Effects of contraction on localization of GLUT4 and v-SNARE isoforms in rat skeletal muscle

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Rose AJ, Jeppesen J, Kiens B, Richter EA. Effects of contraction on localization of GLUT4 and v-SNARE isoforms in rat skeletal muscle. Am J Physiol Regul Integr Comp Physiol 297: R1228–R1237, 2009. First published August 12, 2009; doi:10.1152/ajpregu.00258.2009.—In skeletal muscle, contractions increase glucose uptake due to a translocation of GLUT4 glucose transporters from intracellular storage sites to the surface membrane. Vesicle-associated membrane proteins (VAMPs) are believed to play an important role in docking and fusion of the GLUT4 transporters at the surface membrane. However, knowledge about which VAMP isoforms colocalize with GLUT4 vesicles in mature skeletal muscle and whether they translocate during muscle contractions is incomplete. The aim of the present study was to further identify VAMP isoforms, which are associated with GLUT4 vesicles and examine which VAMP isoforms translocate to surface membranes in skeletal muscles undergoing contractions. VAMP2, VAMP3, VAMP5, and VAMP7 were enriched in immunoprecipitated GLUT4 vesicles. In response to 20 min of in situ contractions, there was a redistribution of GLUT4 (+64 ± 13%), transferrin receptor (TfR; +75 ± 22%), and insulin-regulated aminopeptidase (IRAP; +70 ± 13%) to fractions enriched in heavy membranes away from low-density membranes (−32 ± 7%; −18 ± 12%; −33 ± 9%; respectively), when compared with the resting contralateral muscle. Similarly, there was a redistribution of VAMP2 (+240 ± 40%), VAMP5 (+79 ± 9%), and VAMP7 (+79 ± 29%), but not VAMP3, to fractions enriched in heavy membranes away from low-density membranes (−49 ± 10%, −54 ± 9%, −14 ± 11%, respectively) in contracted vs. resting muscle. In summary, VAMP2, VAMP3, VAMP5, and VAMP7 coimmunoprecipitate with intracellular GLUT4 vesicles in muscle, and VAMP2, VAMP5, VAMP7, but not VAMP3, translocate to the cell surface membranes similar to TfR, TfR, and IRAP in response to muscle contractions. These findings suggest that VAMP2, VAMP5, and VAMP7 may be involved in translocation of GLUT4 during muscle contractions.

DURING EXERCISE, SKELETAL muscle glucose uptake increases (56). In rats, muscle glucose uptake during moderate exercise is actually greater than maximal insulin-stimulated muscle glucose uptake (21). In young men studied in the fasted state, maximal insulin-stimulated leg glucose uptake was ~100 μmol·min⁻¹·kg⁻¹ (leg mass) (6, 45) with ~250 μmol·min⁻¹·kg⁻¹ (leg mass) leg glucose uptake during maximal aerobic exercise (29), indicating the quantitative importance of exercise to stimulate skeletal muscle glucose uptake. It is recognized that the understanding of the molecular mechanisms behind the increased skeletal muscle glucose transport during exercise may be important as a clinically relevant alternative pathway to increase glucose disposal in skeletal muscle in states of insulin resistance (14, 48).

The increase in skeletal muscle glucose uptake during exercise results from a coordinated increase in rates of glucose delivery, surface membrane glucose transport, and intracellular substrate flux through glycolysis (48). Skeletal muscle expresses multiple isoforms of glucose transporters (GLUT) (39), and during exercise, the most important of these is GLUT4, as systemic (51) and muscle-specific (65) GLUT4 knockout (KO) nearly completely abolishes contraction-stimulated glucose uptake in vivo. In addition, in a recent study (12), it has been shown that total GLUT4 KO reduces muscle glucose uptake at rest and during exercise in mice in vivo, even in the face of compensatory increases in capillary density and normal expression of hexokinase II in skeletal muscles, as well as prevailing hyperglycemia during exercise, in GLUT4 KO mice. However, other studies by Wasserman and colleagues (10, 11) have shown that neither partial GLUT4 knockout nor GLUT4 overexpression affect skeletal muscle glucose uptake in running mice, suggesting that the increase in surface membrane permeability with exercise is only a permissive step or that only a fraction of the total GLUT4 pool is necessary for the increase in surface membrane permeability, with exercise.

