The Transferrin Receptor Defines Two Distinct Contraction-Responsive GLUT4 Vesicle Populations in Skeletal Muscle

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Insulin and contraction increase glucose transport in an additive fashion in skeletal muscle. However, it is still unclear whether they do so by inducing the recruitment of GLUT4 transporters from the same or distinct intracellular compartments to the plasma membrane and the T-tubules. Using the transferrin receptor as a recognized marker of recycling endosomes, we have examined whether insulin and/or contraction recruit GLUT4 from this pool to either the plasma membranes or T-tubules, isolated by subcellular fractionation of perfused hindlimb muscles. Either stimulus independently increased GLUT4 translocation from an intracellular fraction to both the plasma membrane and T-tubules. The combination of insulin and contraction induced a marked (approximately threefold) and almost fully additive increase in GLUT4 content, but only in the plasma membrane. Insulin did not stimulate transferrin receptor recruitment from the GLUT4-containing intracellular fraction to either the plasma membrane or the T-tubules. In contrast, contraction stimulated the recruitment of the transferrin receptor from the same GLUT4-containing intracellular fraction to the plasma membrane but not to the T-tubules. Contraction-induced recruitment of the transferrin receptor was also observed from immunopurified GLUT4 vesicles. It is concluded that muscle contraction stimulates translocation of GLUT4 from two distinct intracellular compartments: 1) a population of recycling endosomes that is selectively recruited to the plasma membrane and 2) from GLUT4 storage vesicles that are also insulin-responsive and recruited to both the plasma membrane and the T-tubules. The lack of additive translocation of GLUT4 to the T-tubules may be linked to the failure of GLUT4-containing recycling endosomes to be recruited to these structures. *Diabetes* 49:183–189, 2000

I t is known that the maximal effects of insulin and contraction on glucose transport are additive in mammalian skeletal muscle, strongly suggesting that these stimuli act via separate pathways (1,2). When skeletal muscle is activated by either of these stimuli, GLUT4-enriched vesicles are translocated from intracellular storage site(s) to the cell surface by a mechanism that is still not fully understood. One important question that remains unclear is whether insulin and contraction recruit GLUT4 from one or more intracellular storage compartments. It has been suggested that these stimuli induce the translocation of GLUT4 from different intracellular pools in skeletal muscle, based on the indirect observations made in some studies that exercise or muscle contraction failed to reduce GLUT4 content in an insulin-sensitive intracellular membrane fraction (3–7). However, other studies showed that exercise did decrease GLUT4 content in intracellular membrane fractions that are also responsive to insulin (8–11). It is possible that the latter intracellular membrane fractions contained a mixture of distinct exercise- and insulin-sensitive GLUT4 pools, which would explain their responsiveness to both stimuli. The existence of more than one intracellular GLUT4 storage compartment in muscle cells is also supported by the work of Coderre et al. (12), who reported the isolation of distinct insulin- and exercise-sensitive GLUT4 intracellular pools based on differences in sedimentation coefficients determined by sucrose velocity gradient analysis. Analysis of these membrane pools by SDS-PAGE and silver staining has shown that they have a similar polypeptide composition. Thus, while there is some evidence to suggest that there are distinct insulin- and contraction-sensitive intracellular GLUT4 pools, it is not known whether there is a selective recruitment of GLUT4 from these pools, by either insulin or contraction, to the plasma membrane or the T-tubules.

Immunoelectron microscopic studies have shown that GLUT4 is mainly found in tubulo-vesicular organelles clustered in the cytoplasm of fat and muscle cells. However, a significant proportion of GLUT4 can also be observed in several structures thought to represent elements of the endocytic pathway (13–15). The localization of GLUT4 into endosomal structures has been recently supported by the observation that a substantial part of GLUT4 is colocalized with endosomal markers such as the transferrin receptor (TfR) and IGF-II/mannose-6-phosphate receptor (16,17). Accordingly, compartmental ablation of TfR-containing endosomes, with a transferrin-horseradish peroxidase conjugate, revealed that

~40% of intracellular GLUT4 is colocalized with the TfR in 3T3-L1 adipocytes (18). Whether these TfR-associated GLUT4 vesicles are recruited by insulin is still unclear. Kandror and Pilch (17) recently reported that GLUT4 recycles along with the TfR between the endosomal compartment and the plasma membrane in rat adipose cells. On the other hand, ablation of the TfR$^+$ compartment with the transferrin-horseradish peroxidase conjugate delayed but did not block GLUT4 appearance at the plasma membrane of adipocytes (19). This result suggests that in 3T3-L1 adipocytes, insulin rapidly activates the movement of GLUT4-containing endocytic vesicles to the cell surface, or alternatively, that GLUT4 transits through the endosomal system before its arrival at the plasma membrane.

