Journal of Cellular Biochemistry 52:1–7 (1993)

PROSPECTS

Role of Transverse Tubules in Insulin Stimulated Muscle Glucose Transport

G. Lynis Dohm, Patricia L. Dolan, Wilhelm R. Frisell, and Ronald W. Dudek

Departments of Biochemistry and Anatomy and Cell Biology, School of Medicine, East Carolina University, Greenville, North Carolina

Abstract Although the strongest evidence for recruitment of glucose transporters in response to insulin comes from studies with adipocytes, studies in muscle seem in general to confirm that glucose transporters are also translocated to the cell membrane in muscle in response to insulin. However, the observation that transverse tubule (T-tubule) membranes contain approximately five times more glucose transporter than sarcolemma raised a question as to where glucose transport occurs in muscle. The T-tubule membrane system is continuous with the surface sarcolemma and is a tubule system in which extracellular fluid is in proximity with the interior of the muscle fiber. The purpose of this Prospects article is to evaluate the possibility that the T-tubule membrane may represent a major site of glucose transport in skeletal muscle.

Using immunocytochemical techniques we have located GLUT4 glucose transporters on the T-tubule membrane and in vesicles near T-tubules. Since T-tubules form channels into the interior of the muscle fiber, glucose could diffuse or be moved by some peristaltic-like pumping action into the transverse tubules and then be transported across the membrane deep into the interior of the muscle fiber. This mode of transport directly into the interior of the cell would be advantageous over transport across the sarcolemma and subsequent diffusion around the myofibrils to reach the interior of the muscle. Thus, in addition to the role of the T-tubule in ion fluxes and contraction, this unique membrane system can also provide a pathway for the delivery of substrates into the center of the muscle cell where many glycolytic enzymes and glycogen deposits are located.

Key words: muscle, glucose transport, T-tubules, insulin

Much of what we know about regulation of glucose transport was discovered in adipocytes. Using membrane isolation techniques Cushman and Wardzala [1] and Suzuki and Kono [2] measured glucose transporters in the plasma membrane fraction and found that the number of transporter molecules was increased in response to insulin. There was a concurrent decrease in the number of transporters in an intracellular membrane fraction (called low density microsomes), demonstrating a movement of glucose transporters from an intracellular compartment to the cell membrane. To gain further evidence in support of the recruitment or translocation mechanism, we and others [3–6] fractionated membranes of skeletal muscle to demonstrate the movement of glucose transporters from a microsomal fraction to the sarcolemma. These studies demonstrated that the number of glucose transporters in a sarcolemma membrane fraction, measured by cytochalasin B binding, was doubled in response to insulin. However, since the rate of glucose transport is increased by more than 10 fold in some types of muscle, the two fold increase in transporters suggested that there was also an increase in the intrinsic activity of the sarcolemmal glucose transporters. Further studies in which glucose transport and number of transporters were measured in isolated sarcolemmal vesicles seemed to confirm this conclusion [7].

Although studies with isolated membranes seemed in general to confirm that glucose transporters were translocated in muscle in response to insulin, there were other observations that raised questions. First, because of the complex nature of muscle membranes it was difficult to obtain a sufficient yield of highly enriched membranes. Isolation methods for sarcolemmal membranes gave at best 15% yields of marker enzymes and raised the question whether the membranes isolated were representative. A sec-

Address reprint requests to: Dr. G. Lynis Dohm, Department of Biochemistry, School of Medicine, East Carolina University, Greenville, NC 27858.

ond confounding observation was that of Burdett et al. [8] who reported that the concentration of glucose transporters in transverse-tubule membranes (T-tubules) was approximately 5 fold higher than in the sarcolemma. Since T-tubules are contiguous with the exterior sarcolemma of the muscle fiber, this report raised the possibility that glucose transport could be occurring across the T-tubule. The purpose of this Prospects article is to evaluate the possibility that the T-tubule membrane is a major site of glucose transport in skeletal muscle.

Membrane Structure of Skeletal Muscle

One of the difficulties in comparing of the translocation mechanisms in adipocytes and skeletal muscle is that the membrane structures of the two cell types are so dramatically different. The basic structural unit of muscle is the muscle fiber which is covered by a membrane called the surface sarcolemma. Structurally continuous with the surface sarcolemma, and invaginating into the muscle fiber, is the T-tubule membrane system. A scanning electron micrograph of the surface of a skeletal muscle fiber shows a series of pores, which are the T-tubule invaginations of the sarcolemma, appearing as if a rod had been pushed repeatedly and in a consistent pattern into a pliable sheet to form small channels. In addition to providing access of extracellular fluid to the interior of the muscle cell, an important physiological function of the T-tubule system is the transmission of membrane depolarization to the central part of the muscle.

