The effects of muscle contraction and insulin on glucose-transporter translocation in rat skeletal muscle

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The effect of electrically induced muscle contraction, insulin (10 m-units/ml) and electrically-induced muscle contraction in the presence of insulin on insulin-regulatable glucose-transporter (GLUT-4) protein distribution was studied in female Sprague–Dawley rats during hindlimb perfusion. Plasma-membrane cytochalasin B binding increased ~ 2-fold, whereas GLUT-4 protein concentration increased ~ 1.5-fold above control with contractions, insulin, or insulin + contraction. Microsomal-membrane cytochalasin B binding and GLUT-4 protein concentration decreased by approx. 30% with insulin or insulin + contraction, but did not significantly decrease with contraction alone. The rate of muscle glucose uptake was assessed by determining the rate of 2-deoxy[^{3}H]glucose accumulation in the soleus, plantaris, and red and white portions of the gastrocnemius. Both contraction and insulin increased glucose uptake significantly and to the same degree in the muscles examined. Insulin + contraction increased glucose uptake above that of insulin or contraction alone, but this effect was only statistically significant in the soleus, plantaris and white gastrocnemius. The combined effects of insulin + contraction of glucose uptake were not fully additive in any of the muscles investigated. These results suggest that (1) insulin and muscle contraction are mobilizing two separate pools of GLUT-4 protein, and (2) the increase in skeletal-muscle glucose uptake due to insulin + contraction is not due to an increase in plasma-membrane GLUT-4 protein concentration above that observed for insulin or contraction alone.

INTRODUCTION

Like insulin, muscle contraction can increase skeletal-muscle glucose uptake (Ivy and Holloszy, 1981; Young et al., 1983; Richter et al., 1985; Holloszy et al., 1986; Constable et al., 1988; Goodyear et al., 1990b). Several studies have indicated that both muscle contraction and insulin increase glucose transport into skeletal muscle by causing an increase in plasma-membrane insulin-responsive glucose transporter (GLUT-4) number, due to redistribution/translocation from an intracellular pool (Klip et al., 1987; Hirshman et al., 1988; Sternlicht et al., 1989; Douen et al., 1990a,b; Goodyear et al., 1990a; Fushiki et al., 1989).

Douen et al. (1989, 1990a,b) reported that exercise and insulin produced comparable increases in GLUT-4 protein in the plasma membrane. However, insulin, but not exercise, caused a decrease in GLUT-4 protein in the intracellular transporter pool. These results, along with the well-documented additive effect of muscle contraction and insulin on skeletal-muscle glucose uptake (Nesher et al., 1985; Ploug et al., 1987; Constable et al., 1988), suggest that muscle contraction and insulin may mobilize different intracellular pools of GLUT-4 transporters (i.e. contraction- and insulin-sensitive pools) (Nesher et al., 1985; Ploug et al., 1987; Sternlicht et al., 1989).

In contrast with this idea, Goodyear et al. (1991) and Fushiki et al. (1989) reported that exercise and insulin both increased plasma-membrane GLUT-4 protein concentration, concurrently with decreases in intracellular GLUT-4 protein concentration. These results indicate that muscle contraction and insulin are mobilizing the same intracellular pool of GLUT-4 protein. However, exercise causes a variety of hormonal and systemic changes, including an increase in skeletal-muscle insulin sensitivity and increases in muscle blood flow. Thus the differences among the results of Douen et al. (1989, 1990a,b), Goodyear et al. (1991) and Fushiki et al. (1989) are difficult to interpret, since the hormonal milieu to which the muscle was exposed is unknown, and small amounts of insulin that could have been present during exercise may have significant effects on transporter translocation. Hence, the first purpose of this study was to test the hypothesis that insulin- and contraction-sensitive intracellular pools of GLUT-4 protein exist in skeletal muscle. This was accomplished by using the perfused-hindlimb technique combined with electrically induced muscle contraction to assess the effects of insulin, muscle contraction, and the combination of insulin and muscle contraction on GLUT-4 protein translocation. By using this experimental paradigm, the effects of contraction itself on GLUT-4 protein translocation in the absence of insulin could be examined.

