

## Glucose transporter protein content and glucose transport capacity in rat skeletal muscles

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HENRIKSEN, ERIK J., RAYMOND E. BOUREY, KENNETH J. RODNICK, LASZLO KORANYI, M. ALAN PERMUTT, AND JOHN O. HOLLOSZY. *Glucose transporter protein content and glucose transport capacity in rat skeletal muscles*. *Am. J. Physiol.* 259 (Endocrinol. Metab. 22): E593–E598, 1990.—The relationships among fiber type, glucose transporter (GLUT-4) protein content, and glucose transport activity stimulated maximally with insulin and/or contractile activity were studied by use of the rat epitrochlearis (15% type I-20% type IIa-65% type IIb), soleus (84-16-0%), extensor digitorum longus (EDL, 3-57-40%), and flexor digitorum brevis (FDB, 7-92-1%) muscles. Insulin-stimulated 2-deoxy-D-glucose (2-DG) uptake was greatest in the soleus, followed (in order) by the FDB, EDL, and epitrochlearis. On the other hand, contractile activity induced the greatest increase in 2-DG uptake in the FDB, followed by the EDL, soleus, and epitrochlearis. The effects of insulin and contractile activity on 2-DG uptake were additive in all the muscle preparations, with the relative rates being FDB > soleus > EDL > epitrochlearis. Quantitation of the GLUT-4 protein content with the antiserum R820 showed the following pattern: FDB > soleus > EDL > epitrochlearis. Linear regression analysis showed that whereas a relatively low and nonsignificant correlation existed between GLUT-4 protein content and 2-DG uptake stimulated by insulin alone, significant correlations existed between GLUT-4 protein content and 2-DG uptake stimulated either by contractions alone ( $r = 0.950$ ) or by insulin and contractions in combination ( $r = 0.992$ ). These results suggest that the differences in maximally stimulated glucose transport activity among the three fiber types may be related to differences in their content of GLUT-4 protein.

insulin; contractile activity; 2-deoxy-D-glucose uptake; antiserum R820; glucose transporter-4 protein; messenger ribonucleic acid

IN SKELETAL MUSCLE, glucose transport can be stimulated both by insulin and by contractile activity. The maximal effects of insulin and contractions on glucose transport are additive in mammalian skeletal muscle, providing evidence that these stimuli act via separate pathways (6, 25, 30). A number of studies have provided evidence that maximally insulin-stimulated glucose uptake is greater in type I (slow-twitch oxidative) than in type IIa (fast-twitch oxidative glycolytic) or type IIb

(fast-twitch glycolytic) skeletal muscle fibers (2, 15, 27). A similar pattern was observed in response to exercise in one study (16), with type I fibers showing the greatest increase in sugar uptake and type IIb fibers the smallest. However, in two other studies, the opposite pattern was seen, with type IIb fibers showing the largest increase in sugar uptake in response to contractile activity (27, 28).

James et al. (17), using a rabbit antiserum (R820) against the glucose transporter specific to adipocytes and skeletal muscle (GLUT-4), found that muscles consisting of predominantly type I and IIa fibers possess a greater amount of GLUT-4 protein than muscles consisting of type IIb fibers. In this context, the purpose of the present study was to test the hypothesis that glucose transport stimulated maximally by insulin and contractile activity in combination is related to the GLUT-4 protein content in skeletal muscle.

### MATERIALS AND METHODS

*Animals and muscle preparation.* Male Wistar rats (Sasco, Omaha, NE) weighing 60–80 g were used. Food was restricted to 3 g after 5:00 P.M. of the evening before the experiment. Animals were anesthetized with 5 mg/100 g body wt pentobarbital sodium administered intraperitoneally, and muscles were removed in the following order: epitrochlearis, soleus, extensor digitorum longus (EDL), and flexor digitorum brevis (FDB). These muscles were chosen because they represent a wide range of fiber-type compositions: epitrochlearis, 15% type I-20% type IIa-65% type IIb (24); soleus, 84-16-0% (1); EDL, 3-57-40% (1); and FDB, 7-92-1% (4). Soleus and EDL muscles were divided into two strips by splitting the proximal tendon and then gently pulling the two parts of the split tendon apart so that the muscle was separated longitudinally with the tendons still attached at both ends. All of the muscles used for incubations in this study were of approximately the same wet weight (12–15 mg).

