLUT4 pool(s) in cardiomyocytes and the proteins essential for recruitment to the cell surface in response to insulin.

On the other hand, many cells types, such as endothelial cells and erythrocytes and also fat and muscle cells, contain another transporter isoform, GLUT1, which is thought to be at least in part responsible for the basal uptake of glucose (19, 20). In heart and skeletal muscle, the content of GLUT1 mRNA and protein was shown to largely decrease during postnatal development (21, 22), whereas the reverse is true for GLUT4 (21–23). However, the adult heart still appears to contain considerable amounts of GLUT1, in contrast to skeletal muscles (24, 25). Moreover, recent studies have shown that in heart muscle cells GLUT1 is recruited to the plasma membrane by several

* This work was supported in part by Grant Fi 551/1-2 from the Deutsche Forschungsgemeinschaft, Grant PB92/0805 from the Dirección General de Investigación Científica y Técnica, Grant GRQ94-1040 from Generalitat de Catalunya, Spain, and Grants HA94-125 and HA95-125 from the Deutscher Akademischer Austauschdienst in conjunction with Acciones Integradas Hispano-Alemañas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Recipient of a predoctoral fellowship from the Ministerio de Educación y Ciencia, Spain.

** Supported in part by a grant from Fondo de Investigaciones Sanitarias.

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[1] The abbreviations used are: GLUT, glucose transporter; SCAMP, secretory carrier membrane protein; LDM, low density microsome; PM, plasma membrane; ATB-BMPA, 2-*N*-[4(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine; PBS, phosphate-buffered saline.

types of glucose transport stimuli, including insulin (26, 27), metformin (26), serotonin (27), and catecholamines (28). Thus, it is conceivable that GLUT1 plays an important role in the regulation of glucose uptake in the heart under certain physiological conditions.

In view of the importance of glucose carriers in cardiac function, we have used freshly isolated cardiac myocytes from adult rats: 1) to quantify the effect of insulin on the recruitment of GLUT4 and GLUT1 carriers to the cell surface; 2) to compare the amounts of these proteins expressed in intact cardiac myocytes; and 3) to explore the nature of the intracellular GLUT4 compartment.

MATERIALS AND METHODS

Chemicals—^{125}I-Protein A and ^{125}I-sheep anti-mouse antibody were purchased from ICN (Meckenheim, Germany). ^{125}I-Goat anti-mouse antibody, ECL, and 2-deoxy-D-[^{3}H]glucose were from Amersham Corp. The photoaffinity label (^{3}H-ATB-BMPA) used to quantify the glucose transporters was prepared as described elsewhere (29). All chemicals for media used for cell isolation, glucose transport assays, and labeling experiments were from Merck; antipain, α-hemolysin, protein A-Sepharose, γ-globulin, goat-anti mouse IgG, and goat anti-mouse IgM coupled to agarose were obtained from Sigma; aprotinin, pepstatin, and leupeptin were from ICN; Thesit™ and bovine serum albumin (fraction V, fatty acid free) were purchased from Boehringer Mannheim); purified bovine insulin was a kind gift from Prof. Axel Wollmer (Aachen, Germany). All chemicals were the highest purity grade available. Concentrated stock solutions of insulin (in medium A, see below) were stored at −20 °C in appropriate aliquots and diluted just prior to addition to the isolated cardiomyocytes. Immobilon polyvinylidene difluoride was obtained from Millipore. All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad.

Antibodies—Antisera directed against the C-terminal peptides of either GLUT1 or GLUT4 (and used to purify the photoaffinity-labeled transporters) were raised in rabbits in the laboratories of G. H. (GT1 and GT4), and A. Z. (OSCRX against GLUT4) or were a kind gift from Dr. Samuel W. Cushman and Dina R. Yver (Bethesda, MD; 9301 pA and 8105p αG4). A polyclonal antibody generated against the C terminus of GLUT1 obtained from Biogenesis Inc. was used for immunoblotting assays. Monoclonal antibodies 1F8 (against GLUT4) and 3F8 (against SCAMPs) were kindly provided by Dr. Paul F. Pilch (Boston University). A rabbit polyclonal antibody against rat β_1-integrin was kindly given by Dr. Carles Enrich (University of Barcelona) (30). Monoclonal antibody NCL-DYS 1 against the midrod of dystrophin was obtained from NovoCastra. The polyclonal antibody against the rat α_1-subunit of the Na^+-K^+-ATPase was from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibody 18B11, against TGN 38, was kindly given by Dr. Ignacio Sandoval (Centro de Biologia Molecular, Madrid, Spain).

Isolation of Cardiomyocytes and Glucose Transport Assays—Cardiomyocytes from adult female Sprague-Dawley rats (180–220 g, fed *ad libitum*) were obtained as described previously (31). Treatment of cardiomyocytes for all experiments was performed in medium A containing 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM HEPES, 1 mM $CaCl_2$, and 2% bovine serum albumin (fatty acid free, pH 7.4) at 37 °C, equilibrated with oxygen. The rate of 2-deoxy-D-glucose uptake was determined as described elsewhere (31).