Additionally, neither Douen et al. (1989, 1990a,b) nor Goodyear et al. (1991) measured glucose uptake in the different fibre types of the rat under a maximally saturating concentration of glucose to determine the relationship between maximal muscle glucose uptake and plasma-membrane GLUT-4 protein concentration. Hence, the second purpose of this study was to determine if the rates of contraction- and insulin-stimulated glucose uptake under maximally stimulating conditions remain fully additive in the three fibre types of the rat, and if changes in skeletal-muscle glucose uptake relate to changes in glucose-transporter distribution.

EXPERIMENTAL

Animals

Some 35 female Sprague–Dawley rats (approx. 14 weeks old) obtained from Harlan Sprague–Dawley (Indianapolis, IN, U.S.A.) were randomly assigned to a basal (no insulin present), insulin, muscle contraction, or insulin + contraction group. All rats were housed three to a cage and were given laboratory chow

Abbreviations used: 2-DG, 2-deoxyglucose; KpNPPase, K^+-stimulated *p*-nitrophenol phosphatase; CB, cytochalasin B.

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and water *ad libitum*. The temperature of the animal room was maintained at 21 °C, and an artificial 12 h light/dark cycle was set. All experimental procedures were approved by the University of Texas Animal Care Committee.

Surgical preparation and hindlimb perfusion

Rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt.), following a 12 h fast. The surgical procedure for hind-limb perfusion of the rats and the perfusion apparatus used were similar to those previously described by Ruderman et al. (1971). Additionally, both sciatic nerves of the muscle-contracted rats were surgically exposed and attached to miniature electrodes. After completion of the surgical preparation, cannulas were inserted into the abdominal aorta and vena cava of the rats, and their hindlimbs were washed out with 50 ml of Krebs–Henseleit buffer (pH 7.4). Immediately thereafter, the cannulae were placed in line with the perfusion system and the hindlimbs were allowed to stabilize during a 15 min non-recirculating wash-out period. The perfusion medium consisted of Krebs–Henseleit buffer (pH 7.4) containing 4% dialysed BSA (Cohn fraction V; U.S. Biochemicals), 30% time-expired washed human red blood cells, 0.3 mM pyruvate and 0.5 mM glucose. Perfusate flow rate during the wash-out period was 15 ml/min. After the wash-out, perfusate samples were taken from the venous line for determination of insulin, and the rats were killed with an intracardiac injection of pentobarbital sodium. Insulin analysis indicated that the wash-out procedure effectively lowered the insulin concentration in the hind limbs below detection (< 2.5 μ-units/ml).

For determination of basal and insulin-stimulated glucose uptake, the perfusate contained 28 mM glucose, 2-deoxy-D-[G-^{3}H]glucose (2-DG) (7.5 μCi/mmol of glucose), 2 mM mannitol (60 μCi of D-[1-^{14}C]mannitol/mmol) and 0.3 mM pyruvate. The concentration of glucose used was selected because it was approx. 4-fold higher than the average K_m for insulin-stimulated muscle glucose uptake (Kubo and Foley, 1986), thereby ensuring that maximum velocity of uptake ($V_{max.}$) was measured. When insulin- and insulin + contraction-stimulated glucose uptakes were determined, the perfusate also contained 10 m-units/ml insulin. Perfusion times were 10 min for basal and 7 min for insulin-, contraction- and insulin + contraction-stimulated rats. Perfusate flow rate was maintained at 15 ml/min during measurement of glucose uptake under all four conditions.

For contraction- and insulin + contraction-stimulated glucose uptakes, the rats were surgically prepared as described previously (Brozinick et al., 1993). Briefly, the skin was reflected from both hindlimbs of the rat, and the Achilles tendons were then clipped from the feet. Jeweller's chains were clipped to both calcaneus/Achilles-tendon interfaces. The chains were attached to an apparatus that allowed for adjustment of muscle tension, with one chain in line with an isometric force transducer (Harvard Instruments, Millis, MA, U.S.A.), which was in turn connected to a chart recorder. Both hindlimbs of the muscle-contracted rats were then immobilized in a specially designed acrylic cradle, and the muscles of the triceps seri group were adjusted to achieve maximum twitch tension.

After the 15 min wash-out period, muscle contraction was initiated by stimulating the sciatic nerves with 200 ms trains of 100 Hz, with each impulse in a train being 0.1 ms. The trains were delivered at a rate of 1/s at a supra-maximum voltage (8–12 V) for 2 × 5 min separated by a 1 min rest period. Immediately before initiation of contraction, the perfusate flow rate was increased to 25 ml/min to ensure proper oxygen and substrate delivery to the hindlimb muscles. During contraction for the insulin + contraction rats, the perfusate was the same as in the wash-out period, except that it contained 10 m-units/ml insulin. Once the second stimulation period was completed, the perfusate was changed over to that used for the measurement of basal glucose uptake and the flow rate was decreased to 15 ml/min. Time of perfusion was set for 7 min. All perfusions were performed at 37 °C.