Few studies have been conducted in skeletal muscle and the results are more controversial. Subcellular fractionation and morphological studies have previously shown that GLUT4 and the TfR are not (20) or are partly colocalized in muscle (16,21,22). Whereas insulin was found to cause a small but detectable translocation of the TfR in one study (16), it failed to do so in others (21,22). On the other hand, muscular contraction was reported to increase TfR content in a fraction enriched with both plasma membranes and T-tubules (22). It is therefore possible that insulin and/or contraction mobilize GLUT4 from endocytic vesicles in skeletal muscle.

In previous studies, we have shown that insulin and contraction recruit GLUT4 to the plasma membrane and T-tubules (10,11,23). Whether GLUT4 is selectively recruited from the endocytic vesicles to either cell surface compartments is not known. Therefore, in the present study, we have compared the independent effects of insulin and/or contraction as well as the combined effects of these stimuli on the subcellular distribution of GLUT4 and the TfR using a unique subcellular fractionation protocol that allows the isolation and separation of plasma membranes, T-tubules, and GLUT4-enriched intracellular membranes in distinct fractions. Our results are consistent with the existence of two distinct contraction-sensitive intracellular GLUT4 vesicle populations that are recruited to the plasma membrane (TfR$^+$) or the T-tubules (TfR$^-$) in skeletal muscle. Moreover, only the TfR$^-$ GLUT4 vesicles are recruited by insulin to both the plasma membrane and the T-tubules.

RESEARCH DESIGN AND METHODS

Insulin and/or contraction treatment protocols. Overnight-fasted male Sprague-Dawley rats (275–325 g body weight) were used in these studies. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight). They were surgically prepared for hindquarter perfusion as described previously (24) and heparinized with 1,000 IU heparin injected into the inferior vena cava. Then, catheters were inserted into their aorta and inferior vena cava. The rats were killed by injecting 0.5 ml of 0.76 mol/l KCl and were immediately placed in the perfusion apparatus. Thereafter, the perfusion procedure was carried out as we have described (24). The perfusate was a glucose-free medium containing Krebs-Henseleit solution, bovine erythrocytes (obtained in a local abattoir 1–2 days before perfusion) at a hematocrit of 30%, 0.15 mmol/l pyruvate, and 5% bovine serum albumin (BSA) dialyzed (cutoff at 10–15 kDa) for 48 h with Krebs-Henseleit solution. The perfusate was gassed with 95% O_2 and 5% CO_2 and adjusted with NaOH to pH 7.45. Perfusate temperature was maintained at 37°C. The first 25 ml of the perfusate that passed through the preparation was discarded, and then the perfusate was delivered at a flow of 12.5 ml/min.

In half of the experiments, hindquarters were perfused for 15 min before insulin (20 mU/ml, cell-free medium) was added for the last 25 min of perfusion. A 40-min perfusion period was also used in the contraction experiments. However, in these groups, muscles were perfused for 29 min in the absence of insulin. During the last 11 min indirect muscle stimulation, via the sciatic nerve, was performed for 2 × 5-min bouts with 1-min rest (200 ms trains of 100 Hz, 0.1 ms duration delivered every second at 50–100 V). To induce isometric force production, the knee joint was fixed with a steel pin under the tibiopatellar ligament and the Achilles tendon was attached to an immovable hook. Immediately after insulin and/or contraction stimulation period, skeletal muscles (gactrocnemius, soleus, and plantaris) were removed, frozen in liquid nitrogen, and stored at –80°C.

Glucose transport measurements. Glucose transport was measured as previously described (24,25), using near-saturated concentrations of 3-O-methyl-D-glucose (^{3}H-3-O-MG; 40 mmol/l, 8 µCi). Extracellular space was determined by the inclusion of sorbitol (2 mmol/l, 16 µCi ^{14}C-sorbitol) in the perfusate. At the end of the perfusion periods, the red and white gasctrocnemius and soleus muscles were removed, trimmed of connective tissue and visible blood, and frozen in liquid nitrogen. Samples were stored at –80°C until analysis. The muscle samples were homogenized in ice-cold perchloric acid (0.6 N) and precipitated by centrifugation. The supernatant was used for the determination of transport.

Glucose transport was determined in muscles (red and white gactrocnemius and soleus) with well-known muscle fiber composition. This permitted calculation of the glucose transport across the entire perfused muscle bed that was sampled for the membrane studies (see SUBCELLULAR MEMBRANE FRACTIONATION). Calculations of glucose transport were based on the mass of selected muscle fibers within each muscle as reported in detail elsewhere (26).