Photoaffinity Labeling of Glucose Transporters with [^{3}H]ATB-BMPA—The labeling of glucose transporters was performed according to a method developed previously (12, 29), which was adapted and extensively validated in cardiomyocytes (27, 28). In brief, the labeling of cell surface transporters was carried out as follows. Cardiomyocytes (~5 mg protein/sample in a total volume of 6 ml) were incubated for 30 min at 37 °C in the absence (control) or in the presence of insulin (10 nM). Parallel samples were used for the determination of glucose transport. The cells allotted to the photoaffinity labeling were then washed and resuspended in 500 μl of medium A (with or without insulin); 60 μl of the nonpermeant, photoreactive bismannose compound ^{3}H-ATB-BMPA (300 μCi, 60 μM final concentration) were added immediately before the samples were irradiated for 3 min with UV light under continuous gentle shaking. Following irradiation the cells were washed with medium A and then solubilized at 4 °C with 2% Thesit™ (in a phosphate buffer containing the proteinase inhibitors antipain, aprotinin, pepstatin, and leupeptin, 1 μg/μl each).

Preliminary experiments were performed to ensure that amounts of antibodies used to immunoprecipitate GLUT1 and GLUT4 from homogenized or permeabilized cardiomyocytes were saturating with respect to glucose transporter recovery. First, larger amounts of anti-GLUT1 antisera (up to 300 μl of GT1 or 50 μl of 9301pA) or GLUT4 antisera (up to 300 μl of GT4 or 50 μl of p8105 αG4) or longer incubation times (up to 18 h) did not result in increased GLUT1 or GLUT4 signals. Second, no detectable amount of glucose transporters could be recovered from a second immunoprecipitation with the same antiserum. Third, the total signals obtained were independent of the antiserum used (GT4 or GT1, raised in the laboratory of G. H., *versus* p8105 αG4 or 9301pA). Finally, as shown in a previous study, the order of addition of antibodies has no influence on the results (13).

Determination of Total Cellular Amounts of GLUT4 and GLUT1 by Quantitative Western Blot Analysis—Nonstimulated cardiomyocytes (~30 mg protein/sample) were washed once with TES buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and then homogenized with a Potter (clearance, 0.2 mm) in a total volume of 4.7 ml. The samples were then spun down for 30 min at 50,000 *g* at 4 °C, and the pellet of this centrifugation was resuspended in 350 μl of TES buffer. This crude membrane fraction was used to perform immunoblot analysis, as described in a separate section below. The absolute amounts of GLUT4 and GLUT1 were quantified by comparing the signals obtained from several dilutions of crude membranes with those of known standards. These standards were purified intracellular membranes from isolated adipocytes (GLUT4) or from human erythrocytes (GLUT1) in which the amount of D-glucose-displaceable cytochalasin B binding sites had been determined as described elsewhere (32).

Preparation of Purified Membrane Fractions from Cardiomyocytes—Cardiomyocytes were incubated for 30 min at 37 °C in the presence or absence of insulin (10 nM) and were washed once with TES buffer and then immediately frozen in liquid nitrogen in a ratio of 10^7 cells/2.7 ml of TES. Membrane fractionation was performed as described previously (26).

Protocols of Vesicle Immunoisolation—Protein A-purified monoclonal anti-GLUT4 antibody (1F8) or a corresponding amount of nonspecific antibodies (γ-globulins) was coupled to acrylamide beads (Reacti-gel GF 2000, Pierce) at a concentration of 1 mg of antibody/ml of resin according to the manufacturer's instructions. Before use, the beads were saturated with 1% bovine serum albumin in PBS (134 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, and 1.46 KH_2PO_4, pH 7.4) for at least 30 min (at room temperature) and washed with PBS. Intracellular membranes (low density microsomes (LDMs)) were incubated with beads overnight at 4 °C (50 μg of LDMs, 20-μl beads). The beads were spun down; the supernatant was taken for later analysis; the beads were washed five times with PBS; and the adsorbed material was eluted with electrophoresis sample buffer (0.1 M Tris-HCl, 20% glycerol, and 2% sodium dodecyl sulfate, pH 6.8), incubated for 5 min at 95 °C, cooled, and microcentrifuged. The supernatant fraction from the vesicle immunoadsorption assay and the immunoadsorbed extract were subjected to immunoblot analysis.

In some assays, antibodies 1F8 (5–7 μg) and 3F8 (3 μg) were incubated overnight at 4 °C with goat anti-mouse IgG or goat anti-mouse IgM coupled to agarose (75 μl of bead volume). Beads were collected by a 6-s spin in a Microfuge and washed in PBS. LDM preparations (15–25 μg of proteins) were incubated with 1F8- or 3F8-agarose overnight at 4 °C in the absence of detergents (0.1% bovine serum albumin and 1 mM EDTA in PBS; final volume, 200 μl). The agarose beads and vesicles bound to them were collected by a 6-s spin in a Microfuge. The vesicles that were bound to the immobilized antibody were washed in PBS. The adsorbed material was eluted with electrophoresis sample buffer.

The immunotitration experiments illustrated in Figs. 4 and 5 were performed with antibodies bound to acrylamide beads prepared as described above. LDM membranes (50 μg) were incubated overnight at 4 °C with different mixtures of two batches of beads (one batch with 1F8 and one with γ-globulin as nonspecific antibodies), corresponding to varying amounts of 1F8 (0–7 μg) in a constant total bead volume of 20 μl. The adsorbed and nonadsorbed membranes were then processed as described above.

Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis was performed on a membrane protein following the method of Laemmli (33). Proteins were transferred to Immobilon as previously reported (34) in buffer consisting of 20% methanol, 200 mM glycine, and 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% nonfat dry milk and 0.02% sodium azide in PBS for 1 h at 37 °C and were incubated with antibodies in 1% nonfat dry milk and 0.02% sodium azide in PBS. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished using ^{125}I-protein A for 4 h at room temperature. Detection of the immune complex with monoclonal

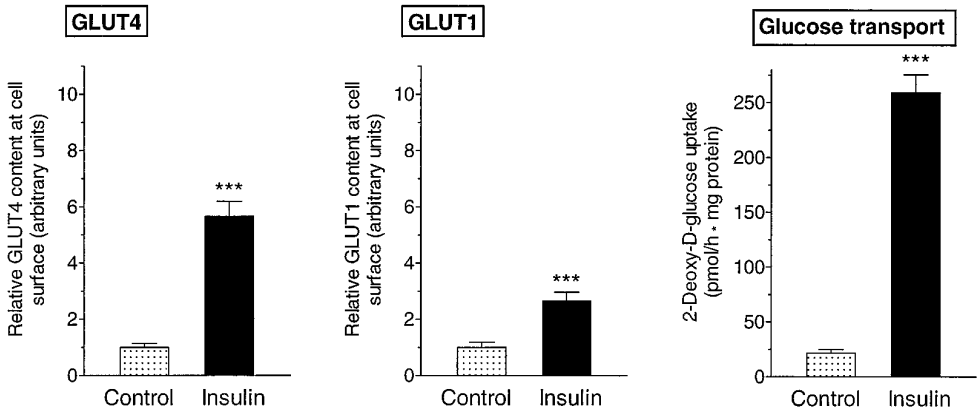

FIG. 1. **Quantitative analysis of the effects of insulin on cell surface labeling of GLUT4 and GLUT1 and on glucose transport in intact cardiomyocytes.** Cardiomyocytes were exposed to insulin (10 nM) for 30 min at 37 °C. The samples were then subjected to labeling, solubilization, immunoprecipitation, and gel electrophoresis, and the amount of labeled transporters was determined as described under "Materials and Methods." The rate of 2-deoxy-D-glucose uptake was measured in parallel samples (*right panel*). Results shown are means of 16–18 independent experiments ± S.E. (*bars*). The labeling data are expressed as values normalized to control. The statistical significance of the differences from control was assessed by paired Student's *t* test; ***, $p < 0.001$ *versus* control.

antibodies was performed using sheep anti-mouse ^{125}I-labeled antibody. Antibody 3F8 was detected using horseradish peroxidase linked to a goat anti-IgM mouse secondary antibody and visualized using an ECL system. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RESULTS

Effects of Insulin on Cell Surface Content of GLUT4 and GLUT1 and Quantification of Total Glucose Transporter Content of Cardiomyocytes—To quantify the effect of insulin on cell surface GLUT4 and GLUT1 we used the selective, nonpermeant, photoreactive bismannose compound ^{3}H-ATB-BMPA, which has proven to give a more accurate picture of cell surface changes than classical membrane fractionation methods combined with Western blot analysis (12, 14, 16). ATB-BMPA labeling was performed according to a protocol that has previously been successfully used in adipocytes (12, 13, 35) or skeletal muscles (14–16) and that we have recently adapted to isolated cardiac muscle cells (27, 28).

Fig. 1 summarizes the quantitative data on the effects of insulin on the content of glucose transporters at the surface of cardiomyocytes. The hormone induced 5.7- and 2.7-fold increases in the amounts of GLUT4 and GLUT1, respectively, in this compartment (Fig. 1, *left* and *middle panels*). By comparison, insulin caused a 12.0 ± 0.74-fold stimulation of glucose transport in the same experiments (Fig. 1, *right panel*). It is worth mentioning that in the basal, *i.e.* nonstimulated, state and in terms of absolute signals, the level of GLUT4 labeling at the surface of cardiomyocytes already exceeded that of GLUT1 labeling by a factor of ~2.5 (not shown), so that with insulin treatment, there was about four times more GLUT4 than GLUT1 at the cell surface. Thus, the insulin-dependent increase in glucose transport is largely explained by a recruitment of glucose transporters (mainly GLUT4). The difference in the extent of GLUT translocation and glucose transport stimulation might be due either to a change in intrinsic activity of recruited transporters or to a slightly higher accuracy of the transport assay in comparison with the labeling method.

It should be noted that the quantitative evaluation of the effects of insulin and the comparison of GLUT1 and GLUT4 relies on the assumption that the hormone does not modify the reactivity of the cell surface transporters, and that both isoforms display the same labeling efficiency. In this context, it was previously shown that the K_d of ATB-BMPA binding is similar for both carrier isoforms and is not altered by insulin (36). Importantly, the K_i value found for the inhibition of glucose transport by ATB-BMPA in cardiomyocytes (~180 μM; not shown) was similar to that determined in erythrocytes (29), rat adipocytes (13), and 3T3-L1-adipocytes (36). The K_i was unchanged with insulin treatment (13, 36). Moreover, incorporation efficiency of ATB-BMPA into GLUT1 and GLUT4 was consistently found to be the same in different cell types, including rat adipocytes (13, 37), 3T3-L1-adipocytes (36), and *Xenopus* oocytes (37), and to be very high under the conditions used (which were the same as in this study; Refs. 13, 29, and 36). Finally, insulin had no effect on the total level of ATB-BMPA incorporation as determined in cardiomyocytes permeabilized with the pore-forming agent α-hemolysin or by sonication (data not shown). This finding is in line with previous investigations, which have shown that the total amount of labeled GLUT1 and GLUT4 is the same in basal or insulin-stimulated 3T3-L1 adipocytes (38) or muscle (14). Overall, these observations indicate that the labeling of ATB-BMPA to cell surface glucose transporters is independent of the cell type and is not affected by insulin.