Immediately after each perfusion, the hindlimbs of the rats were washed out with 30 ml of Krebs–Henseleit buffer containing 0.3 mM pyruvate to remove intravascular erythrocytes. The soleus (slow-twitch red fibres), plantaris (10% slow-twitch red, 45% fast-twitch red, 45% fast-twitch white) and red (fast-twitch red) and white (fast-twitch white) portions of the gastrocnemius were removed and blotted on gauze dampened with Krebs–Henseleit buffer. The muscles were freeze-clamped in tongs cooled in liquid N_2 and stored at −80 °C until analysed for 2-DG uptake. These muscles were chosen because they represent the hindlimb muscle fibre-type composition of the rat (Ariano et al., 1973).

Determination of 2-DG uptake

The perfused muscle samples were weighed, homogenized in 1 ml of 10% trichloroacetic acid at 4 °C and centrifuged in a microcentrifuge (Fisher Scientific, Houston, TX, U.S.A.) for 10 min. Duplicate 0.3 ml samples of the supernatant were transferred to 20 ml scintillation vials containing 10 ml of Scintiverse E (Fisher Scientific) and vortex-mixed. For determination of perfusate specific radioactivity, well-mixed samples of the arterial perfusate were obtained during perfusion. The samples were deproteinized in 10% trichloroacetic acid and treated the same as the muscle samples. The samples were counted for radioactivity in a LS-350 liquid-scintillation spectrophotometer (Beckman, Fullerton, CA, U.S.A.). Efficiency and channel crossover were determined by counting radioactivity of ^{3}H and ^{14}C standards of known d.p.m. The accumulation of intracellular 2-[^{3}H]DG, which is indicative of muscle glucose uptake, was calculated by subtracting the concentration of 2-[^{3}H]DG in the extracellular space from the total muscle 2-[^{3}H]DG concentration. The extracellular 2-DG was quantified by measuring the concentration of [^{14}C]mannitol in the trichloroacetic acid supernatant.

Membrane preparation

Plasma and intracellular membranes were prepared as described in detail by Hirshman et al. (1990). Briefly, approx. 6 g of mixed rat skeletal muscle, consisting of the biceps femoris, extensor digitorum longus, flexor halicus longus, red and white gastrocnemius (RG and WG), plantaris, peroneus longus, semimembranosus, semitendinosus, soleus and tibialis anterior was removed at the end of perfusion, cleaned of fat and connective tissue, and weighed. These muscles were minced in a buffer consisting of 255 mM sucrose, 100 mM Tris and 0.2 mM EDTA, pH 7.6. The minced muscles were homogenized (Brinkman PT-10 Polytron) at a slow speed and then homogenized again with a Potter–Elvehjem Teflon/glass tissue grinder and adjusted to a volume of 25 ml with homogenization buffer. A 0.5 ml sample of this homogenate was removed for measurement of marker enzymes and protein, and then centrifuged at 34000 ***g*** for 20 min. The resulting pellet was used for purification of plasma membranes, whereas the supernatant was used for purification of microsomal membranes.

Microsomal and plasma-membrane suspension was weighed to determine the exact volume. A sample of each suspension was removed for determinations of marker enzymes and protein, and

Table 1 Body and muscle weights of rats used in membrane preparations, and protein recovery

Values are means ± S.E.M. for the numbers of experiments in parentheses: * significantly different from basal ($P < 0.05$).

	Basal (10)	Contraction (8)	Insulin (8)	Insulin + contraction (9)
Body wt. (g)	198.4 ± 4.2	203.8 ± 10.2	204.8 ± 10.1	205.1 ± 9.3
Muscle wt. (g)	6.43 ± 0.20	6.78 ± 0.33	6.83 ± 0.25	6.51 ± 0.06
Total protein				
Homogenate (mg)	811.2 ± 24.5	871.9 ± 53.5	864.7 ± 30.0	775.4 ± 37.2
Plasma membrane (mg)	1.21 ± 0.11	1.25 ± 0.06	1.36 ± 0.09	1.23 ± 0.07
Microsomal membrane (mg)	0.953 ± 0.051	0.714 ± 0.091	0.791 ± 0.108	0.621 ± 0.051*

the remainder was used for equilibrium D-glucose-inhibitable cytochalasin B (CB) binding and Western blotting. All samples were stored at −80 °C until assayed.