Subcellular membrane fractionation. Plasma membranes, T-tubules, and intracellular membranes were isolated from 7–8 g of muscles (gactrocnemius, soleus, and plantaris) using the procedure developed in our laboratory (23,27). This subcellular fractionation protocol has been extensively characterized with immunologic and enzymatic markers (10,11,23,27). In brief, this technique allows the simultaneous and separated isolation of plasma membrane and transverse tubule vesicles from the same muscle homogenate. In addition, the use of a strong salt treatment (lithium bromide) yields an intracellular fraction that is greatly enriched with GLUT4 but devoid of plasma membranes and transverse tubule markers. Membrane protein concentrations were determined by the bicinchoninic acid assay (Pierce) using bovine serum albumin as standard. 5′-nucleotidase activity was determined as previously described (23,27).

Immunoadsorption of GLUT4-containing vesicles. For each sample, 80 µl of Dynabeads (M-280 sheep anti-rabbit IgG; Dynal, Lake Success, NY) were washed twice for 5 min with 400 µl of PBS (pH 7.4) containing 0.1% nonfat milk (NFM), once with 0.2 mol/l Tris (pH 7.4) for 4 h at 37°C, and rinsed twice with PBS/NFM for 5 min at 4°C. The coated beads were coupled to the GLUT4 antibody as follows: for each sample, 5 µl of GLUT4 polyclonal antibody (or pre-immune serum) were incubated with the beads overnight at 4°C with constant rotation. The coated beads were then washed four times with 400 µl PBS/NFM to remove unbound antibodies and were incubated again overnight at 4°C with 50 µg of the intracellular membrane fraction (L-IM) in a volume adjusted to 500 µl with PBS/NFM containing protease inhibitors. The beads were washed three times with PBS/NFM containing protease inhibitors and separated from unbound material by attraction to the magnet (Dynal). The supernatant (unbound material) and washes were pooled and centrifuged at 190,000g for 1 h. The sedimented unbound material (Sn) and the beads (immunoprecipitate [IP]) were resuspended in Laemmli sample buffer and incubated 1 h at 37°C before SDS-PAGE and Western blot analysis.

Western blot analysis. Membranes (10–25 µg) were subjected to SDS-PAGE on 7.5 or 13% polyacrylamide gels as described by Laemmli (28) and electrophoretically transferred to polyvinylidene difluoride (PVDF) filter membranes for 2 h. PVDF membranes were then incubated for 1 h at room temperature with buffer I (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl) containing 0.04% NP-40 and 0.02% Tween-20 and 3% bovine serum albumin (fatty acid free BSA; Sigma, St. Louis, MO), followed by overnight incubation at 4°C with primary antibodies as described in figure legends. The PVDF membranes were then washed for 30 min, followed by a 1-h incubation with either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (NEN Life Science, Boston, MA) in buffer I containing 1% BSA. The PVDF membranes were washed for 30 min in buffer I, and the immunoreactive bands detected by the enhanced chemiluminescence method. A muscle standard (an unrelated crude membrane fraction) was run on every gel for comparison of samples from different immunoblots.

Antibodies. A monoclonal antibody generated against the TfR was purchased from Zymed Laboratories (San Francisco, CA). Polyclonal antibodies against the GLUT4 glucose transporter and annexin II were purchased from Biogenesis (Brentwood, NH) and Santa Cruz Biotech (Santa Cruz, CA), respectively. A monoclonal (IIF7) antibody generated against the α_1-subunit of the dihydropyridine receptor (DHPr) was kindly provided by Dr. K. Campbell (University of Iowa, Iowa City, IA). The monoclonal antibody Mck-1 against the α_1-subunit of the Na/K-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts General Hospital, Charlestown, MA).

Data analysis. Autoradiographs were analyzed by laser scanning densitometry using a tabletop Agfa scanner (Arcus II; Agfa-Gavaert, Mortsel, Belgium) and quantified with the National Institutes of Health Image program. All data are expressed as means ± SE. The effect of insulin and/or contraction on the measured parameters were compared by a two-way analysis of variance (ANOVA).

TABLE 1
Individual and combined effects of insulin and electrically stimulated contraction on 3-O-MG transport by the perfused lower leg muscle bed

Conditions	Glucose transport [μmol 3-O-MG $\cdot$ g^{-1} $\cdot$ (5 min)$^{-1}$]
Control	0.53 ± 0.26[a]
Insulin	2.79 ± 0.19[b]
Electrical stimulation	3.22 ± 0.37[b]
Insulin + electrical stimulation	5.22 ± 0.64[c]

3-O-MG transport was measured and calculated as described in RESEARCH DESIGN AND METHODS. Values not sharing a common superscript are different at $P < 0.05$. 3-O-MG, 3-O-methyl-D-glucose.

RESULTS

The individual or combined effects of insulin or electrically stimulated contraction on glucose transport are shown in Table 1. Consistent with previous findings, those stimuli induced an additive increase in glucose transport in the same hindlimb muscles (i.e., gactrocnemius, soleus, and plantaris muscles) that were sampled for subcellular fractionation experiments.