Although the results presented above point to GLUT4 as the major transporter responsible for insulin-dependent glucose uptake, reports indicating a relatively high expression of GLUT1 in cardiac tissue (24, 25) prompted us to directly compare the total amounts of GLUT4 and GLUT1 in isolated cardiomyocytes by Western blot analysis. For this purpose, nonstimulated cardiomyocytes were homogenized as described under "Materials and Methods," and their content in GLUT4 and GLUT1 was estimated by comparing the cell samples with known standards obtained from intracellular adipocyte membranes (GLUT4 standard) or erythrocyte membranes (GLUT1 standard). Using this method, we found contents of 1.2 pmol of GLUT4 and 0.47 pmol of GLUT1/mg of protein ($n = 2$). In other words, GLUT1 makes up nearly 30% of the total glucose transporter content in these cells. This substantial level of GLUT1 expression was essentially confirmed by experiments in which we have attempted to determine the total amounts of GLUT1 and GLUT4 by using ATB-BMPA in cardiomyocytes permeabilized with either α-toxin or by sonication. These experiments even yielded a higher proportion of GLUT1 (up to 50%; data not shown), although we cannot rule out that the labeling efficiency of intracellular GLUT4 might be lower than that of GLUT1.

Characterization of GLUT4-containing Vesicles and Co-localization of GLUT4, GLUT1, and SCAMPs in Intracellular

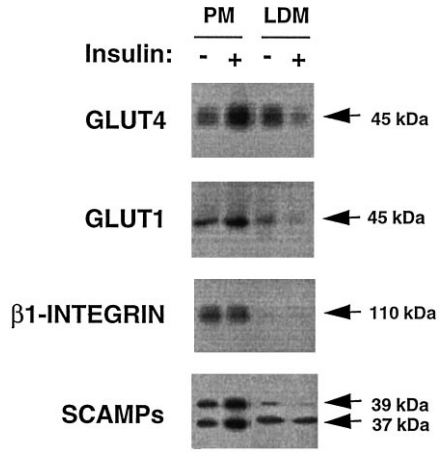

FIG. 2. **Effect of insulin on GLUT4, GLUT1, β_1-integrin, and SCAMP distribution in intracellular and plasma membranes from cardiomyocytes.** Isolated cardiomyocytes were incubated for 30 min with or without insulin (10 nM), as indicated, before PM and LDM fractions were obtained as described under "Materials and Methods." GLUT4, GLUT1, β_1-integrin, and SCAMPs were detected by immunoblot analysis by using specific antibodies. Equal amounts of membrane proteins (5 μg for GLUT4, 15 μg for GLUT1, 30 μg for β-integrin, and 10 μg for SCAMPs) from PMs or LDMs were laid on gels. Representative autoradiograms, obtained after various times of exposure, of six to eight separate experiments are shown.

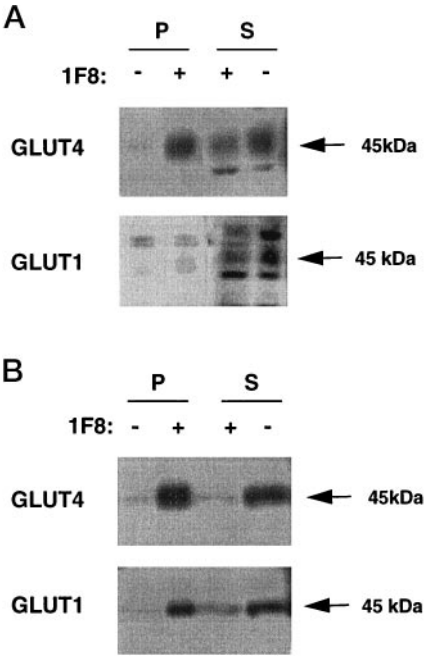

FIG. 3. **Immunoadsorption of GLUT4 and GLUT1 in intracellular membranes from unstimulated cardiomyocytes.** LDM membranes obtained from nonstimulated cardiac myocytes were immunoadsorbed with 1F8-agarose beads (*A*) or with 1F8-acrylamide beads (*B*) (+) or with beads covered with nonspecific antibodies (−). After the incubation, the adsorbed fraction (pellet, *P*) and nonadsorbed fraction (supernatant, *S*) were electrophoresed and immunoblotted to determine the abundance of GLUT4 and GLUT1. Representative autoradiograms, obtained after various times of exposure, of six to eight separate experiments are shown.

Membranes—In view of the fact that GLUT4 and GLUT1 showed differential recruitment to the cardiomyocyte surface in response to insulin, and regarding the high level of GLUT1 expression in these cells, we decided to characterize the intracellular insulin-sensitive GLUT4 pool and the degree of co-localization of GLUT4 and GLUT1 in this compartment. In addition, the distribution of SCAMPs has also been examined, because these proteins have been reported to co-localize with GLUT4 in isolated rat adipocytes (39, 40).

To this end, subcellular fractionation of isolated rat cardiomyocytes was performed as previously reported (26). This procedure results in the isolation of several membrane fractions. One of these fractions (PM, see "Materials and Methods") was enriched in the plasma membrane marker enzyme ouabain-sensitive *p*-nitrophenylphosphatase (41) by a factor of 13.5 (2.40 *versus* 0.17 nmol/h/μg of protein), whereas the specific activity of the sarcoplasmic reticulum marker, the EGTA-sensitive Ca^{++}-ATPase (42), was decreased by a factor of 3.6 (0.41 *versus* 1.88 nmol/h/μg of protein) when compared with crude cell homogenates. PM was also highly enriched in cell surface markers such as β_1-integrin (Fig. 2), the α_1-subunit of the Na^+-K^+-ATPase, and dystrophin, as determined by immunoblot analysis (not shown). Another fraction (LDMs) contained substantial amounts of GLUT4, GLUT1, and TGN 38 (a trans-Golgi marker) and was nominally free from plasma membrane markers or EGTA-sensitive Ca^{++}-ATPase activity (data not shown).