*Muscle incubations.* To allow the soleus and EDL muscles to recover from the splitting procedure, all muscles were incubated initially for 60 min in 2 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB; 19) containing 8 mM glucose, 32 mM mannitol, 0.1% bovine

serum albumin (radioimmunoassay grade) before a 30-min incubation in identical medium in the absence or presence of 2 mU/ml pork insulin (Squibb, Princeton, NJ). The gas phase in the flasks was 95% O<sub>2</sub>-5% CO<sub>2</sub>. The flasks were shaken in a Dubnoff incubator at 35°C.

**Muscle stimulations.** For electrical stimulation, the distal end of the muscle was attached to a vertical Lucite rod containing two platinum electrodes (13). The proximal end was clipped to a jeweler's chain and attached to a Grass model FTO3C isometric force transducer. The mounted muscle was immersed in 20 ml KHB and continuously oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub> at 35°C. For activation of glucose transport activity by contractions, the muscles were stimulated with supramaximal square-wave pulses of 0.2-ms duration with a Grass S11 stimulator. Ten tetanic contractions were produced by stimulating at 100 Hz for 10 s at a rate of one contraction/min for 10 min. This stimulation protocol was found to elicit a maximal effect of muscle contractions on sugar transport, as evidenced by the finding that increasing the number of tetani from 10 to 15 had no further effect on glucose transport activity (Henriksen and Holloszy, unpublished data).

**Measurement of glucose transport activity.** Glucose transport activity was measured by use of the glucose analogue 2-deoxy-D-glucose (2-DG) and a modification (32) of the procedure used previously in frog sartorius muscle (13, 23). After the initial incubation periods or electrical stimulation, the muscles were rinsed in the absence of glucose for 10 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol and insulin, if present during the previous incubation period. The muscles were then incubated for 20 min at 29°C in 1.5 ml of KHB containing 1 mM 2-deoxy-[1,2-<sup>3</sup>H]glucose (1.5 mCi/mmol) and 39 mM [U-<sup>14</sup>C]mannitol (8 μCi/mmol) (ICN Radiochemicals, Irvine, CA), in the absence or presence of insulin. The gas phase in the flasks during both the rinse and incubation periods was 95% O<sub>2</sub>-5% CO<sub>2</sub>. The muscles were then processed, and the extracellular space and intracellular 2-DG concentrations were determined as described previously (32). Under these conditions 2-DG uptake is linear (Henriksen and Holloszy, unpublished observations). Glucose transport activity is expressed as μmol 2-DG·ml intracellular water<sup>-1</sup>·20 min<sup>-1</sup>.

**Measurement of GLUT-4 protein.** Muscles were frozen in situ using an aluminum clamp cooled in liquid N<sub>2</sub> and then homogenized in 30 vol of ice-cold phosphate-buffered saline (PBS, pH 7.4) containing 0.5% deoxycholate and 0.5% Triton X-100, using a Brinkmann homogenizer. To remove connective tissue, whole homogenates were centrifuged at 1,000 g for 5 min at 4°C. The protein concentration in the supernatant was assayed using a modification (26) of the Lowry method (22). An aliquot containing 100 μg of protein from each muscle sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) with a 10% acrylamide gel and transferred (Polyblot, Hayward, CA) to nitrocellulose filter paper.

GLUT-4 protein was detected by use of the antiserum R820, which is specific for the COOH-terminal peptide

sequence (residues 498-509) of this protein (17). The nitrocellulose papers were blocked for 90 min with PBS containing 50 mg/ml nonfat dried milk (Carnation, Los Angeles, CA) and then exposed to 10 μg/ml R820 for 1 h at 37°C. Thereafter, the blots were washed with PBS, incubated with 2.5 μCi <sup>125</sup>I-protein A (Amersham, Arlington Heights, IL) in 10 ml PBS for 1 h at room temperature, dried, and exposed to Kodak XAR-5 film at -70°C. The labeled bands were traced on the nitrocellulose, cut out, and counted in a gamma counter. Areas of equal size were excised from unlabeled areas, and counts from these bands were subtracted from the total counts. Results were corrected for standards to allow direct comparison of counts from different blots.