Protein and marker-enzyme assays

Homogenate and membrane protein were determined for each preparation (Table 1) by the Coomassie Brilliant Blue method (Bio-Rad protein assay, Richmond, CA, U.S.A.) as described by Bradford (1976), with crystalline BSA as the standard. K^+-stimulated *p*-nitrophenol phosphatase (KpNPPase) specific activity was measured as a marker for plasma membranes by the method of Bers et al. (1980). To determine if there was sarcoplasmic-reticulum contamination of the membrane fractions, both homogenate and membrane fractions were assayed for Ca^{2+}-stimulated ATPase (Ca^{2+}-ATPase) activity by the method of Seiler and Fleischer (1982). The validity of the Ca^{2+}-ATPase assay was verified in a preparation that was enriched in sarcoplasmic reticulum.

CB binding

The concentration of glucose transporters in the membrane fractions was measured by equilibrium D-glucose-inhibitable [^{3}H]CB binding as described by Wardzala et al. (1978). Briefly, Scatchard plots were generated from five-point binding studies, in which membranes were incubated with CB concentrations of 21, 42, 63, 83 and 125 nM in the absence or presence of 500 mM D-glucose. Cytochalasin E was added at a final concentration of 2000 nM to lower non-specific binding. Bound CB was separated from free by centrifugation at 48000 ***g*** for 40 min for plasma membranes, and 220000 ***g*** for 2 h for the microsomal membranes. A portion of the supernatant (0.025 ml) and the dissolved pellet were counted for radioactivity by liquid-scintillation spectrophotometry, and the counts were corrected for channel cross-over by counting standards of known d.p.m., to determine free and bound CB binding. Tracer amounts of [^{14}C]urea were added to account for trapped [^{3}H]CB in the pellet. The total number of glucose transporters ($B_{max.}$ or R_0) and the dissociation constant (K_d) were determined from a linear plot derived by subtracting curves generated in the presence of D-glucose from those generated in the absence of D-glucose.

Western blotting

Plasma and microsomal membrane protein (30 μg of sample protein) along with molecular-mass markers (Bio-Rad) were subjected to SDS/PAGE run under reducing conditions with a 12%-acrylamide resolving gel as described previously (Laemmli, 1970). Membrane samples from each group of rats (basal, insulin, contraction, and insulin + contraction) were run in adjacent lanes. Resolved proteins were transferred to poly(vinylidene difluoride) (PVDF) sheets (Bio-Rad) via the method of Towbin et al. (1979), utilizing the buffer system of Kyhse-Andersen (1984). Immunoblotting was performed as previously described (Banks et al., 1992). Briefly, the PVDF sheets were blocked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5, at 25 °C) and 5% non-fat milk. Next, the sheets were washed in TBS with 0.05% Tween-20 (TTBS) and then incubated with the polyclonal anti-GLUT-4 antibody R349 (given by Dr. Mike Mueckler, Washington University, St. Louis, MO, U.S.A.). The PVDF was then washed in TTBS and incubated with ^{125}I-labelled goat anti-rabbit IgG (New England Nuclear, Boston, MA, U.S.A.). Finally, the sheets were washed and air-dried. Antibody binding was detected by autoradiography that was performed at −70 °C for 24 h. Labelled bands were traced, cut out and counted in a γ-radiation counter (Beckman model 5500). Radioactivity of each band was corrected for background radiation, and the counts were expressed as a percentage of a standard (30 μg of heart homogenate protein), run on each gel.

Muscle glycogen analyses

To verify which muscles are innervated by the sciatic nerve, the left sciatic nerves of a separate group of rats ($n = 6$) were electrically stimulated *in vivo*. Muscle samples were moved from the contracted left hindlimb and the control right hindlimb, frozen in liquid N_2 and stored at −80 °C until analysed for glycogen concentration by the procedure of Lo et al. (1970).

Statistical analysis

The data were analysed by ANOVA to test the effects of treatment (control, insulin, contraction, and insulin + contraction) on muscle glucose uptake and glucose-transporter distribution. When a significant F ratio was obtained, the Newman–Keuls *post hoc* test was employed to identify statistically significant differences ($P < 0.05$) between the means.