In keeping with prior observations (26) and with the data shown in Fig. 1, the incubation of cardiomyocytes with insulin caused a significant increase in GLUT4 and GLUT1 in plasma membranes by 138 ± 35% and 61 ± 18%, respectively (Fig. 2). The results indicate, in agreement with data illustrated in Fig. 1, that insulin causes a greater recruitment to the cell surface of GLUT4 than GLUT1. Under the same conditions, no alterations in the abundance of β_1-integrin (Fig. 2), the α_1-subunit of the Na^+-K^+-ATPase, or dystrophin were detected (data not shown). Concomitantly, there was a significant drop in the abundance of GLUT4 and GLUT1 in LDMs after insulin treatment (levels after insulin accounted for 40 ± 8% and 48 ± 6% of control values, respectively; $n = 6-8$ observations).

Western blot analysis of plasma and LDM membranes with anti-SCAMP antibodies revealed two distinct bands showing apparent molecular masses of 37 and 39 kDa (Fig. 2), as previously shown in adipocytes and skeletal muscle (39, 40). Acute insulin treatment resulted in a redistribution of SCAMP 39 from LDMs to the plasma membranes (Fig. 2). Thus, insulin caused a significant increase in the abundance of SCAMP 39 in plasma membranes (94 ± 5% increase) and a concomitant significant decrease in LDMs (levels in the insulin-treated group accounted for 55 ± 8% of the unstimulated control group) (Fig. 2). A similar tendency was found with regard to SCAMP 37, which did not reach significance in PM (37 ± 34% increase in PM and 26 ± 7% decrease in LDMs after insulin treatment) (Fig. 2).

We next characterized the degree of co-localization of GLUT4, GLUT1, and SCAMPs in LDMs. To this end, we performed two different types of vesicle immunoadsorption assays with LDMs obtained from unstimulated cardiomyocytes: 1) immunoadsorption of GLUT4-containing vesicles with 1F8 antibody (monoclonal antibody against GLUT4) noncovalently bound to agarose beads, and 2) immunoisolation of GLUT4 vesicles with 1F8 covalently linked to acrylamide beads. Immunoadsorption of GLUT4 vesicles with 1F8-agarose resulted in the specific recovery of near 60% of total GLUT4 present in LDMs (Fig. 3 and Table I); however, on adsorption with 1F8-acrylamide, approximately 80% of all GLUT4 was specifically recovered in the immunoprecipitates (Fig. 3 and Table I). Based on this, we reasoned that this different efficiency of the two protocols to immunoisolate GLUT4 vesicles may be used to characterize the extent of co-localization of GLUT4 with other proteins such as GLUT1 and SCAMPs in LDMs. As shown in Fig. 3 and Table I, after immunoadsorption with 1F8-agarose, only 15% of GLUT1 originally contained in LDMs was immunoadsorbed. Similarly, only 24% of SCAMP 39 was adsorbed to

TABLE I
Recovery of GLUT4, GLUT1, and SCAMPs on immunoisolation of intracellular membranes from unstimulated cardiac myocytes

LDM membranes obtained from nonstimulated cardiac myocytes were immunoadsorbed with 1F8-agarose beads, 1F8-acrylic beads, 3F8-agarose beads, or beads linked to nonspecific antibodies. After the incubation, the adsorbed and nonadsorbed fractions were electrophoresed and immunoblotted to determine the abundance of GLUT4, GLUT1, and SCAMPs. Autoradiograms were subjected to scanning densitometry. Data are means ± S.E. of three to six experiments (with the exception of data on GLUT1 after immunoadsorption with 3F8-agarose beads, which are the mean of two observations) and expressed as percentages of specific immunoadsorption.

Immunoadsorption agent	GLUT4	SCAMP 39	GLUT1
	% of specific immunoadsorption		
1F8-agarose	60.1 ± 2.2	24.2 ± 7.1	15.2 ± 2.7
1F8-acrylamide	79.2 ± 0.9	55.4 ± 7.1	63.7 ± 6.6
3F8-agarose	36.6 ± 5.7	63.0 ± 15.6	24.7

1F8-agarose (Table I). When using 1F8 coupled to acrylic beads, four times as much GLUT1 and more than twice as much SCAMP 39 were specifically immunoprecipitated when compared with 1F8-agarose (*i.e.* 64% of all GLUT1 contained in LDMs and 55% SCAMP 39; Fig. 3 and Table I). Thus, this contrasts with the relatively small increase of 20% in GLUT4 recovery (*i.e.* 80% of total GLUT4 with 1F8-acrylamide *versus* 60% with 1F8-agarose; Fig. 3 and Table I).

Taken together, these results might be interpreted in terms of the existence of two distinct intracellular GLUT4 membrane populations in LDMs from unstimulated cardiomyocytes: GLUT4 vesicles showing a low GLUT1 and SCAMP 39 content and GLUT4 vesicles showing a high GLUT1 and SCAMP 39 content.