Similar to the action of insulin, it has been shown that exercise/muscle contraction increases the surface membrane (i.e., plasma membrane and t-tubuli) content of GLUT4 in skeletal muscles of mammals using several different methods and models (5, 7, 9, 15, 30, 31, 34, 36, 40, 49, 50, 54). The molecular events involved in stimulating GLUT4 movement within muscle cells are complex (62). Some work has focused on the early signaling events involved in stimulating skeletal muscle glucose transport during contractions (48). Less attention, however, has been paid to the effects of exercise/contraction on the distal mechanisms in GLUT4 translocation. Earlier studies have provided evidence that there are distinct contraction and insulin-responsive GLUT4-containing vesicle “pools” in skeletal muscle (5, 7, 34, 40) and that the molecular signals that trigger increased glucose transport and GLUT4 translocation to the surface membrane are different when comparing insulin and contraction stimulation (20, 33, 36, 61). Indeed, insulin, but not contractions, results in changes in distribution of the Rab4 protein (52), and contractions, but not insulin, alter the distribution of the transferrin receptor (34), although the latter is controversial (64). Even so, there are likely to be similarities between the two stimuli, as both exercise and insulin probably recruit GLUT4 vesicles that contain insulin-responsive aminopeptidase (5), as well as stimulate higher sarcocellular content of GTP-binding proteins (9). Also, both insulin and contraction-stimulated glucose uptake in skeletal
skeletal muscle involves signaling through TBC1D4/AS160 (28), probably via partially distinct mechanisms (27). Although it is not known whether the higher surface membrane GLUT4 with contractions results from a slower endocytosis or faster exocytosis of GLUT4 vesicles in differentiated mammalian skeletal muscle, insights from work with cultured muscle cells suggest that both mechanisms may contribute (57). In particular, when cultured L6 myotubes were stimulated by K⁺-induced depolarization, the resulting increase in cell surface GLUT4 was a combination of a small increase in GLUT4 exocytosis and a larger decrease in endocytic rate (57).

 Trafficking between different intracellular membrane compartments involves transport vesicle intermediates that are generated at donor membranes and are then delivered to specific acceptor membranes (for review, see Ref. 18). The final step of this process is the physical fusion of vesicular trafficking intermediates with the target membrane compartment, which is mediated by the interactions of vesicle-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and target SNAREs (4, 53). Specific interaction between a vesicle-SNARE (v-SNARE) and the cognate target-SNARE (t-SNARE) leads to the formation of a SNAREpin complex, in which four SNARE motifs assemble into a twisted parallel four-helical bundle (for review, see Ref. 18). Of the seven described v-SNARE isoforms (18), VAMP2 (synaptobrevin 2) (41, 55), VAMP3 (cellubrevin) (55), VAMP5 (myobrevin) (63), and VAMP7 (tetanus toxin insensitive VAMP, TI-VAMP) (43), but not VAMP1 (synaptobrevin 1) (55), have been shown to be expressed in skeletal muscle. An earlier study of humans showed that exercise increases the plasma membrane content of VAMP2 in skeletal muscle (29); however, no studies have looked at the effects of exercise/contraction on the localization of other VAMP isoforms. Hence, the aim of this study was to further identify VAMP isoforms that are associated with intracellular GLUT4 vesicles and to examine which VAMP isoforms translocate to surface membranes in skeletal muscles undergoing contractions.

METHODS

Materials. All materials were from Sigma-Aldrich unless stated otherwise.

Animals. Male Wistar rats were used for experimentation in the fed state. The animals were fed standard laboratory chow and consumed water ad libitum and were kept on a constant 12:12-h light-dark cycle. All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes (council of Europe no. 123, Strasbourg, France, 1985).