RESULTS

The rats of the four groups tested did not differ in body weight, muscle weight or total muscle protein concentration (Table 1). Plasma-membrane and microsomal-membrane proteins were also similar across groups, with the exception that the microsomal-membrane protein of the insulin + contraction group was significantly less than that of the basal group.

Electrically induced contraction decreased glycogen levels in all muscles tested (Table 2), providing evidence that the muscles utilized for membrane purification were activated during sciatic-nerve stimulation.

Table 2 Muscle glycogen from Sprague–Dawley rats with contracted and resting muscles

Values are means ± S.E.M. of 6 muscles per group: * significantly different from non-contracted ($P < 0.05$). Abbreviations: RG, red gastrocnemius; WG, white gastrocnemius; EDL, extensor digitorum longus; TA, tibialis anterior; SM, semimembranosus; FHL, flexor hallicus longus; PL, peroneus longus; BF, biceps femoris.

	Glycogen (μmol/g)	
	Non-contracted	Contracted
Soleus	29.9 ± 2.4	21.8 ± 2.2*
Plantaris	31.8 ± 3.0	10.8 ± 2.1*
RG	30.4 ± 1.8	12.7 ± 2.0*
WG	32.2 ± 2.3	8.6 ± 1.9*
EDL	27.0 ± 2.0	10.5 ± 1.8*
TA	29.4 ± 3.4	16.3 ± 2.9*
SM	30.5 ± 2.1	16.3 ± 1.9*
FHL	20.5 ± 2.6	12.5 ± 1.9*
PL	24.9 ± 1.2	9.8 ± 2.5*
BF	30.5 ± 3.1	15.9 ± 2.8*

Table 3 Glucose uptake in the soleus, plantaris, red gastrocnemius (RG) and white gastrocnemius (WG) of Sprague–Dawley rats during basal conditions, insulin stimulation, and after muscle contraction

Values are means ± S.E.M.: * significantly different from basal; † significantly different from contraction; ‡ significantly different from insulin ($P < 0.05$).

	Glucose uptake (μmol/h per g)			
	Basal	Contraction	Insulin	Insulin + contraction
Soleus	3.44 ± 0.89	36.0 ± 4.76*	46.2 ± 3.16*	53.4 ± 5.13*†
Plantaris	3.62 ± 4.76	29.3 ± 2.63*	30.8 ± 3.58*	40.1 ± 2.61*†‡
RG	3.37 ± 0.77	45.0 ± 2.93*	43.0 ± 3.98*	50.1 ± 3.37*
WG	2.57 ± 0.88	15.3 ± 1.46*	21.35 ± 2.32*	28.98 ± 6.93*†‡

Basal, insulin-, contraction- and insulin + contraction-stimulated glucose uptakes are shown in Table 3. Under basal conditions there were no differences between fibre types. However, fibre-type differences were apparent with insulin-, contraction- and insulin + contraction-stimulated uptakes. During stimulation by contraction or insulin + contraction, the red gastrocnemius (RG) showed the highest uptake, followed by the soleus, plantaris, and white gastrocnemius (WG). In contrast, during insulin stimulation the soleus displayed the highest uptake, followed by the RG, plantaris and WG.

Insulin + contraction resulted in a significant increase in glucose uptake above that stimulated by contraction alone in the soleus, and a significant increase in glucose uptake above that stimulated by either insulin or contraction alone in the plantaris and WG. Insulin + contraction did not result in a significant increase in glucose uptake above that stimulated by either insulin or contraction in the RG.

Specific activities, recoveries and fold enrichments of the plasma-membrane marker enzyme KpNPPase are given in Table 4. KpNPPase activity was enriched ~ 48-fold in the plasma membranes, and ~ 9-fold in the microsomal membranes, compared with the crude homogenate. Recovery of KpNPPase was ~ 7.5 % in plasma membranes and ~ 0.8 % in the microsomal membrane fraction. Muscle contraction and insulin had no influence on percentage recovery or fold enrichment.

Ca^{2+}-ATPase, a sarcoplasmic-reticulum marker enzyme, was not detectable in the microsomal membranes, and was only barely detectable in the basal and insulin-stimulated plasma-membrane preparations. This demonstrates that there was virtually no contamination of the plasma membranes with sarcoplasmic reticulum, as the fold enrichments of Ca^{2+}-ATPase were less than 1 for the groups in which it could be detected (results not shown).