Within the muscle fiber are several long cylindrical contractile units called myofibrils (see Figure 1A). The myofibrils are surrounded by the tubular compartments of the sarcoplasmic reticulum which serve as a reservoir for the release and accumulation of calcium during muscle contraction. Continuous with the sarcoplasmic reticulum and in tight apposition to the T-tubule membrane are the terminal cisternae. As a unit, the T-tubule and the closely apposed terminal cisternae on either side is called the triad (Figure 1B).

Although there are other membrane structures inside a muscle cell (muscle fiber) the main point of this discussion is that the T-tubule membrane system is continuous with the exterior surface sarcolemma and that it is a tubule system in which extracellular fluid is in proximity with the interior of the muscle fiber.

Immunolocalization of GLUT 4 in Skeletal Muscle

To overcome the problems associated with cellular membrane isolations we decided to study the cellular localization of GLUT4 glucose transporter by immunolabeling the GLUT4 with a primary antibody and then detecting the primary antibody with colloidal gold labeled IgG. The gold is visualized by electron microscopy to locate GLUT4 within the muscle fiber. In human muscle under basal and insulin stimulated conditions we found only a very slight labeling of GLUT4 on the surface sarcolemma [9]. However, specific labeling for GLUT4 was clearly evident in the region of the triad and in vesicles in the cytoplasm. There were two labeling motifs in the triad region; a portion of the GLUT4 was associated with the T-tubule membrane and the remainder was clearly in vesicles in close proximity to the T-tubules (see Figure 2). We [9] also isolated sarcolemmal and T-tubule membranes by the methods of Burdett et al. [8] and confirmed by Western blot analysis that there was a heavy concentration of GLUT4 associated with triad membranes. However, in contrast with the cytochalasin B binding results of Burdett et al. [8] we found negligible amounts of the GLUT4 glucose transporter associated with the sarcolemmal membranes isolated by this method. Earlier we had found GLUT4 associated with sarcolemmal membranes isolated by two other isolation procedures [3] but those sarcolemmal fractions have more contamination by T-tubule marker enzymes than the membranes isolated by the method of Burdett et al. [8]. In fact, there appeared to be a correlation between the contamination by T-tubules and the amount of GLUT4 (unpublished observation).

Subsequent to the publication of our results there have been two papers published on the immunolocalization of GLUT4 in skeletal muscle [10,11] and one describing GLUT4 in heart [12]. Using immunogold labeling Rodnick et al. [10] showed that in unstimulated muscle, GLUT4 was not present in surface membranes but there was labeling in "tubulo-vesicular structures" clustered in the trans Golgi reticulum. In response to insulin and exercise GLUT4 underwent translocation to the sarcolemma. They also reported that GLUT4 was "occasionally" labeled between the myofibrils near the transverse tubules.