**Measurement of GLUT-4 mRNA.** Total RNA was extracted from tissues using the guanidine thiocyanate method of Chomczynski and Sacchi (5). The specificity of the cRNA probes was validated by use of Northern analysis (data not shown). Quantitation of mRNA was performed by means of dot blot hybridization with probes for the GLUT-4 gene (17) or β-actin. The clone from a rat heart cDNA library was subcloned in Bluescript SK<sup>+</sup> (Stratagene, La Jolla, CA), an RNA expression vector. Transcription of uniformly labeled <sup>32</sup>P-cRNA and synthetic mRNA with T<sub>3</sub> or T<sub>7</sub> RNA polymerase was performed according to the manufacturer's instructions. Aliquots of total RNA (0.5-5.0 μg) and dilutions of synthetic mRNA (0.5-1,000 pg) and of cDNA (1-1,000 pg) as standards were dissolved in 15% formaldehyde in ×10 SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and blotted onto Nytran membranes. Membranes were then hybridized to the corresponding cRNA probes for 16-18 h (GLUT-4 at 60°C, β-actin at 55°C) in 50% formamide in ×5 SSPE (0.9 M NaCl, 5 mM EDTA, and 50 mM NaH<sub>2</sub>PO<sub>4</sub>; pH 7.4), 0.2% SDS, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, and 200 μg/ml denatured, single-stranded salmon sperm DNA) and then washed (GLUT-4 at 65°C, β-actin at 52°C) in ×0.1 SSC and 0.1% SDS. Blots were exposed to Kodak XAR5 film at -80°C. The amount of mRNA was measured by densitometric analysis, comparing the intensity of the sample dot to standards.

**Statistical analysis.** The significance of differences between two groups was evaluated using the unpaired Student's *t* test. When multiple comparisons were made, a factorial analysis of variance was performed, with post hoc analysis using Scheffe's *F* test. Correlations were analyzed using univariate linear regression.

## RESULTS

**Glucose transport rates.** As shown in Fig. 1, maximally insulin-stimulated rates of 2-DG uptake differed greatly among the four muscles. The relative pattern of insulin-stimulated 2-DG uptake in the four muscles was: soleus > FDB > EDL > epitrochlearis, with the soleus having a roughly threefold greater rate of 2-DG uptake than the epitrochlearis. The relative magnitudes of the increases in 2-DG uptake induced by maximally effective contractile activity in the four muscles were: FDB > EDL = soleus > epitrochlearis (Fig. 1). The increases above basal

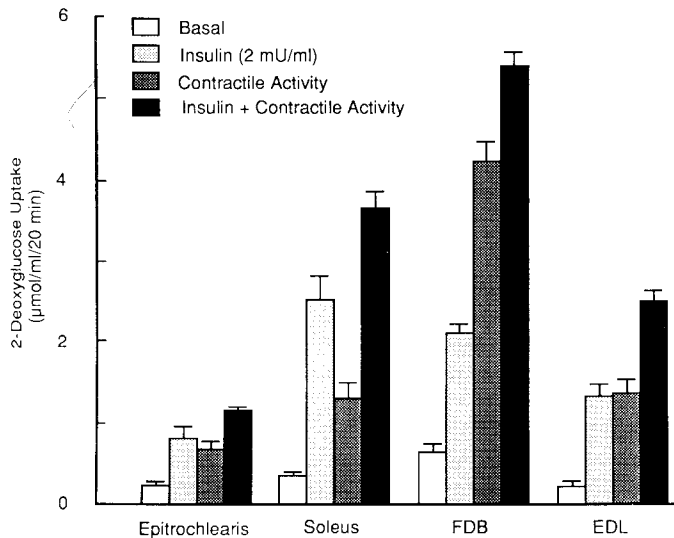


FIG. 1. Effects of insulin, contractions, or insulin and contractions in combination on 2-deoxy-D-glucose (2-DG) uptake in epitrochlearis, soleus, extensor digitorum longus (EDL), and flexor digitorum brevis (FDB). Muscles were initially incubated at 35°C for 60 min in Krebs-Henseleit bicarbonate (KHB) containing 8 mM glucose and 32 mM mannitol before being treated with or without insulin for 30 min. Muscles were then either washed immediately for 10 min at 29°C in glucose-free medium containing 40 mM mannitol and insulin, if present previously, or were first stimulated electrically to contract before subsequent incubations. Glucose transport activity was then assessed by measuring the intracellular accumulation of 2-DG at 29°C for 20 min. Values are means  $\pm$  SE for 6–12 muscles/group.