Glucose transporter number (R_0), as estimated by CB binding, increased 2.0-, 2.3- and 2.1-fold in plasma membranes from contraction-, insulin-, and insulin + contraction-stimulated rats respectively (Table 5). The dissociation constant (K_d) also increased significantly in plasma membranes from all three groups. R_0 in microsomal membranes decreased 34 % with insulin stimulation and 28 % with insulin + contraction stimulation, but did not significantly decline with contraction alone. K_d in microsomal membranes did not change with contraction or insulin, but did significantly decrease with insulin + contraction.

Table 5 also shows the relative amount of GLUT-4 protein in each membrane preparation, expressed as a percentage of a heart standard run on each gel. Plasma-membrane GLUT-4 protein concentration increased 1.6-, 1.8- and 1.5-fold for contraction, insulin and insulin + contraction respectively. Microsomal membrane GLUT-4 protein concentration decreased by 29 and 30 %

Table 4 KpNPPase specific activities, percentage recoveries, and enrichments of subcellular membrane fractions from skeletal muscle of basal, muscle-contracted, insulin-treated, or muscle-contracted and insulin-treated rats

Values are means ± S.E.M. for the numbers of experiments in parentheses. There were no significant differences between groups.

KpNPPase	Basal (10)	Contraction (8)	Insulin (8)	Insulin + contraction (9)
Homogenate				
Specific activity (nmol/min per mg)	3.26 ± 0.10	3.75 ± 0.23	3.82 ± 0.26	3.73 ± 0.29
Plasma membrane				
Specific activity (nmol/min per mg)	150.5 ± 11.4	178.1 ± 9.3	175.8 ± 12.6	172.7 ± 6.2
Recovery (%)	7.14 ± 1.00	7.34 ± 0.73	7.61 ± 0.99	7.74 ± 0.75
Enrichment (fold)	46.4 ± 3.6	49.1 ± 4.4	48.0 ± 5.4	48.9 ± 4.3
Microsomal membrane				
Specific activity (nmol/min per mg)	33.8 ± 6.3	37.2 ± 9.1	37.9 ± 6.0	37.2 ± 8.5
Recovery (%)	1.05 ± 0.22	0.79 ± 0.11	0.86 ± 0.14	0.77 ± 0.20
Enrichment (fold)	10.3 ± 1.8	9.9 ± 1.7	9.8 ± 1.3	9.4 ± 2.1

Table 5 Glucose-transporter number, dissociation constants, and GLUT-4 protein concentration of subcellular membrane fractions from skeletal muscle of basal, muscle-contracted, insulin-treated, or muscle-contracted and insulin-treated rats

GLUT-4 protein concentration is expressed as a percentage of a rat heart standard. Values are means ± S.E.M. for the numbers of experiments in parentheses: * significantly different from basal ($P < 0.05$). R_0, glucose-transporter number; K_d, dissociation constant.

	Basal (10)	Contraction (8)	Insulin (8)	Insulin + contraction (9)
Plasma membranes				
R_0 (pmol/mg)	7.5 ± 0.5	14.7 ± 1.1*	17.3 ± 1.6*	15.3 ± 0.9*
K_d (nM)	80.2 ± 9.0	146.2 ± 10.2*	124.5 ± 9.0*	142.2 ± 10.7*
GLUT-4 (%)	164.6 ± 17.9	266.1 ± 31.1*	293.7 ± 34.8*	254.1 ± 15.5*
Microsomal membranes				
R_0 (pmol/mg)	14.1 ± 1.5	11.9 ± 0.6	9.3 ± 0.6*	10.2 ± 1.6*
K_d (nM)	128.5 ± 21.1	110.0 ± 13.9	89.7 ± 12.1	68.7 ± 12.9*
GLUT-4 (%)	565.7 ± 22.0	531.2 ± 27.8	401.0 ± 61.1*	394.6 ± 48.3*

with insulin and insulin + contraction respectively, but did not decrease significantly with contraction.