Subcellular fractionation technique. In the present study, the subcellular fractionation method used was based on the technique shown by Zhou et al. (64), but with minor modifications. Briefly, gastrocnemius and plantaris muscles from rat hindlimb were removed and dissected free from connective tissue, nervous tissue, and visible fat. The muscles were minced with scissors and homogenized on ice three times (5 s each) using a Polytron homogenizer (PT3100; Kinematica) set at maximum speed 13,000 rpm in 15 ml of buffer containing 20 mM HEPES, 250 mM sucrose, 2 mM EDTA, 10 mM sodium fluoride, 20 mM sodium pyrophosphate, 3 mM benzamidine, 10 μg/ml apro tinin A, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4. A small aliquot, 500 μl, of the homogenate was frozen and stored at –80°C, for later production of lysate. The remaining homogenate was centrifuged at 2,000 g for 10 min (Universal 320R, Hettich Zentrifugen). The supernatant was transferred to new tubes, and the pellet was resuspended in 5 ml of homogenization buffer, rehomogenized, and centrifuged again at 2,000 g for 10 min. The pellet, which contained mainly unhomogenized pieces of tissue, was discarded, and the two supernatants were pooled, filtered through doubled layerd gauze, and centrifuged at 12,000 g, in a Sorvall SS34 rotor (Thermo Fisher Scientific) for 20 min. The 12,000 g pellet (P1) was resuspended in PBS with the cocktail of protease and phosphatase inhibitors listed above and saved for later analysis by Western blotting. The supernatant was centrifuged at 180,000 g for 90 min. The supernatant from the 180,000 g spin (cytosol fraction) was stored, and the pellet was resuspended in PBS with protease and phosphatase inhibitors, mechanically homogenized using a tightly fitting Potter-Elvehjem Teflon pestle, and loaded on a 10–30% (wt/wt) continuous sucrose gradient, and centrifuged at 276,000 g for 55 min in an AH-650 Sorvall swing-out rotor (Thermo Fisher Scientific). Gradients were harvested using a syringe and separated into eight fractions, starting from the bottom of the tube. The pellet of the sucrose-gradient centrifugation (P2) was resuspended in PBS with the cocktail of protease and phosphatase inhibitors listed above and analyzed together with the gradient fractions. All centrifugations were performed at 4°C.

Immunoprecipitation of GLUT4 vesicles. Intracellular GLUT4 vesicles were immunoenriched essentially as outlined by Kandror and Pilch (26). A rat (180–200 g) was anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The posterior hindlimb (i.e., gastrocnemius and plantaris) muscles were removed in situ by 10.220.33.1 on June 19, 2017 http://ajpregu.physiology.org/ Downloaded from
contracted gastrocnemius-plantaris muscle and the contralateral control gastrocnemius-plantaris muscle were removed and dissected free from visible fat and nervous tissue and used for subcellular fractionation, as described above. After experimentation, the animals were killed by cervical dislocation while unconscious.

Insulin stimulation. Rats (180–200 g) \( (n = 3) \) were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The vasculature of the left hindlimb was occluded, and the gastrocnemius and plantaris muscles (control) were removed and used for subcellular fractionation, as described above. Subsequently, insulin (Actrapid; Novo Nordisk) was administered by tail vein injection to give a dose of 10 U/kg body wt. After 10 min of insulin stimulation, the right gastrocnemius and plantaris muscle were removed and used for subcellular fractionation.

Before and after insulin injection, blood samples were collected from the tail vein for blood glucose measurements (Bayer Glucometer; Bayer AG). After experimentation, the animals were killed by cervical dislocation while unconscious.

Tissue lysate preparation. The 500-μl aliquot of homogenate saved prior to centrifugation and subcellular fractionation was supplemented with NP40 and sodium chloride to final concentrations of 1% NP40 and 150 mM NaCl, respectively. These homogenates were rehomogenized using a polytron at maximum speed (PT1200, Kinematic) and mixed thoroughly by end-over-end rotation at 4°C for 60 min, and finally centrifuged at 13,000 g for 20 min at 4°C. The clarified supernatant (i.e., lystate) was removed and stored at −80°C until required. A small aliquot of each lystate was taken and diluted for protein concentration determination prior to storage.