We next investigated the effect of insulin and electrical stimulation on GLUT4 subcellular distribution in mixed hindlimb muscles (gactrocnemius, soleus, and plantaris). Surface and intracellular membrane compartments of resting and stimulated muscles were isolated from several rats and the identity of the fractions was confirmed using specific enzymatic and immunologic markers (Table 2 and Fig. 1). As expected, the enzymatic activity of 5'-nucleotidase was markedly enriched in plasma membrane fractions (Table 2). This activity was small in T-tubule–enriched fractions and nondetectable in intracellular membranes. The α_1-subunit of the Na/K-ATPase, an immunologic marker of the plasma membrane, was also predominantly enriched in the plasma membrane fraction in all muscle groups (Fig. 1). In contrast, the DHPr was detected mainly in T-tubule fractions, in accordance with the specific localization of this protein to the tubular extensions in skeletal muscle. Importantly, the L-IM fraction was depleted of these muscle cell surface markers.

The subcellular distribution of enzymatic and immunologic markers is in good agreement with our previous observations (10,11,23,27). The distribution or the content of the above markers was in general not affected by insulin and/or electrical stimulation. Only very minor differences in plasma membrane recovery and in the activity of 5'-nucleotisase activity of the T-tubule fractions were observed.

The subcellular distribution of GLUT4 and the TfR, as well as the effects of insulin and/or contraction on the content of these proteins in surface and internal fractions, is shown in Figs. 1 and 2. Representative Western blots of immunoreactive GLUT4 or TfR contents are illustrated in Fig. 1, whereas scanning data of relative concentrations of these proteins in the different fractions from control and stimulated muscles are presented in Fig. 2. As expected, GLUT4 was mainly enriched in the L-IM fraction in resting muscle. Interestingly, the TfR, a well-recognized marker of recycling endosomes, was also found to be enriched in this GLUT4-enriched intracellular fraction.

Insulin or contraction both stimulated the recruitment of GLUT4 from the L-IM fraction to the plasma membrane and T-tubule–enriched fractions. When used in combination, insulin and contraction resulted in a significantly additive increase in GLUT4 content in the plasma membranes. In that situation, GLUT4 was almost tripled in the plasma membrane as compared with resting condition (Fig. 2A, left panel). In marked contrast, the combination of these stimuli failed to increase T-tubule GLUT4 content more than did their individual effects (Fig. 2A, middle panel). Consistent with the lack of additive GLUT4 translocation to the tubular fraction, the combination of insulin and contraction caused only a small but not significant further reduction in GLUT4 protein amounts in the L-IM fraction (Fig. 2A, right panel).

The effects of these stimuli on TfR subcellular distribution were more selective than those observed on GLUT4. Muscle contraction stimulated TfR translocation from the L-IM fraction to the plasma membranes but not to the T-tubules (Fig. 1 and Fig. 2B). Furthermore, insulin failed to change the distribution of the TfR in the muscle. In accordance with the selective effects of contraction on TfR distribution, the combined effect of insulin and contraction was not different than that of contraction alone.
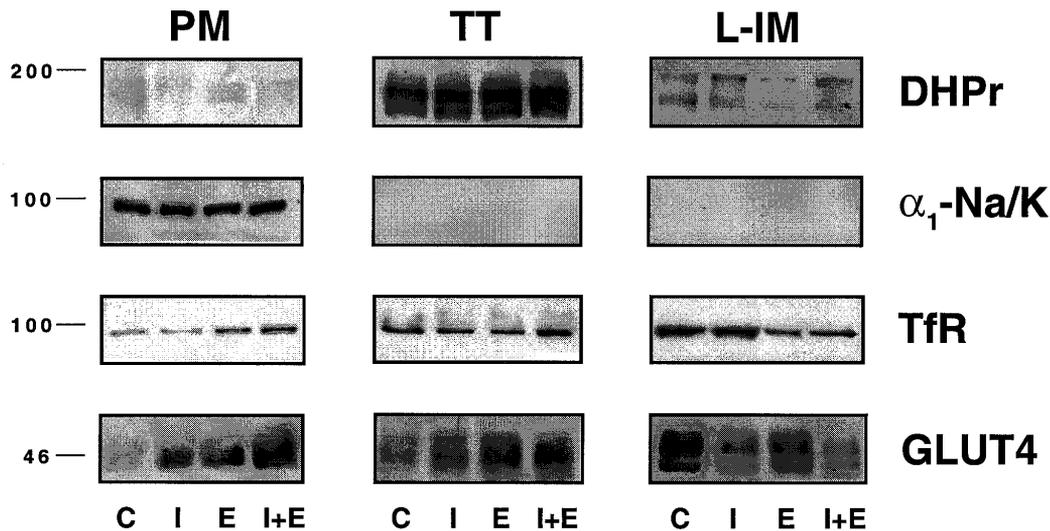
TABLE 2
Protein recovery and 5'-nucleotidase activity of isolated membrane fractions