Immunoadsorption of LDMs was also carried out with antibodies directed against SCAMPs (antibody 3F8 linked to agarose beads). With this approach, nearly 63% of SCAMP 39 but only 37% of GLUT4 and 25% of GLUT1 were recovered (Table I). This finding suggests the existence of a subpopulation of LDMs that is enriched in SCAMP 39 compared with GLUT4. This type of analysis could not be extended to GLUT1 vesicles, since the anti-GLUT1 antibodies used showed little efficiency in immunoadsorbing GLUT1 vesicles (data not shown).

The conclusion that LDMs may contain at least two populations of GLUT4-containing vesicles, on the grounds of the immunoprecipitation experiments described above, is limited by the fact that it is based on the comparison of data obtained from two different protocols (adsorption with 1F8-agarose *versus* adsorption with 1F8-acrylamide; Table I). To directly examine the hypothesis of different vesicle populations, we performed immunotitration experiments using varying amounts of 1F8 (bound to acrylamide) to adsorb GLUT4 vesicles in LDMs from nonstimulated cells and determined the amount of recovered GLUT4, GLUT1, and SCAMPs by Western blot analysis. We found that about 10 times less 1F8 antibody (~0.7 μg) was required to reach a saturating degree of GLUT4 adsorption than was the case for either GLUT1 (7 μg of 1F8; Fig. 4) or SCAMPs (Fig. 5). These observations thus confirm the existence of an intracellular vesicle pool that is enriched in GLUT4 but poor in GLUT1 and SCAMPs.

Finally, it was verified that the GLUT4-containing vesicles immunoadsorbed with 1F8-acrylamide, as described above, represents an insulin-sensitive pool. Therefore, the action of insulin on the abundance of GLUT4, GLUT1, and SCAMP 39 was studied in GLUT4 vesicles immunoadsorbed with a saturating amount of 1F8-acrylamide (to obtain GLUT4 vesicles with a high content in GLUT1 and SCAMP 39; see above). In this fraction, there was an insulin-dependent reduction in the amount of GLUT4 (−46%), GLUT1 (−45%), and SCAMP 39 (−59%) (Fig. 6*A*). Similarly, insulin reduced the GLUT4 content in GLUT4 vesicles obtained with 1F8-agarose (55% decrease; Fig. 6*B*).

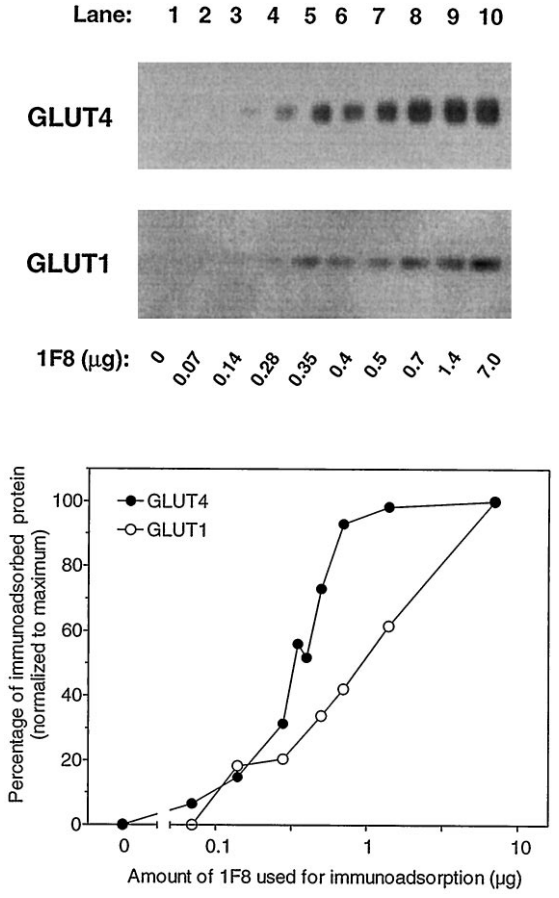

FIG. 4. **Immunotitration of GLUT4 and GLUT1 in intracellular membranes from unstimulated cardiomyocytes.** LDM membranes obtained from nonstimulated cardiac myocytes were immunoadsorbed with increasing amounts of 1F8 acrylic beads (corresponding to the 1F8 quantities indicated). GLUT4 and GLUT1 were detected in the adsorbed fractions by Western blotting. *Upper panel,* representative autoradiograms. *Lower panel,* quantitative analysis. The amounts of immunoadsorbed GLUT4 and GLUT1 were determined by densitometry of autoradiograms such as those shown above and normalized to the maximal amount precipitated with a saturating dose of 1F8 in the same experiment. Data are means from two to five independent experiments. (Note that the percentage values plotted here are not expressed in the same way as those shown in Table I, which were normalized to the total amount in LDM; *e.g.* 100% GLUT1 as plotted in this figure would be equivalent to ~64% in Table I.)

DISCUSSION

Quantification of GLUT1 and GLUT4 and Insulin-dependent Recruitment to the Plasma Membrane—A first issue addressed in this study is the direct comparison of the contents of GLUT4 and GLUT1 in isolated rat cardiomyocytes and of the relative contribution of these isoforms to the effect of insulin on glucose uptake. As shown in Fig. 1, GLUT4 is recruited to the plasma membrane to a larger extent than GLUT1 in response to insulin, as quantified by ATB-BMPA labeling. This is confirmed by experiments such as that illustrated in Fig. 2, in which the relative effects of insulin on the level of GLUT4 and GLUT1 were evaluated in purified plasma membranes by Western blot analysis. It is also worth mentioning that the absolute level of ATB-BMPA labeling of GLUT4 at the surface of nonstimulated cardiomyocytes already exceeded that of GLUT1 labeling by a