in glucose transport activity induced by contractile activity and by insulin were additive in all four muscles, with the relative rates being FDB > soleus > EDL > epitrochlearis (Fig. 1). This relative pattern of maximally activated glucose transport in the four muscles was also seen when sugar uptake was assayed at 0.1 mM 2-DG (epitrochlearis,  $0.20 \pm 0.02 \mu\text{mol} \cdot \text{ml}^{-1} \cdot 20 \text{ min}^{-1}$ ; EDL,  $0.33 \pm 0.03$ ; soleus,  $0.48 \pm 0.04$ ; FDB,  $0.65 \pm 0.03$ ), indicating that the above rates of 2-DG uptake (assayed at 1 mM) reflect glucose transport activity and not glucose phosphorylation capacity.

Of particular interest are the findings that 1) whereas insulin induced a greater increase in glucose transport activity in the soleus than in the FDB, contractile activity caused a threefold greater stimulation of sugar transport in the FDB than in the soleus; 2) glucose transport activity stimulated by 2 mU/ml insulin was more than twofold greater than contraction-stimulated sugar transport in the soleus; in contrast, insulin stimulated sugar transport was 50% as great as contraction-stimulated transport in the FDB; and 3) although insulin-stimulated sugar transport was about twofold greater in the soleus than in the EDL, contraction-stimulated sugar transport rates were similar in these two muscles. Although the magnitude of the effects of insulin and contractile activity on sugar transport was markedly different in the soleus and in the FDB, the effects of these two individual stimuli were similar in the EDL and in the epitrochlearis.

**GLUT-4 protein quantitation.** It has recently been demonstrated that adipose tissue and skeletal muscle express a distinct glucose transporter protein (14, 17) that is recognized by the antiserum R820 (17). We used

this antiserum to compare the relative abundance of the GLUT-4 protein in epitrochlearis, soleus, FDB, and EDL muscles. An autoradiogram of a representative immunoblot is presented in Fig. 2A. Equal amounts of protein (100  $\mu\text{g}$ /lane) were used. Quantitation of the GLUT-4 protein using immunoblots of five to six muscles per group showed that the FDB possessed the greatest content of this protein, followed by the soleus, EDL, and the epitrochlearis (Fig. 2B).

**Relationship between GLUT-4 protein content and glucose transport activity.** The correlations between the amount of GLUT-4 protein detected by R820 and the rates of 2-DG uptake stimulated by insulin alone (Fig.