Analytical techniques. Protein concentration of the lysates, the P1, P2, and cytosol fractions was determined in triplicate using the bicinchoninic acid (BCA) method using BSA standards (Pierce) and BCA assay reagents (Pierce). A maximal coefficient of variance of 5% was accepted between replicates. All samples were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting for protein expression and phosphorylation. The primary antibodies used were anti-transferrin receptor (Zymed Laboratory, Invitrogen), anti-IRAP (provided by Dr. Susanne R. Keller, University of Virginia) anti-Na+K+-ATPase pan-α subunits (Affinity Bioreagents), anti-Glut1 (Affinity Bioreagents), anti-DHPRα1 (Affinity Bioreagents), anti-FABPc (provided by Prof. J. Glatz, University of Maastricht, The Netherlands), anti-atriadin (Affinity Bioreagents), anti-F1-ATPaseβ (Santa Cruz Biotechnology), anti-VAMP2 (Affinity Bioreagents), anti-VAMP3 (Synaptic Systems), anti-VAMP5 (Synaptic Systems), anti-VAMP7 (Santa Cruz Biotechnology) and anti-Glut4 (Affinity Bioreagents). The primary phospho-specific antibodies were anti-phospho-PKB Ser473 (Cell Signalling Technology), and anti-phospho-ERK1/2 Thr202/Tyr204 (Cell Signalling Technology). Secondary antibodies were from DakoCytomation (Denmark). Band intensity was quantified by Kodak imaging software (Kodak ID 3.5). Preliminary experiments demonstrated that the amounts of protein loaded were within the dynamic range for the conditions used and the results obtained (data not shown).

Calculations and statistics. For fractionation work, the sum of arbitrary units from immunoblot scans of P1+P2 and F1–F8 was divided by the value for the lystate to account for differences in the extraction efficiency of individual samples. Delta values (i.e., fold of \( x \)) were calculated by the following equation: \( [\text{Vol} - \text{Vol} x] / \text{Vol} x \). Statistical analyses were performed using SigmaStat 3.5 for Windows. For signaling work, paired t-tests were performed. For fractionation work, a two-way ANOVA for repeated measures was used with Student-Newman-Keuls multiple-comparison post hoc tests used when the ANOVA revealed significant interactions between variables. Graphs were constructed using SigmaPlot v.3.5. Differences were considered to be significant when \( P \) was less than 0.05.

RESULTS

Characterization of fractionation technique. Preliminary experiments were performed to characterize the various fractions produced by the density-based separation of cellular membranes of skeletal muscle. As shown in Fig. 1, the various fractions were probed using antibodies detecting proteins known to be located in certain subcellular compartments or organelles. These included the recycling endosomes (transferrin receptor) (34), both surface (i.e., plasma membranes and t-tubuli) and intracellular membranes (Na+/K+-ATPase) (19), surface membranes (GLUT1) (37), t-tubuli (dioxyxynucleotidase receptor α1) (22), sarcoplasmic reticulum (triadin) (16), β-subunit of the mitochondrial F1-ATPase (mitochondria) (17), and cytosol (cytosolic fatty acid binding protein) (13). As can be seen from Fig. 1, markers of dense organelles, such as mitochondria and sarcoplasmic reticulum, were enriched in the heavy membrane fractions P1 and P2. Also, importantly, markers of surface membranes were enriched in P1 and P2. A marker of recycling endosomes was enriched in the low-density membrane fractions F1–F8, but these fractions were devoid of the markers for dense organelles. The cytosolic fraction was enriched with a cytosolic marker (FABPc) and was devoid of markers of both dense and light membrane fractions; the cytosolic marker was not detected in the membrane fractions. Altogether, this work shows that this fractionation technique effectively allows distinction between surface and intracellular membrane compartments.

v-SNARE isoforms are enriched in GLUT4 immunoprecipitates from low-density membranes. As shown in Fig. 2, immunoprecipitation with GLUT4 antibodies was able to remove approximately half of the GLUT4 from the sample pooled from fractions F2–F5. Importantly, no GLUT4 was detected in the protein-G beads when no antibodies were included in the immunoprecipitation procedure. Of the v-SNARE isoforms

![Fig. 1. Characterization of skeletal muscle fractions using Western blot analysis. Rat posterior hindlimb muscles (i.e., gastrocnemius and plantaris) were excised and fractionated based upon a protocol of density-dependent separation slightly modified from Zhou et al. (64). Protein concentrations of lystate (L), pellet 1 (P1), P2, and cytosol (C) were determined, and equal protein amounts of these as well as equal volume amounts of fractions 1–8 (F1–F8) were resolved by SDS-PAGE, and membranes were immunoblotted using antibodies specific for subcellular protein markers. These protein markers included transferrin receptor (TIR), pan α-subunit of Na+,K+-ATPase (NKAα), glucose transporter 1 (GLUT1), dihydroxyxynucleotidase receptor α (DHPRα1), cytosolic fatty acid binding protein (FABPc), triadin/trisk95, and β-subunit of F1-ATPase (F1-ATPase-β). Images of immunoblots are shown.](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00675.2009)
tested, VAMP2, VAMP3, VAMP5, and VAMP7 were enriched in the GLUT4 IP and were partially immunodepleted from the supernatant. Importantly, no v-SNARE was detected in the IP run without the GLUT4 antibodies added. Furthermore, NKAα/H9251 was not detected in the GLUT4 IP.