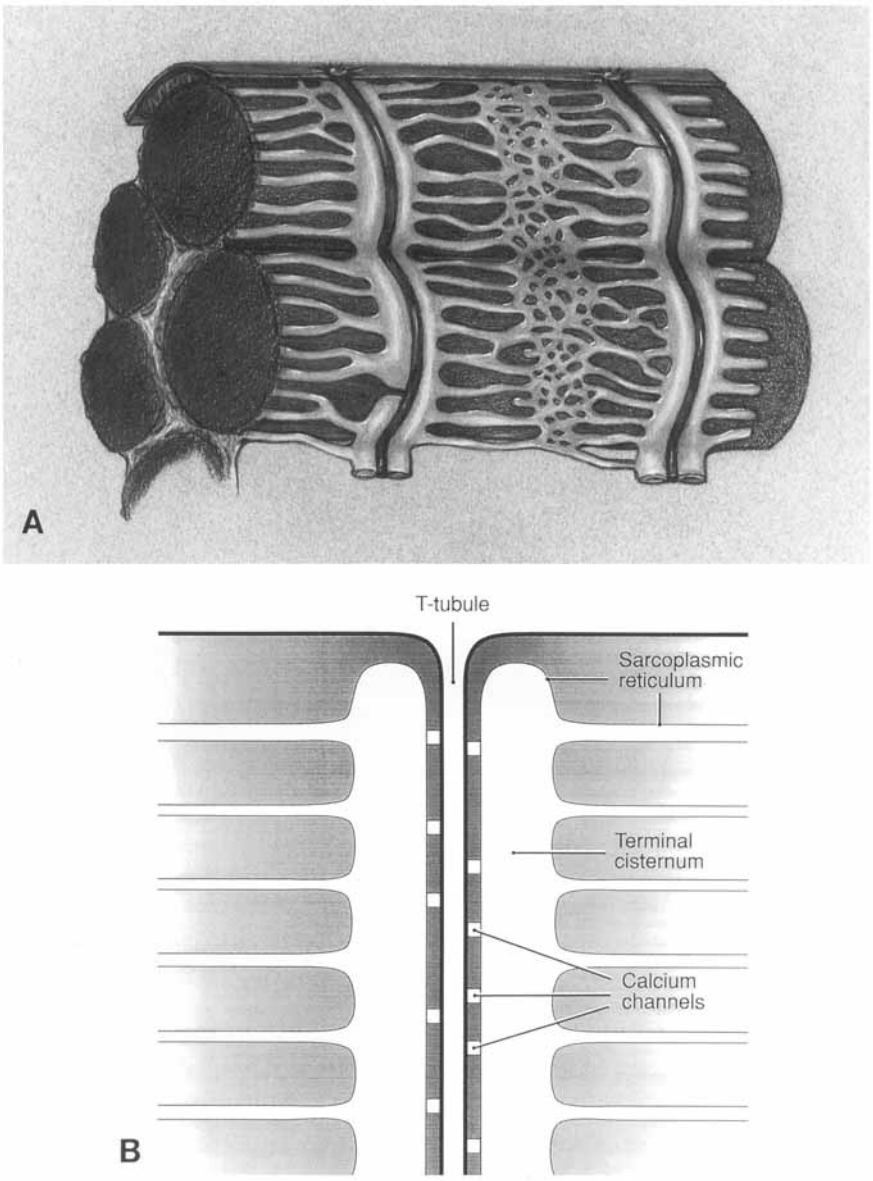

Fig. 1. Membrane structure of skeletal muscle **A:** Longitudinal section showing myofibrils surrounded by sarcoplasmic reticulum The muscle fiber is covered by the sarcolemma and the T-tubules invaginate and are flanked on either side by terminal cisternae **B:** Diagram demonstrating the juxtaposition of the T-tubule with sarcolemma and the terminal cisternae

Bornemann et al. [11] used a different technique to immunolabel the GLUT4 transporter in muscle. They prelabeled the muscle with primary antibody and visualized the GLUT4 by a horseradish peroxidase-coupled secondary antibody and diaminobenzidine. The muscle fibers were then embedded in epoxy resin and studied by electron microscopy. They reported strong immunoreactivity in subsarcolemmal clusters of vesicles and cisternae, Golgi-like structures, and triadic junctions. When the rats were injected with insulin 15 min before fixing the muscle, the sarcolemma was also labeled.

In heart the membrane structure is somewhat different from that in skeletal muscle but Slot et al. [12] reported similar results to those obtained within skeletal muscle [10]. In basal myocytes there was very little GLUT4 in the different domains of the plasma membrane (sarcolemma, intercalated disk, and transverse tubular system). After stimulation by insulin and exercise approximately 42% of GLUT4 was found