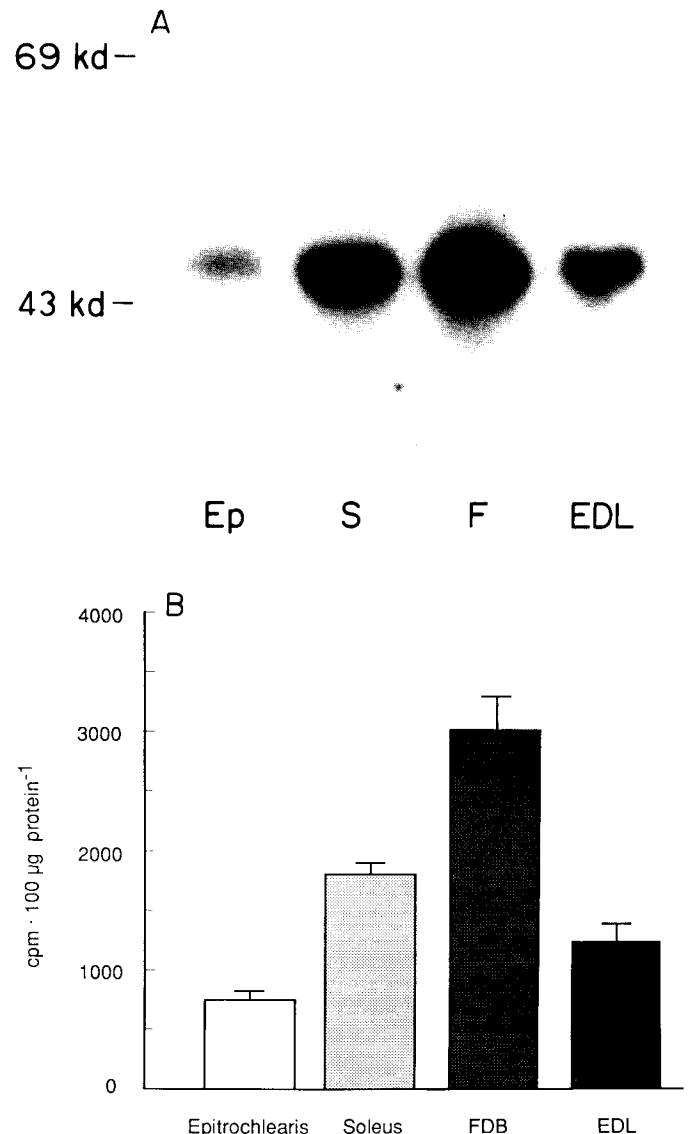


FIG. 2. Glucose transporter (GLUT-4) protein content in epitrochlearis, soleus, EDL, and FDB muscles. A: representative autoradiogram of immunoblot of GLUT-4 protein in epitrochlearis (Ep), soleus (S), EDL, and FDB (F) muscles. Protein (100  $\mu\text{g}$ ) from each muscle was analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antiserum R820 against GLUT-4 protein. Immunolabeled bands were then visualized with  $^{125}\text{I}$ -labeled protein A. B: bands from 5–6 individual immunoblots were counted for radioactivity as described in MATERIALS AND METHODS. Each bar represents means  $\pm$  SE.

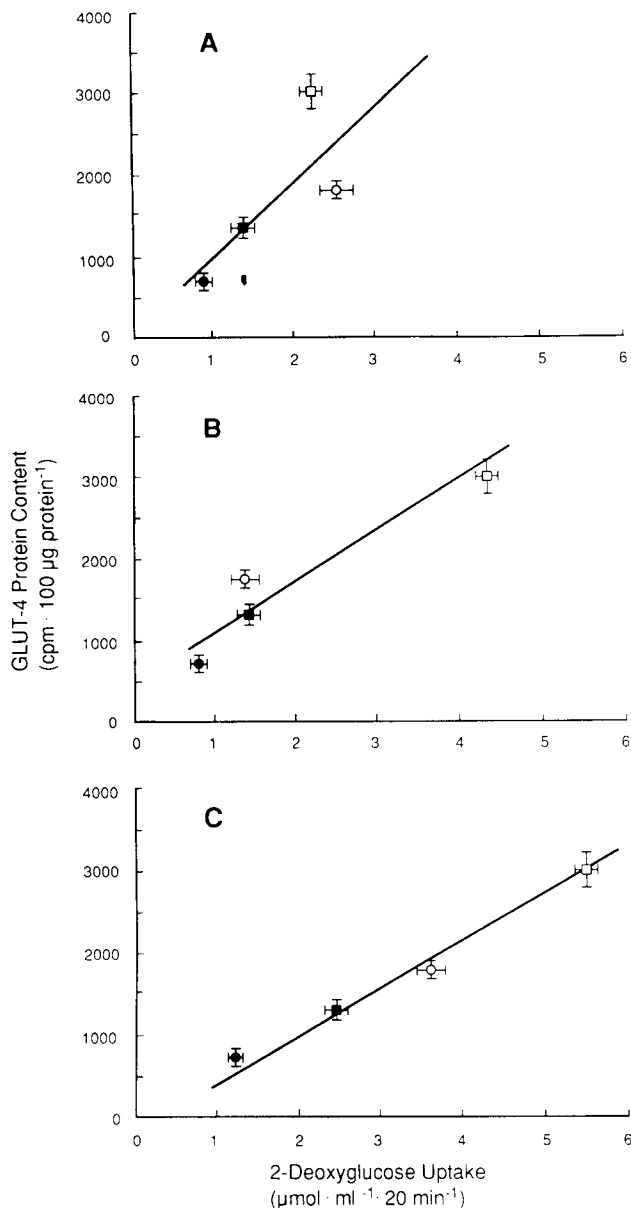


FIG. 3. Relationship between stimulated glucose transport and GLUT-4 protein content in epitrochlearis, soleus, EDL, and FDB muscles. Data shown in Figs. 1 and 2 for epitrochlearis (●), soleus (○), EDL (■), and FDB (□) were analyzed using linear regression to determine correlation between mean values for GLUT-4 protein content and 2-DG uptake stimulated by insulin alone (A), contractions alone (B), and insulin and contractions in combination (C). Regression equations and correlation coefficients: A,  $y = 910x + 86$ ,  $r = 0.720$ ; B,  $y = 592x + 537$ ,  $r = 0.950$ ; C,  $y = 551x - 74$ ,  $r = 0.992$ .