Fractions	Conditions	Protein recoveries (μg/mg crude unfractionated membranes)	5'-Nucleotidase (nmol $\cdot$ mg^{-1} $\cdot$ min^{-1})
Plasma membranes	Control	20.07 ± 1.06	709.47 ± 78.27
	Insulin	16.62 ± 0.85	620.75 ± 56.72
	Contraction	14.68 ± 0.57	586.78 ± 37.54
	Insulin + contraction	13.52 ± 0.76	622.88 ± 63.32
T-tubules	Control	169.23 ± 15.28	84.12 ± 4.77
	Insulin	179.57 ± 8.02	72.83 ± 5.13
	Contraction	189.03 ± 10.31	55.21 ± 3.42
	Insulin + contraction	209.14 ± 11.86	53.94 ± 8.29
L-IM	Control	0.41 ± 0.09	ND
	Insulin	0.33 ± 0.06	ND
	Contraction	0.43 ± 0.07	ND
	Insulin + contraction	0.30 ± 0.06	ND

Datas are means ± SE of 8–10 individual membrane preparations. ND, nondetectable.

FIG. 1. Representative Western blots showing the effects of insulin and contraction and their combined effects on DHPr, α_1-Na/K ATPase, TfR, and GLUT4 content in plasma membranes (PM), transverse tubules (TT), and LiBr-released intracellular membrane (L-IM) fractions. A total of 10–25 µg of membrane proteins isolated from control (C), insulin (I), electrically stimulated contraction (E), and insulin + contraction (I + E) were used for Western blot analysis, as described in RESEARCHDESIGNAND METHODS. Immunoreactive bands were detected by the enhanced chemiluminescence method. Molecular weight standards are shown at the left.

Contraction may stimulate GLUT4 and TfR translocation from the same or different vesicle populations in the L-IM fraction. To address this important issue, we have purified GLUT4 vesicles from this fraction by immunoadsorption. As can be seen in Fig. 3, GLUT4 vesicles (IP fraction) were successfully purified from the L-IM fraction using this procedure. The overall efficiency of GLUT4 vesicle immunoadsorption was 75 ± 5, 77 ± 11, and 81 ± 16% in control, insulin-, and contraction-stimulated conditions, respectively (mean ± SE, n = 3–6), in these experiments. A significant proportion (58 ± 6%) of the TfR protein was found to co-purify with the GLUT4 vesicles in unstimulated muscle. In contrast, annexin II, a protein believed to be involved in endosome binding and/or fusion (29,30), was mostly recovered in the GLUT4-depleted vesicles of the unadsorbed supernatant fraction (Sn). Control experiments with pre-immune serum revealed that nonspecific immunoadsorption of GLUT4 was <10% (data not shown). Muscle contraction reduced TfR content in both purified GLUT4 vesicles and in the unadsorbed fraction without any detectable change in annexin II levels. In marked contrast, insulin failed to affect the TfR content in the GLUT4 vesicles despite the fact that it caused a significant reduction in GLUT4 in the same vesicles. A small but not statistically significant reduction in TfR levels was observed in the GLUT4-depleted vesicles of the unadsorbed fraction from insulin-treated muscle. Interestingly, the amount of TfR co-isolated with GLUT4 was not changed by insulin (57 ± 2 vs. 58 ± 6% for controls) but was significantly augmented by contraction (83 ± 11%, P < 0.05 vs. controls by ANOVA).

DISCUSSION

The principal objective of the present study was to determine whether insulin and contraction induce the recruitment of GLUT4 transporters from the same or distinct intracellular compartments to the plasma membrane and the T-tubules. Our hypothesis was that either one or both of these stimuli might induce translocation of GLUT4 from recycling endo-