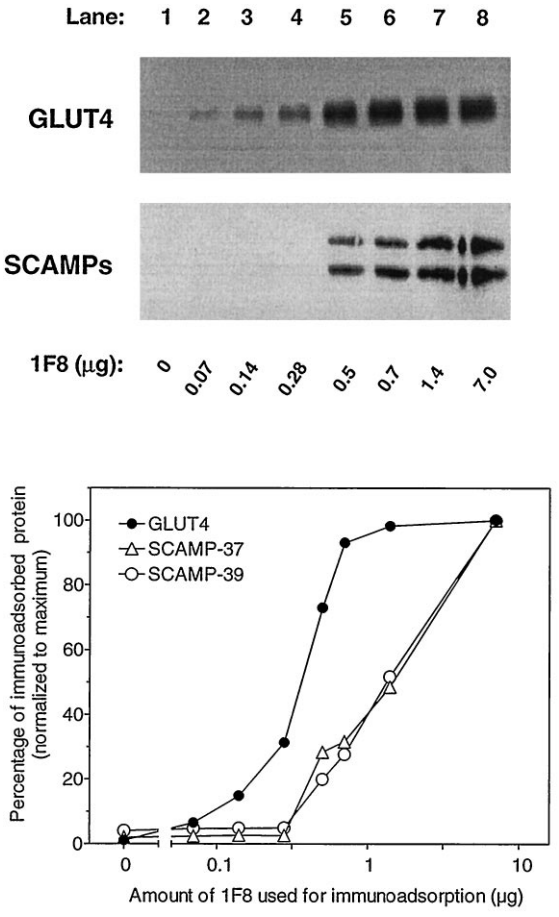

FIG. 5. **Immunotitration of GLUT4 and SCAMPs in intracellular membranes from unstimulated cardiomyocytes.** LDM membranes were immunoadsorbed with increasing amounts of 1F8-acrylamide, and the content of GLUT4 and SCAMPs in the adsorbed material was determined as described in the legend of Fig. 4. *Upper panel,* representative autoradiograms. *Lower panel,* quantitative analysis. Data are means from four independent experiments and expressed as in Fig. 4.

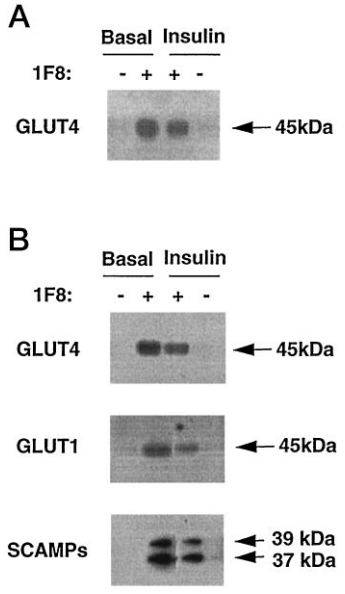

FIG. 6. **Effect of insulin on the content of GLUT4, GLUT1, and SCAMPs in immunoadsorbed intracellular GLUT4-containing vesicles.** LDM membranes obtained from basal (*i.e.* unstimulated) or insulin-treated cardiac myocytes were immunoadsorbed with 1F8 (+) or with antibodies from nonimmune serum (−) coupled to agarose beads (*upper panel*) or to acrylamide beads (*lower panel*) as described under "Materials and Methods." The immunoadsorbed material was subsequently subjected to immunoblot. Representative autoradiograms of three to eight separate experiments are shown.

factor of ~2.5 (not shown), and the preferential recruitment of GLUT4 by insulin will accentuate the importance of GLUT4 as the predominant transporter in the plasma membrane. Thus, these results clearly indicate that GLUT4 is responsible for a large part of insulin-stimulated glucose uptake in these cells. This is in line with prior observations in adipocytes and skeletal muscles (12–14, 16).

On the other hand, we found a high degree of GLUT1 expression in cardiomyocytes, as determined by quantitative Western blot analysis, which confirms qualitative and semiquantitative data obtained by others in heart tissue (24, 25). This high level of GLUT1 expression appears to be a unique property of the heart among insulin-sensitive tissues. Thus, GLUT1 accounts for only a very small percentage of total glucose carriers (~5–10%) in adipocytes (19, 43) and skeletal muscles (20, 25) (*versus* ~30% in cardiomyocytes). This large amount of GLUT1 in cardiomyocytes, along with the observation that it is recruited by a variety of agents in these cells (26–28), suggests that this transporter may play a specific role in the regulation of cardiac glucose transport. Several pathophysiological situations illustrate the possible importance of GLUT1 for cardiac glucose uptake. For instance, it was recently reported that the basal and insulin-dependent glucose transport in isolated perfused rat hearts is decreased following *in vivo* treatment with triiodothyronine (mimicking hyperthyroidism) (44), an intervention known to selectively reduce the myocardial level of GLUT1 but not GLUT4 (45). Similarly, fasting over 24–48 h was shown to dramatically diminish the basal rate as well as the insulin sensitivity of myocardial glucose uptake *in vivo*, with a concomitant reduction in the amount of cardiac GLUT1 (25). It is also worth mentioning that streptozotocin-induced diabetes also causes a decrease in GLUT1 protein in the rat heart (46).

The fact that insulin induces a more modest translocation of GLUT1 than GLUT4 suggests that the specific function of GLUT1 in these cells lies in the mediation of other effects than that of insulin. One possibility worth being explored is that the intracellular GLUT1 pool of heart cells is more responsive to stimuli such as contraction or anoxia than to insulin. In this context, one may speculate that GLUT1 becomes redistributed to this pool after cardiomyocyte isolation (*e.g.* as a consequence of the arrest of contractile activity).