DISCUSSION

Activation of skeletal-muscle glucose uptake by insulin or muscle contraction involves the rapid translocation of specific glucose-transporter proteins to the plasma membrane (Klip et al., 1987; Sternlicht et al., 1988; Douen et al., 1990a,b; Hirshman et al., 1990; Goodyear et al., 1990b, 1991). Six different glucose-transporter isoforms have been identified, but in skeletal muscle the two predominant isoforms are termed GLUT-1 and GLUT-4 (Douen et al., 1990b). The GLUT-1 isoform is found in small amounts in the plasma membrane, and its concentration is not significantly increased by insulin (Douen et al., 1990a,b; Goodyear et al., 1991). Insulin or muscle contraction, however, increases the GLUT-4 protein concentration in the plasma membrane, and this suggests that it is primarily the GLUT-4 isoform that is involved in the regulation of insulin- or contraction-stimulated glucose uptake (Douen et al., 1990a,b; Hirshman et al., 1990).

In the present study, the CB-binding data and Western-blot analysis provide further evidence that insulin, and muscle contraction in the absence of insulin, increase plasma-membrane glucose-transporter concentration. In addition, we found that the combination of contraction + insulin resulted in an increase in plasma-membrane GLUT-4 protein concentration, but did not increase it above that with contraction or insulin alone. These findings agree with those of Goodyear et al. (1990b), who demonstrated that insulin, muscle contraction, and insulin + contraction increased plasma-membrane glucose-transporter number by 1.7-, 2.0- and 1.8-fold respectively.

The increase in insulin-stimulated plasma-membrane GLUT-4 protein concentration is associated with a decline in transporters from an intracellular pool (Wardzala and Jeanrenaud, 1981; Klip et al., 1987; Douen et al., 1989, 1990a,b; Hirshman et al., 1990). Although muscle contraction increases plasma-membrane GLUT-4 protein concentration, the origin of the contraction-sensitive GLUT-4 protein is still in question (Douen et al., 1990b; Goodyear et al., 1990b, 1991). Fushiki et al. (1989) and Goodyear et al. (1991) demonstrated that insulin and exercise both caused a decrease in CB binding and GLUT-4 protein concentration of microsomal membranes isolated from rat skeletal muscle. Similarly, we found that insulin or insulin + contraction caused a 34 and 28% decrease in microsomal CB binding and a 29 and 30% decrease in microsomal GLUT-4 protein concentration, respectively. However, we could not demonstrate a decline in microsomal CB binding or GLUT-4 protein concentration with muscle contraction alone. We have obtained similar results in lean (Etgen et al., 1993) and obese (Brozinick et al., 1993) Zucker rats. A possible explanation for the difference in results could be related to the methods used for activation of muscle contraction. Goodyear et al. (1991) and Fushiki et al. (1989) used acute treadmill exercise instead of electrical stimulation to induce muscle contraction. As a result, neither of these groups was able to control for the presence of insulin during muscle contraction. This observation may be especially relevant with regard to the results of Goodyear et al. (1991), who used rats in the post-prandial state, when plasma insulin levels are generally elevated. The present study utilized the hindlimb-perfusion technique, and thereby removed any possible contributing effects of insulin during the period of muscle contraction. Thus it may be suggested that the presence of even low concentrations of insulin confound the effects of contraction on muscle glucose-transporter distribution (Cartee et al., 1991; Nesher et al., 1985).

A limitation to our finding is the sensitivity of the CB-binding and Western-blot techniques. It is conceivable that muscle contraction results in a small, undetectable, decline in the microsomal GLUT-4 protein concentration. However, it should be pointed out that our results are in agreement with those of Douen et al. (1990a,b), who found that insulin and exercise plus insulin, but not exercise alone, caused a decline in intracellular-membrane GLUT-4 protein concentration and glucose-transporter number in rat skeletal muscle. It was also demonstrated by Cartee et al. (1991) that hypoxia, which activates glucose transport via the same mechanism as muscle contraction, increased plasma-membrane glucose-transporter number and GLUT-4 protein concentration, without a significant decrease in glucose-transporter number or GLUT-4 protein concentration in their intracellular fraction. Taken together, these results support the hypothesis that, although muscle contraction and insulin recruit the same immunologically detectable glucose-transport protein (GLUT-4), two distinct pools of GLUT-4 protein exist, one sensitive to insulin and one to muscle contraction.