Skeletal muscle signaling in response to insulin and contraction stimuli. Signaling responses to the different stimuli are shown in Fig. 3. In response to insulin, there was an 18-fold increase in skeletal muscle phospho-Ser473 protein kinase B, but no change with contraction. In contrast, there was a 7-fold increase in skeletal muscle phospho-Thr-X-Tyr extracellular regulated kinase 1/2 with contraction, but not insulin.

Effects of insulin on blood glucose and skeletal muscle GLUT4 and VAMP2 localization. In response to tail-vein injection of insulin, blood glucose fell from 8.1 ± 0.1 mmol/l to 3.8 ± 0.2 mmol/l (P < 0.01). The distribution of skeletal GLUT4 and VAMP2 in intracellular fractions is shown in Fig. 4. There was redistribution of both GLUT4 and VAMP2 to fractions enriched in heavy membranes (mainly P2) away from low-density membranes. In particular, there was a 64 ± 13% increase in GLUT4 in P1+P2 (P < 0.05) and a 32 ± 7% decrease in GLUT4 in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. In addition, there was a 75 ± 22% increase in TR in P1+P2 (P < 0.05) and a 18 ± 12% decrease in TR in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. Similarly, there was a 70 ± 13% increase in IRAP in P1+P2 (P < 0.05) and a 33 ± 9% decrease in IRAP in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle.

Concerning the v-SNARE isoforms, there was redistribution of VAMP2, VAMP5, and VAMP7, but not VAMP3, to fractions enriched in heavy membranes away from low-density membranes. In particular, there was a 65 ± 21% increase in VAMP2 in P1+P2 (P < 0.05) and a 32 ± 8% decrease in VAMP2 in F1–F8 (P < 0.05) in insulin-stimulated muscle vs. basal muscle.

Effects of contractions on skeletal muscle GLUT4, TfR, IRAP, and v-SNARE localization. Shown in Fig. 5 are the effects of contraction on the distribution of skeletal muscle GLUT4, transferrin receptor (TfR), and insulin-regulated amipopeptidase (IRAP) in intracellular fractions. There was redistribution of GLUT4, TfR, and IRAP to fractions enriched in heavy membranes away from low-density membranes. In particular, there was a 64 ± 13% increase in GLUT4 in P1+P2 (P < 0.05) and a 32 ± 7% decrease in GLUT4 in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. In addition, there was a 75 ± 22% increase in TR in P1+P2 (P < 0.05) and a 18 ± 12% decrease in TR in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. Similarly, there was a 70 ± 13% increase in IRAP in P1+P2 (P < 0.05) and a 33 ± 9% decrease in IRAP in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle.

Concerning the v-SNARE isoforms, there was redistribution of VAMP2, VAMP5, and VAMP7, but not VAMP3, to fractions enriched in heavy membranes away from low-density membranes. In particular, there was a 65 ± 21% increase in VAMP2 in P1+P2 (P < 0.05) and a 32 ± 8% decrease in VAMP2 in F1–F8 (P < 0.05) in insulin-stimulated muscle vs. basal muscle.

Fig. 2. Multiple v-SNARE isoforms coprecipitate with intracellular GLUT4. Rat posterior hindlimb muscles (i.e., gastrocnemius and plantaris) were excised and fractionated. Five hundred microliters of pooled intracellular membrane fractions (F2–F5) were incubated with (+) or without (−) α-GLUT4 antibody (ab). Equal amounts of immunoprecipitate (IP, beads) and post-IP supernatant (s/n) samples were resolved by SDS-PAGE, and membranes were immunoblotted (IB) using an ab specific for glucose transporter 4 (GLUT4), as well as v-SNARE isoform vesicle-associated membrane protein (VAMP) 2, VAMP3, VAMP5, and VAMP7. Samples were also probed for pan α-subunit of Na+–K+–ATPase (NKAα) as a negative control. Images of immunoblots are shown.