3A), by contractions alone (Fig. 3B), or by both stimuli in combination (Fig. 3C) were evaluated with linear regression analysis. Because it was not possible to measure these parameters on the same muscle, these correlations were made using the mean values from different groups of muscles. In muscles stimulated by insulin alone, a relatively low ( $r = 0.720$ , NS) correlation was found. There was a better correlation ( $r = 0.950$ ,  $P < 0.05$ ) between GLUT-4 protein content and 2-DG uptake in muscles stimulated by contractile activity (Fig. 3B). However, there was clearly a discrepancy between GLUT-4 protein content and contraction-stimulated 2-

DG uptake in the soleus. When 2-DG uptake was stimulated maximally by insulin and contractile activity in combination, there was an excellent correlation ( $r = 0.992$ ,  $P < 0.01$ ) between the GLUT-4 protein content and the rate of 2-DG uptake. These relationships were the same whether 2-DG uptake was expressed relative to muscle wet weight or muscle protein (data not shown).

**GLUT-4 mRNA.** Values for total RNA, GLUT-4 mRNA, and  $\beta$ -actin mRNA in the four muscles are shown in Table 1. Total RNA was slightly lower in the epitrochlearis than in the other muscles. Expressed relative to total RNA, GLUT-4 mRNA was higher in the FDB than in the other muscles. When expressed relative to muscle wet weight, significantly less GLUT-4 mRNA was detected in the epitrochlearis and significantly more GLUT-4 mRNA was found in the FDB in comparison with the soleus and EDL. No significant differences among muscles were observed for  $\beta$ -actin mRNA. These relationships are not altered when mRNA is expressed relative to muscle protein instead of muscle wet weight (data not shown). A significant correlation ( $r = 0.980$ ,  $P < 0.02$ ) was found between the GLUT-4 mRNA (pg/mg muscle) and GLUT-4 protein (counts  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>), indicating the probability that expression of this protein in various skeletal muscles is under pretranslational control.

#### DISCUSSION

The present results show that GLUT-4 protein content and glucose transport activity are closely correlated in muscles of different fiber-type composition when glucose transport is maximally stimulated by insulin and contractile activity in combination (Fig. 3C). This finding suggests that the content of GLUT-4 protein may be a major determinant of the capacity of muscle cells to take up glucose. It is interesting in this context that streptozocin-induced diabetes, which causes decreases in the maximal stimulation of glucose transport both by insulin and by contractions (31), also results in a decrease in the muscle GLUT-4 protein content (3).

Lillioja et al. (21) have reported that responsiveness of whole body glucose disposal to insulin in humans is positively correlated with the percent of type I fibers and negatively correlated with the percent of type IIb fibers in the vastus lateralis muscle. The present finding that maximally insulin-stimulated glucose transport was highest in the soleus, which consists of >80% type I fibers (1) and has a high content of GLUT-4 protein (Fig. 2), and was lowest in the epitrochlearis, which consists of 65% type IIb fibers (24) and has the lowest content of this protein (Fig. 2), could help to explain at the cellular level the finding of Lillioja et al. (21). This explanation depends on the assumption, which needs verification, that human and rat skeletal muscle fiber types are similar in their relative contents of the GLUT-4 protein and their responses to insulin.

Insulin causes the translocation of glucose transporters from an intracellular pool to the plasma membrane in adipocytes (7, 29). In skeletal muscle, both insulin (11, 18) and contractile activity (8, 10, 12) cause translocation of glucose transporters to the plasma membrane. Recent

TABLE 1. Total RNA, GLUT-4 mRNA, and  $\beta$ -actin mRNA in epitrochlearis, soleus, EDL, and FDB muscles

Muscle	Total RNA	GLUT-4 mRNA		$\beta$ -Actin mRNA	
	$\mu\text{g}/\text{mg}$ muscle	$\text{pg}/\mu\text{g}$ RNA	$\text{pg}/\text{mg}$ muscle	$\text{pg}/\mu\text{g}$ RNA	$\text{pg}/\text{mg}$ muscle
Epi	0.66 $\pm$ 0.12*	94 $\pm$ 10	53 $\pm$ 10 $\dagger$	1.84 $\pm$ 0.21	1.10 $\pm$ 0.13
Sol	0.89 $\pm$ 0.08	111 $\pm$ 8	95 $\pm$ 6	1.55 $\pm$ 0.12	1.49 $\pm$ 0.11
EDL	1.15 $\pm$ 0.10	89 $\pm$ 12	99 $\pm$ 10	1.41 $\pm$ 0.14	1.60 $\pm$ 0.19
FDB	1.11 $\pm$ 0.13	137 $\pm$ 11 $\ddagger$	146 $\pm$ 11 $\dagger$	1.29 $\pm$ 0.13	1.41 $\pm$ 0.22