somes as well as from the unique GLUT4 storage vesicular compartment that is segregated from the endosomal system. This hypothesis was based on previous studies showing that
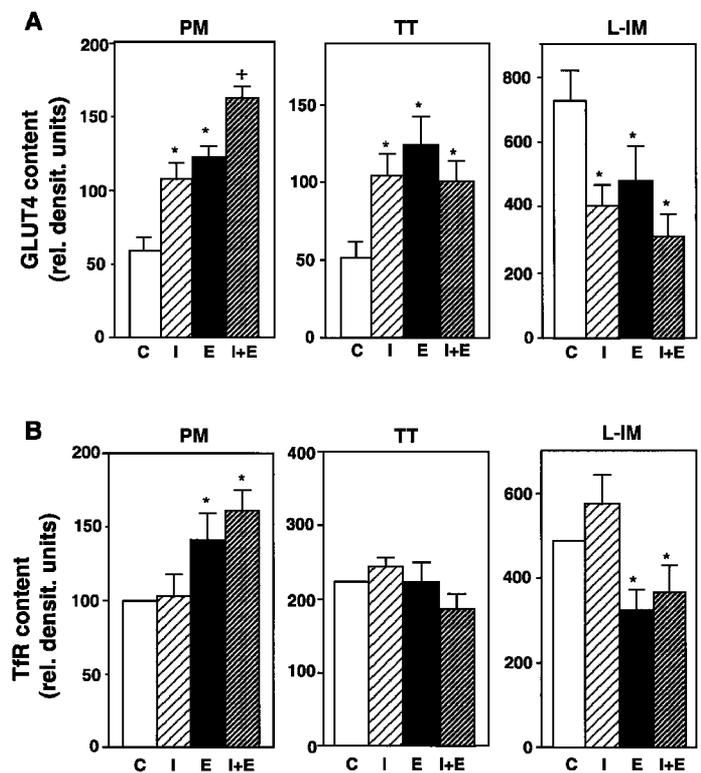

FIG. 2. GLUT4 (A) and TfR (B) content in plasma membrane (PM), T-tubule (TT), and L-IM fractions isolated from control (C), insulin (I), contraction (E), and insulin + contraction (I + E) muscles. Densitometric values are means ± SE of data obtained from 8–11 individual membrane preparations each performed with muscles from 3 animals in each experimental group. *P < 0.05 vs. controls, +P < 0.05 vs. insulin or contraction values alone.
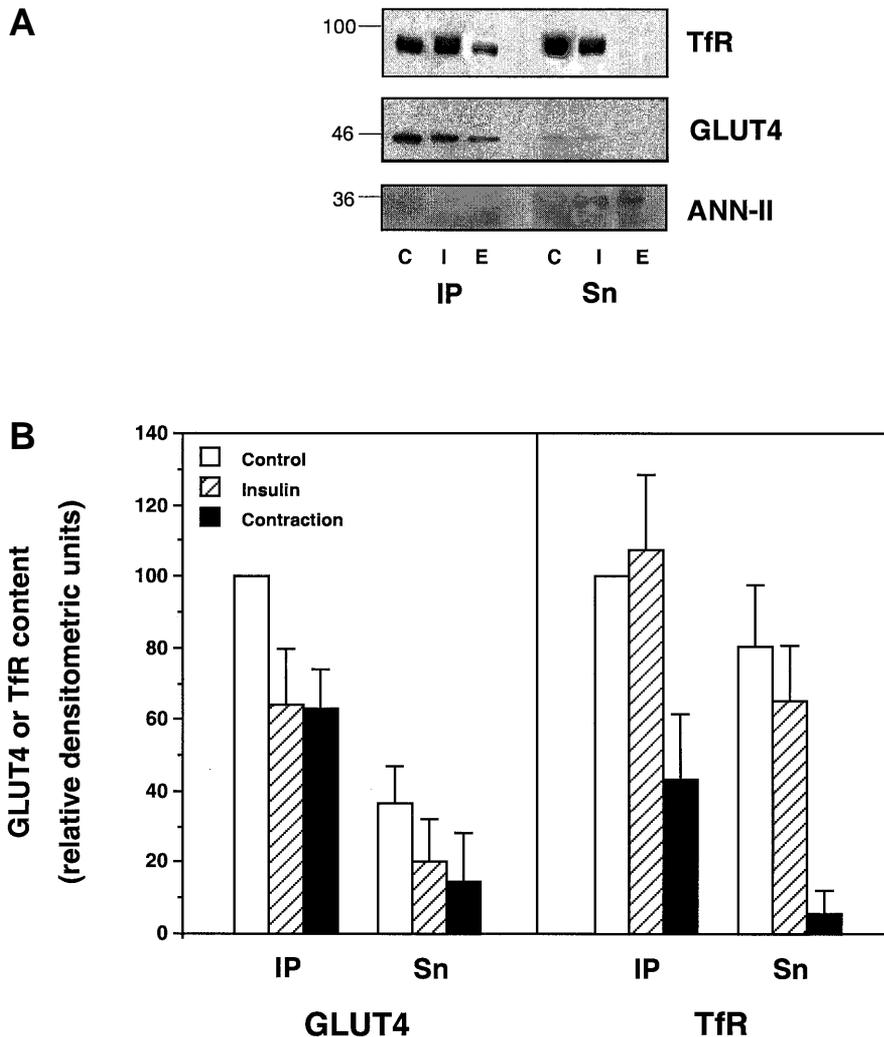
FIG. 3. Effects of insulin or contraction on GLUT4, TfR, or annexin II in immunoadsorbed GLUT4 vesicles. A: Representative Western blot showing the subcellular distribution of GLUT4, TfR, and annexin II in immunoisolated GLUT4-containing vesicles (IP) and unadsorbed fraction (Sn) isolated from control (C), insulin-stimulated (I), or contraction-stimulated (E) muscles. Immunoadsorption of GLUT4-containing vesicles was performed as described under RESEARCH DESIGN AND METHODS. A total of 50 µg of L-IM was subjected to immunoadsorption using sheep anti-rabbit IgG beads coupled to an anti-GLUT4 polyclonal antibody. IP and Sn fractions were then analyzed for GLUT4 and TfR by immunoblotting. Molecular weight standards are shown at the left. B: Means ± SE of 3–6 individual immunoadsorption experiments with L-IM membranes isolated from different animals.