Skeletal muscle signaling in response to insulin and contraction stimuli. Signaling responses to the different stimuli are shown in Fig. 3. In response to insulin, there was an ~18-fold increase in skeletal muscle phospho-Ser473 protein kinase B, but no change with contraction. In contrast, there was an ~7-fold increase in skeletal muscle phospho-Thr-X-Tyr extracellular regulated kinase 1/2 with contraction, but not insulin.

Effects of insulin on blood glucose and skeletal muscle GLUT4 and VAMP2 localization. In response to tail-vein injection of insulin, blood glucose fell from 8.1 ± 0.1 mmol/l to 3.8 ± 0.2 mmol/l (P < 0.01). The distribution of skeletal GLUT4 and VAMP2 in intracellular fractions is shown in Fig. 4. There was redistribution of both GLUT4 and VAMP2 to fractions enriched in heavy membranes (mainly P2) away from low-density membranes. In particular, there was a 65 ± 21% increase in GLUT4 in P1+P2 (P < 0.05) and a 32 ± 8% decrease in GLUT4 in F1–F8 (P < 0.05) in insulin-stimulated muscle vs. basal muscle.

Fig. 3. Diverse skeletal muscle signaling with insulin or contraction stimuli. Unconscious rats were either injected intraperitoneally with insulin (I; n = 3) or subjected to sciatic-nerve induced contractions (C; n = 8) and posterior hindlimb muscles (i.e., gastrocnemius and plantaris) from resting (basal: B), as well as stimulated conditions were excised. Lysate proteins from these samples were resolved by SDS-PAGE, and membranes were immunoblotted using antibodies specific for phosphorylated forms of PKB and ERK1/2. Data are expressed as means ± SE. *Significant difference from basal, P < 0.05. Representative images of immunoblots are shown.
tions enriched in heavy membranes away from low-density membranes (Fig. 6). In particular, there was a 240 ± 40% increase in VAMP2 in P1+P2 (P < 0.05) and a 49 ± 10% decrease in VAMP2 in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. On the other hand, there were no differences in the distribution of VAMP3 when comparing rested and contracted muscle. However, similar to VAMP2, there was a 79 ± 9% increase in VAMP5 in P1+P2 (P < 0.05) and a 54 ± 9% decrease in VAMP5 in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle. Lastly, there was a 79 ± 29% increase in VAMP7 in P1+P2 (P < 0.05) and a 14 ± 11% decrease in VAMP7 in F1–F8 (P < 0.05) in contraction-stimulated muscle vs. resting muscle.

DISCUSSION

The major novel findings of this study were that contractions induced a translocation of skeletal muscle v-SNARE isoforms VAMP2, VAMP5, and VAMP7, but not VAMP3, from intracellular compartments to cell surface membranes (Fig. 6) in concert with GLUT4. Importantly, we were also able to show that all of these v-SNARE isoforms coimmunoprecipitate with...
Fig. 5. Skeletal muscle GLUT4, TIR, and IRAP translocate from intracellular membranes to cell surface membranes during nerve-induced contractions in situ. Unconscious rats were subjected to sciatic nerve-induced contractions (n = 8) and posterior hindlimb muscles (i.e., gastrocnemius and plantaris) from resting (basal), as well as stimulated conditions, were excised and fractionated. Proteins from these fractions were resolved by SDS-PAGE, and membranes were immunoblotted using antibodies specific for glucose transporter 4 (GLUT4; A), transferrin receptor (TIR; B), and insulin-regulated aminopeptidase (IRAP; C). Left: average data of each fraction. Right: difference in abundance between conditions in surface membrane (P1 + P2) and intracellular membranes (F1–F8). Data are expressed as means ± SE. *Significant difference from basal; P < 0.05. Representative images of immunoblots are shown.
GLUT4 from low-density membranes of skeletal muscle (Fig. 2), indicating that these isoforms associate with intracellular GLUT4 vesicles and may participate in the docking and fusion of GLUT4 to the surface membrane.