As has been previously demonstrated (Wallberg-Henriksson and Holloszy, 1984; Nesher et al., 1985; Richter et al., 1985; Ploug et al., 1987; Goodyear et al., 1990b), we found that contractile activity increases skeletal-muscle glucose uptake in the absence of insulin. Several of these studies also indicated that the effects of insulin and muscle contraction on skeletal-muscle

glucose uptake were fully additive (Nesher et al., 1985; Ploug et al., 1987). In contrast with these findings, however, we failed to show a fully additive effect of insulin + contraction on glucose uptake. A possible reason for this may be related to the fact that we measured glucose uptake using the phosphorylatable glucose analogue 2-DG in the presence of glucose, whereas previous studies used the non-phosphorylatable glucose analogue 3-*O*-methyl-D-glucose. Maximal rates of glucose uptake, as elicited in the present study, are associated with a shift in the rate-limiting step for glucose uptake from glucose transport to phosphorylation by hexokinase (Idström et al., 1986; Kubo and Foley, 1986). As a result of a possible shift in the rate-limiting step for glucose uptake, we may have underestimated the effect of contraction + insulin due to counter-transport of 2-DG. In agreement with this explanation, the only other study to measure insulin + contraction-stimulated glucose uptake during hindlimb perfusion with a saturating concentration of glucose also failed to find a fully additive effect of insulin + contraction (Idström et al., 1986).

Although insulin + contraction increased glucose uptake above that with contraction or insulin alone in the soleus, plantaris and white gastrocnemius, glucose-transporter number, as determined by CB binding, or plasma-membrane GLUT-4 protein concentration was no greater in insulin + contraction-stimulated plasma membranes than in contraction- or insulin-stimulated membranes. A limitation to this finding is the lack of an additional effect of contraction plus insulin on glucose uptake in all muscle fibre types. Thus it is possible that muscle, not additionally activated by the combination of insulin plus contraction, was used in the transporter analysis and decreased the probability of demonstrating an additional increase in plasma-membrane transporter concentration. However, in agreement with our results, Douen et al. (1990a,b) showed an additive effect of prior exercise and insulin on glucose uptake in the perfused-hindlimb preparation, but no further increase in plasma-membrane glucose transporters or GLUT-4 protein. This suggests that exercise + insulin increases plasma-membrane glucose-transporter intrinsic activity, as suggested by Sternlicht et al. (1989). In contrast with these results, Goodyear et al. (1990b) found that insulin + contraction did not increase skeletal-muscle glucose uptake, plasma-membrane glucose-transporter number or glucose-transporter intrinsic activity above that with either insulin or contraction alone. The difference between the present results and those of Goodyear et al. (1990b) may stem from the fact that the rats in the latter study were exposed to a high concentration of insulin (30 m-units/ml) for 10 min before the initiation of muscle contraction. Constable et al. (1988) demonstrated that, if isolated epitrochlearis muscles were exposed to a high insulin concentration before contraction, the combined effects of contraction and insulin on glucose transport were not additive. Therefore, it is possible that Goodyear et al. (1990b) might have observed an additive effect of contraction and insulin on glucose uptake if the muscle had been exposed to insulin during or after muscle contraction rather than before.

A finding that differs from previous studies (Goodyear et al., 1990b, 1991; Hirshman et al., 1990), is that muscle contraction, insulin, and insulin + contraction resulted in a significant increase in the dissociation constant (K_d) for CB binding. The reason for the change in the K_d in the present study is unclear, but could be due to the fact that the muscle was exposed to a saturating concentration of glucose. Two previous reports have shown slightly higher K_d values after muscle contraction or exercise (Goodyear et al., 1990a,b), but, to our knowledge, no previous studies have reported an increase in K_d due to insulin or insulin + contraction. This would indicate that, during maximal stimulation of skeletal-muscle glucose uptake by insulin and muscle contraction, a change in transporter affinity and/or intrinsic activity may occur in order to limit accumulation of intracellular glucose in the muscle cell. Any further speculation on this finding, however, will require further study.

In summary, it was found that insulin, contraction and insulin + contraction increased glucose uptake in the soleus, plantaris, and red and white portions of the gastrocnemius. In general, the effect of insulin + contraction was greater than insulin or contraction alone. CB binding and Western blotting indicated that the increases in glucose uptake were all associated with similar increases in plasma-membrane glucose transporters. Insulin and insulin + contraction caused a decrease in intracellular membrane glucose transporters, but contraction alone did not. These results suggest that two distinct pools of glucose transporters exist in mammalian skeletal muscle, one sensitive to insulin, and one sensitive to muscle contraction.

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