GLUT4 and the TfR are partly colocalized in adipocytes and skeletal muscle, suggesting that the transporter is targeted to this recycling pathway in these cells (16–18,21). Using the TfR as a paradigm for recycling plasma membrane receptors, we confirmed that GLUT4 recycles at least in part through this pathway in muscle cells. Indeed, we found that GLUT4 and the TfR are colocalized in the transporter-enriched L-IM fraction and that ~60% of the TfR could be brought down by immunoadsorption of GLUT4 vesicles from unstimulated muscle. The vesicular association of intracellular GLUT4 and TfR reported here is in the same range of values reported in rat adipocytes (61%) and 3T3-L1 adipocytes (40%) (17,18). On the other hand, the extent of colocalization of these proteins is much greater than that previously reported in two other studies in which intracellular membrane fractions were isolated from skeletal muscle (~30%) (16,21). However, it should be noted that different muscle fractionation protocols were used in this and previous studies. Both TfR⁻ and TfR⁺ GLUT4 intracellular fractions have been previously isolated by Aledo et al. (20,21), but the latter fraction was shown to be unresponsive to either insulin or contraction (4,5,20,21), suggesting that the TfR-containing GLUT4 vesicles isolated in this and Aledo's studies are of different nature. On the other hand, the colocalization of GLUT4 and TfR reported here is in closer agreement with the recent observation that 52% of

GLUT4 staining overlapped with TfR staining in whole muscle fibers by confocal microscopy (22).

It was found that contraction, but not insulin, stimulates TfR translocation to the plasma membrane. These results are in line with those of Ploug and colleagues, who also reported that contraction redistributed the TfR to the cell surface but that insulin was without effect (22). In the latter study, however, it was not possible to determine if the TfR was recruited from GLUT4-containing or GLUT4-depleted endosomal vesicles, since the degree of association between these two proteins was assessed at the light microscopy level. The immunoadsorption experiments shown in Fig. 3 indicate that the TfR is recruited both from GLUT4-containing vesicles as well as from transporter-depleted vesicles. Thus, based on the premise that the TfR is a representative marker of recycling endosomes, it can be concluded that GLUT4 is localized to a subpopulation of recycling endosomes and that contraction increases GLUT4 and the TfR at the muscle plasma membrane at least in part through a selective mobilization of these vesicles. The endosomal compartment is known to be a complex structure and different endosome populations have been previously identified on the pathway of TfR recycling in CHO cells (31). The complexity of the endosomal apparatus has also been documented in adipocytes, based on the finding that the TfR and insulin receptor are recycled through different

endosomes (17). More studies will be needed to determine whether the GLUT4-containing and GLUT4-depleted recycling vesicles identified in the present study represent structurally distinct endosomes that share the TfR along its recycling pathway, or if these vesicles can be further distinguished on the basis of some functional differences. We would favor the latter hypothesis, based on the observation that contraction recruits the TfR more dramatically from the GLUT4-depleted recycling endosomes than from GLUT4$^+$ vesicles.

Insulin failed to affect TfR distribution in the present study. Only a small but variable decrease in TfR content was observed in the GLUT4-depleted vesicles from the supernatant of the immunoadsorptions. This decrease was too small to be detected in the L-IM fraction and this may explain why we could not detect an increase in TfR levels in the plasma membrane. We found a similar lack of TfR redistribution in muscle that has been stimulated with insulin for only 4 min (in vivo injection), ruling out the possibility that insulin may induce greater effect on TfR recycling at earlier time points (data not shown). A lack of significant effect of insulin on TfR recycling is consistent with recent reports that insulin failed to induce TfR translocation (22) or caused only a marginal redistribution of the receptor in skeletal muscle (16), and with the fact that the hormone increases cell surface appearance of endosomal proteins severalfold less than does GLUT4 in adipocytes (32,33). This small insulin-dependent reduction in TfR content from the GLUT4-depleted vesicles may represent the known stimulation by insulin of constitutive recycling of cell surface receptors within the endosomal system (32,33). The endosomal nature of this GLUT4-depleted fraction is supported by the presence of annexin II in these vesicles. The lack of effect of insulin on annexin II is consistent with a previous study conducted in adipocytes (34). These data further underscore the complexity of the endosomal system and they also suggest that this multicomponent organelle has evolved differently in skeletal muscle and adipose cells.