The tissue subcellular fractionation technique applied in the present study was modified from the method used by Zhou et al. (64). A great advantage of this method is that, aside from the first low-speed centrifugation step, nothing is discarded during fractionation. Furthermore, with the modification from the present study, cellular signaling is measured directly on the homogenate being fractionated, which allows the possibility to make links between translocation and signaling. To characterize the fractions, immunoblotting of marker proteins was used (Fig. 1), which showed similar distribution pattern of cell surface membrane-, cytosolic soluble- and intracellular vesicle membrane proteins, as previously described (64). This was done on all fractionations and indicated a successful separation of cell surface membranes, soluble cytosolic proteins, and intracellular membranes. Furthermore, 5′-nucleotidase activity, a plasma membrane marker, was previously measured and showed ~15–20-fold higher activity in the P2 fraction compared with the intracellular fraction (64). Zhou et al. (64) investigated insulin-stimulated GLUT4 trafficking but not contractions. Therefore, in verification of the protocol in our hands, we subjected rats to marked hyperinsulinemia in accordance with the protocol of Zhou et al. (64) and observed a significant insulin-induced PKB Ser473 phosphorylation (Fig. 3) and translocation of both GLUT4 and VAMP2 from the intracellular compartments to the cell surface membrane (Fig. 4). This was in accordance with previous findings by Zhou et al. (64), and altogether, this confirmed that the fractionation technique was effective. The electrical stimulation protocol was applied as a model of exercise. It is hard to compare directly to voluntary exercise, but on the basis of changes in muscle nucleotide status and lactate levels (46), contractions were of a relatively high intensity at least from a metabolic point of view.

The 50–80% increase in cell surface membrane (i.e., plasma membrane and t-tubuli) content of GLUT4 in rat skeletal muscle with contractions corresponded to a 25–40% decrease
of GLUT4 in intracellular low-density membranes (Fig. 4), indicative of a translocation phenomenon. This result is in agreement with other studies using different methods and models (5, 7, 9, 15, 30, 31, 34, 36, 39, 49, 50, 54), in which GLUT4 translocation to surface membranes in mammalian skeletal muscles was demonstrated during exercise/contractions. In particular, using a more complicated fractionation technique, Roy and Marette (50) showed that skeletal muscle GLUT4 redistributes to both plasma (±95%) and t-tubular (±60%) from intracellular (±40%) membranes after exercise (50). Also, similar to the present study (Fig. 5), others have observed a translocation of the transferrin receptor (34) and IRAP (5). However, a new finding in the present study was the translocation of VAMP5 and VAMP7 to surface membranes in response to muscle contractions (Fig. 6).

The composition of intracellular GLUT4 vesicles in skeletal muscle is not fully understood (62). In the present study, we were able to show that multiple v-SNARE isoforms, expressed in skeletal muscle, are enriched in intracellular GLUT4 vesicles (Fig. 2). Similarly, others have found that both VAMP2 and VAMP3 coimmunoprecipitate with GLUT4 storage vesicles from skeletal muscle cells (42). Here, this information is extended in that VAMP2, VAMP3, VAMP5, and VAMP7 were detected in GLUT4 immunoprecipitates from low-density membranes from mature rat skeletal muscle. This adds to the body of evidence that other proteins such as IRAP (1, 25), SCAMPs (25), and perhaps transferrin receptor (1) are enriched in intracellular GLUT4 vesicles in skeletal muscle. Furthermore, we have shown that VAMP7 is expressed in mature skeletal muscle, similar to what has been shown before for cultured L6 myotubes (43).