Values are means  $\pm$  SE for 5-7 muscles/group. GLUT-4, glucose transporter; Epi, epitrochlearis; Sol, soleus; EDL, extensor digitorum longus; FDB, flexor digitorum brevis.  $P < 0.05$  vs. EDL\*; all other muscles $\dagger$ ; EPI and EDL $\ddagger$ .

studies using either the monoclonal antibody 1F8 (11) or the antiserum R820 (9) have provided evidence that it is the GLUT-4 protein that is translocated by insulin (11) or exercise (9). The strong relationship between GLUT-4 protein content and contraction-stimulated 2-DG uptake (Fig. 3B) provides further evidence that R820 recognizes not only an insulin-regulatable, but also a contractile activity-regulatable, glucose transporter protein in muscle.

Studies on the rat epitrochlearis muscle, which consists predominantly of type IIB fibers (24), have shown that the maximal effects of insulin and contractile activity on glucose transport activity are additive (6, 30). The present results show that the maximal effects of insulin and contractions on glucose transport are also additive in muscles made up predominantly of type I (soleus) or type IIA (FDB) fibers. One possible explanation for this finding is that there are two separate pools of glucose transporters in muscle, with one pool being available for translocation by the action of insulin but not contractions, whereas the other pool is susceptible to translocation by contractions but not insulin.

Attempts to directly evaluate this possibility have not been successful because of methodological limitations encountered in fractionating skeletal muscle. These include a large (>90%) and variable loss of plasma membrane and, likely, cross-contamination of the plasma membrane and intracellular glucose transporter fractions (11, 18). However, the present results provide strong, albeit indirect, evidence for the hypothesis that insulin and contractions stimulate the translocation of glucose transporters from two separate pools. This evidence includes the findings that 1) there is an excellent correlation between the content of glucose transporter protein and glucose transport activity after maximal stimulation by insulin and contractions in combination (Fig. 3C), but not by insulin alone (Fig. 3A), and 2) there are marked differences between type I and type IIA fibers in the relative magnitudes of the increases in glucose transport activity by insulin and by contractions (Fig. 1). In the case of type I fibers (soleus), insulin caused a more than 2-fold greater increase in glucose transport activity than did contractions, whereas in type IIA fibers (FDB) contractions caused a 2.5-fold greater increase in glucose transport activity than did insulin. These differences could be explained in the context of the hypothesis that there are two separate intracellular pools of glucose transporters if the relative sizes of these pools are different, i.e., a large insulin-regulatable and a small contraction-regulatable pool of glucose transporters in the so-

leus, with the converse, a large contraction-regulatable pool and a small insulin-regulatable pool of glucose transporters, being present in the FDB. Indeed, Douen et al. (9) have recently provided evidence supporting the existence of distinct glucose transporter pools recruitable by insulin or exercise. However, our data cannot rule out the alternative possibility that a single pool of glucose transporters may exist in skeletal muscle, with differential recruitment in response to insulin or contractile activity in various muscles.

Both GLUT-1 and GLUT-4 proteins have been detected in skeletal muscle (9). However, the GLUT-1 protein is located primarily in the plasma membrane, and only the GLUT-4 protein is translocatable in response to insulin or exercise (9). Because glucose transport capacity in muscle appears to be a function of both number and intrinsic activity of the glucose transporter proteins (11), future work should attempt to assess the possibility that intrinsic activity of either or both glucose transporter isoforms may be altered by insulin and/or contractions and that this activity may differ among muscles of different fiber-type compositions.

In conclusion, the present results show that there are large differences among skeletal muscle fiber types in their content of GLUT-4 protein detectable by the antiserum R820. These differences appear to have physiological significance, as evidenced by our finding of an excellent correlation between glucose transport activity stimulated by insulin and contractions and glucose transporter protein content in muscles of different fiber-type composition.

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