Although GLUT4 and the TfR were recruited to the cell surface by contraction stimulation, only the former was increased in both the plasma membrane and the T-tubules, the two cell surface components of the myocytes. The fact that GLUT4 was still recruited to the T-tubules in contracted muscle despite the lack of TfR mobilization indicate that a TfR$^-$ GLUT4 storage pool is translocated to the tubules by contraction. The simplest interpretation of these data is therefore that contraction mobilizes two distinct intracellular vesicle populations: a TfR$^+$ population of vesicles that is recruited only to the plasma membrane, and a TfR$^-$ population of vesicles that is translocated to the T-tubules. However, we cannot rule out that the TfR$^-$ GLUT4 vesicles are also recruited to the plasma membrane by contraction. It is also not possible to resolve from the present results if contraction and insulin induce the translocation of the same or different TfR$^-$ GLUT4 vesicles. On the other hand, the lack of additive effect of these stimuli on GLUT4 translocation to the T-tubules suggests that the same vesicles are mobilized and that the lack of additive translocation of GLUT4 to these structures is linked to the failure of TfR$^+$ GLUT4 vesicles to be recruited to the tubules.

It was observed that the amount of TfR co-isolated with GLUT4 was significantly augmented in contraction-stimulated muscles but not after insulin treatment. There are two possible explanations for the finding that contraction increased GLUT4-TfR vesicular association. First, contraction may have induced the translocation of more TfR$^-$ GLUT4 vesicles (to the plasma membranes and/or to the T-tubules) as compared to its selective effect to recruit TfR$^+$ recycling endosomes to the plasma membrane. Another possibility is that contraction caused the fusion of TfR$^-$ GLUT4 storage vesicles with TfR$^+$ recycling endosomes, thereby enhancing the colocalization of these two proteins. This latter possibility would be compatible with a model in which the contraction-responsive GLUT4 vesicles that are recruited to the plasma membrane traffic through the TfR-containing recycling endosomes.

The physiological relevance of this unique action of contraction to activate the translocation of GLUT4 from recycling endosomes remains speculative at this time. One possibility is that contracting myofibers need to obtain glucose very rapidly in order to cope with the energy demands, which are suspected to increase dramatically when contraction is initiated. Interestingly, studies in adipocytes have shown that the externalization rate of the TfR and other recycling proteins is faster than that of GLUT4 (35,36). Accordingly, Martin et al. recently reported that ablation of the recycling endosomal system with a transferrin-horseradish peroxidase conjugate delayed (but did not block) insulin-stimulated mobilization of GLUT4 to the plasma membrane of fat cells (19). These data suggest that the GLUT4 located in this recycling compartment is likely to reach the cell surface more rapidly than transporters not residing in it. Such a rapidly accessible GLUT4 pool would allow myofibers to quickly increase glucose transport when contraction is triggered. A need for a rapid stimulation of glucose transport is less critical when insulin is the only stimulus, presumably because glucose is not used as an immediate substrate but is mainly stored as glycogen in this condition.

It is interesting that a significant proportion of the TfR in the L-IM fraction is recruited from vesicles devoid of GLUT4 in working muscle. This suggests that the effect of contraction on TfR redistribution is not merely a consequence of moving GLUT4-containing vesicles to the cell surface. Rather, these results may suggest that muscular contraction stimulates the recycling of the TfR from multiple recycling endosomes, some also containing GLUT4. The activation of TfR recycling in contracted muscle may be important to maintain the levels and activities of iron-containing proteins involved in the respiratory capacity of muscle mitochondria. Indeed, iron deficiency has been reported to impair muscle performance and to decrease the capacity for aerobic metabolism by reducing the concentrations of myoglobin and several iron-containing proteins (e.g., cytochrome C oxidase) (37–39). Thus, recycling endosomes may fulfill the dual functions of providing both glucose and transferrin to contracting myofibers.

In summary, the present data show that contraction stimulates the translocation of GLUT4 from at least two distinct populations of intracellular vesicles: one population of TfR-enriched recycling vesicles that are selectively translocated to the plasma membrane, and one population of TfR$^-$ vesicles that are recruited to the T-tubules (and possibly the plasma membrane) and that may represent a specialized GLUT4 storage compartment. Insulin failed to activate GLUT4 translocation from the TfR-containing recycling vesicles. Our data further suggest that the lack of additive translocation of GLUT4 to the T-tubules may be linked to the failure of TfR$^+$ GLUT4 vesicles to be recruited to the tubules.

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