The precise mechanism(s) behind the movement of GLUT4 from intracellular membranes to surface membranes during muscle contractions is poorly understood. Earlier work by Marette and coworkers (34) using adult rat skeletal muscle has provided evidence that muscle contraction stimulates translocation of GLUT4 from two distinct intracellular compartments: one being a population of recycling endosomes and another from GLUT4 storage vesicles that are also insulin-responsive. Similar results were found using microscopy of rat soleus muscles (40) in that GLUT4 translocation to surface membranes with contraction is from both large structures, including multivesicular endosomes located in the TGN region, and small tubulovesicular structures. Studies conducted by Klip and coworkers have shown that the increase in muscle cell surface GLUT4 from K+-induced depolarization was a combination of a small increase in GLUT4 exocytosis and a larger decrease in endocytotic rate (57). Unlike work on insulin stimulation (62), no study has provided direct evidence that the GLUT4 translocation occurring with muscle contractions involves SNARE proteins. However, there are some indications that this may occur. In this study, it was shown that of the v-SNARE isoforms expressed, skeletal muscle VAMP2, VAMP5, and VAMP7, but not VAMP3, translocate from intracellular membranes to surface membranes during contractions (Fig. 6). Indeed, previous work by our group has shown a higher abundance of GLUT4 and VAMP2 proteins in plasma membrane vesicles obtained from skeletal muscle of humans after exercise compared with rest (30, 31), indicating that these two proteins may be functionally linked. On the other hand, others have observed that there is no defect in contraction-stimulated glucose uptake ex vivo in skeletal muscles of VAMP3 knockout mice (60). The lack of VAMP3 translocation (Fig. 6) in skeletal muscle during contraction supports this finding, even though VAMP3 was associated with GLUT4 vesicles (Fig. 2; Ref. 42). Nonetheless, VAMP5 may be needed for some other stimuli than contraction and insulin-stimulated GLUT4 translocation (42, 60), as VAMP3 ablation was found to prevent GTPγS [a PI-3 kinase-independent signaling cascade (8)] stimulated GLUT4 translocation (38). Thus, VAMP3-associated GLUT4 vesicles could potentially be important in alternative physiological situations, such as osmotic stress and uncoupling of oxidative phosphorylation, which was shown to induce GLUT4 translocation, via a PI-3 kinase-independent mechanism (3).

As VAMPs 2, 5, and 7 reside in intracellular GLUT4 vesicles (Fig. 2) and translocate along with GLUT4 to cell surface membranes during contractions (Figs. 5 and 6), this probably indicates that they play a functional role in GLUT4 docking at the surface membrane. As mentioned earlier, the increase in muscle cell surface GLUT4 from K+-induced depolarization, which is thought to be part of the contraction-signaling pathway to increase GLUT4 translocation (48), was a combination of a small increase in GLUT4 exocytosis and a larger decrease in endocytotic rate (57). Other stimuli, such as insulin, involve a larger increase in the exocytotic rate to mediate the increase in cell surface GLUT4 in muscle cells, and this selectively requires VAMP2 (43). Another stimulus, osmotic shock, involves a large decrease in endocytotic rate to mediate the increase in cell surface GLUT4, and this selectively requires VAMP7 (43). Thus, the increase in skeletal muscle surface membrane VAMP2 and VAMP7 during contractions implicates that both an accelerated exocytotic and decelerated endocytotic rate of GLUT4 traffic may occur during contractions, similar to what was observed for K+ stimulation (57).

A previous study showed that VAMP5 was heavily expressed in skeletal muscle and heart and was localized mainly to plasma membranes but also intracellularly in mature skeletal muscle, and it was hypothesized that it may play a role in GLUT4 trafficking (63). Here, we show that VAMP5 is localized to intracellular GLUT4 vesicles and translocates to surface membranes in skeletal muscle during contractions. While VAMP5 is not involved in insulin-stimulated GLUT4 trafficking of adipocytes (58), further studies are required to delineate its potential role in GLUT4 trafficking in skeletal muscle.

In addition to their role in regulation of GLUT4 vesicle trafficking, the possibility for other putative effects of the contraction induced v-SNARE protein translocation could be an attractive target to pursue. Several other important proteins in skeletal muscle function and metabolism, such as Na+/K+-ATPase and FAT/CD36, have been shown to increase in cell surface membranes in response to insulin (19, 32, 35) and muscle contractions (Jepsen J, Rose A. J. and Kiens B, unpublished observations) (2, 23, 24). The Na+/K+-ATPase and FAT/CD36 are important in regulating membrane potential and fatty acid metabolism, respectively, in skeletal muscle, and they respond to aforementioned physiological stimuli by translocation from intracellular vesicles to cell surface membranes (2, 23, 24). However, the protein composition and mechanism of translocation of these intracellular vesicles are unknown, but
as several v-SNAREs also translocate with these stimuli, they are attractive candidates.

**Perspectives and Significance**

In the present study, we were able to show that the v-SNAREs VAMP2, VAMP3, VAMP 5, and VAMP7 associated with intracellular GLUT4-containing vesicles and that VAMP2, VAMP5, and VAMP7 translocate along with GLUT4, IRAP and the transferrin receptor from intracellular membranes to heavy membranes enriched in plasma membranes and t-tubuli. These findings suggest that VAMP2, VAMP5, and VAMP7 may be involved in the docking and fusion of GLUT4-containing vesicles to the surface membrane during contraction in mature skeletal muscle.

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