Characterization of Intracellular GLUT4-containing Membranes—We have further explored the intracellular insulin-sensitive GLUT4 pool by performing vesicle immunoisolation analysis in LDMs from rat cardiomyocytes. Immunoadsorption assays with 1F8-agarose resulted in a high recovery of GLUT4 but in a low recovery of GLUT1 and SCAMP 39; in contrast, immunoadsorption with 1F8-acrylamide led to a large increase in the amount of GLUT1 and SCAMP 39 recovered, with a relatively modest increase in the degree of GLUT4 recovery (Table I). In other words, immunoadsorption with 1F8-agarose yields GLUT4 vesicles with a low GLUT1 and low SCAMP 39 content (GLUT4 pool 1), whereas immunoadsorption with 1F8-acrylamide yields, in addition, GLUT4 vesicles with a high GLUT1 and high SCAMP 39 content (GLUT4 pool 2). Furthermore, immunotitration experiments with LDM membranes show that a much smaller amount of 1F8 is required to reach a saturating degree of GLUT4 recovery than was the case for GLUT1 (Fig. 4) or SCAMPs (Fig. 5). Thus, our data are compatible with the existence of at least two different intracellular

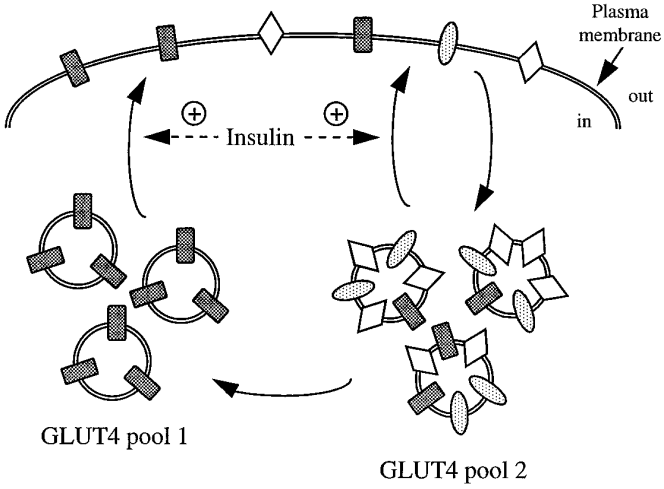

FIG. 7. **Hypothetical model of GLUT4 and GLUT1 distribution in rat cardiomyocytes.** According to this model, intracellular vesicles containing GLUT4 (*filled rectangles*) consist of at least two pools in nonstimulated cardiomyocytes: one (pool 1) with a low content of GLUT1 (*open diamonds*) and SCAMPs (*dotted ovals*) and the other (pool 2) with a relatively high content of GLUT1 and SCAMPs. Both pools are acutely recruitable by insulin. For details see "Discussion."

GLUT4 populations (see Fig. 7).

Immunocytochemical studies have localized GLUT4 in several distinct intracellular sites in adipocytes (47) and cardiomyocytes (18). Furthermore, kinetic studies are indicative of the existence of two intracellular GLUT4 pools in adipocytes. First, mathematical analysis of kinetic data of GLUT4 endocytosis and exocytosis obtained with the photoaffinity reagent ATB-BMPA (6, 35) predicts that GLUT4 localizes to at least two distinct intracellular compartments in fat cells (48). Second, data of subcellular trafficking of both GLUT4 and GLUT1 and chimeric transporters in adipocytes (49) are best explained by a model postulating two intracellular pools (50). According to the predictions of this model, GLUT4 is internalized into an endosomal compartment and then sorted into an insulin-recruitable compartment; in contrast, GLUT1 is endocytosed into the endosomal compartment and recycles from this compartment to the cell surface (50). Further support to the idea that GLUT4 is present in separate intracellular compartments comes from very recent compartment ablation analysis; thus, ablation of the endosomal compartment in 3T3-L1 adipocytes reduces by 40% the amount of cellular GLUT4 (51).

In view of all this and considering that SCAMPs are known markers of the endosomal compartment (52), we propose that GLUT4 pool 2 is of endosomal nature and might be similar to the endosomal GLUT4 pool predicted in adipocytes (48, 50) (see Fig. 7). Our finding that insulin depletes GLUT1 and SCAMP 39 from GLUT4 pool 2 is consistent with the model proposed by Yeh *et al.* (50), according to which GLUT1 would recycle from the endosomal compartment to the cell surface in adipocytes. Furthermore, GLUT4 pool 1 might be analogous to the insulin-recruitable compartment proposed by others on the basis of kinetic (48, 50) and immunoadsorption (53) experiments. In keeping with this, we found that GLUT4 was markedly depleted from the 1F8-agarose immunoprecipitates after insulin treatment (Fig. 6).

In this context, other experiments further support the notion of a different intracellular location of GLUT4 and GLUT1. Thus, quantitative Western blot analysis showed that the molar ratio of GLUT1:GLUT4 is much smaller in LDMs from nonstimulated cardiomyocytes (0.1:1; not shown) than that found in crude homogenates of these cells (0.4:1; see "Results"). This suggests the existence of an additional GLUT1 pool that is not part of LDMs (and is therefore distinct from pools 1 and 2).

Acknowledgments—The expert and devoted technical assistance of Christiane Löken and Ilinca Ionescu is gratefully acknowledged. We also thank Dr. Samuel W. Cushman and Dina R. Yver for providing some of the antibodies used in the labeling experiments.

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