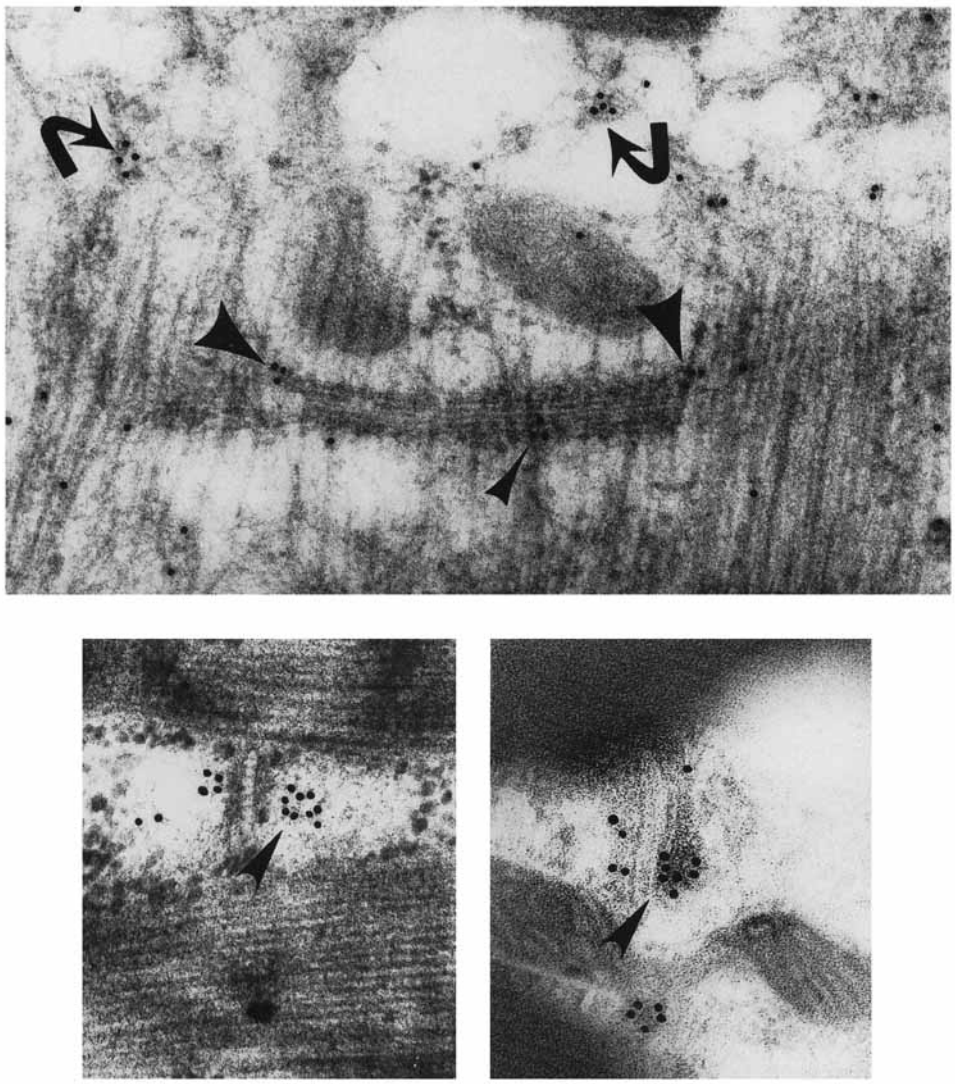

Fig. 2. Immunocytolocalization of GLUT4 protein on or near the T-tubule. **Top panel:** Longitudinal section through a T-tubule can be seen near the center of the micrograph. GLUT4 labels either on the T-tubule membrane (arrow heads) or in cytoplasmic vesicles (curved arrows). **Bottom left panel:** GLUT4 containing vesicles can be seen near the T-tubule. **Bottom right panel:** GLUT4 containing vesicle appears to be fused to the T-tubule.

in the plasma membrane with each domain of the plasma membrane contributing equally. The remainder of the labeling was in tubulo-vesicular elements at the same site as prior to stimulation.

Our observation that there was little GLUT4 on the sarcolemma even in the insulin stimulated state is in contrast to the other two published muscle studies and led us to investigate possible difficulties with our methods that might explain these discrepancies. Our initial studies were with human muscle but subsequent studies in our lab have confirmed that with our methods we get the same results in rat muscle. The use of the GLUT4 monoclonal antibody 1F8 has been criticized [12] but we have confirmed our original observations using three different polyclonal antibodies raised against the carboxyl-terminal peptide of GLUT4 and one polyclonal antibody raised against the amino terminal peptide of GLUT4. We have also been criticized for using osmicated tissue for our immunocytochemical localization. However, in subsequent studies we have used unosmicated samples that have been either aqueous or quick-freeze fixed and have observed similar location of GLUT4. Thus, the question of GLUT4 localization on the sarcolemma after insulin treatment remains unre-

solved. However, there remains agreement among the studies that there is GLUT4 in the triad region of muscle.

Intracellular Glucose Transporters

The intracellular location of glucose transporters can also be addressed by the antibody detection techniques discussed above. We observed glucose transporters in vesicles close to the T-tubule and near the Golgi region. Some vesicles appeared to be attached to T-tubules but there was no evidence that they were associated with terminal cisternae or sarcoplasmic reticulum (see Figure 2).

Two research groups have isolated intracellular membranes from skeletal muscle to try to characterize the cellular compartment associated with transporters. Douen et al. [13] used isopycnic centrifugation to isolate a membrane fraction that lost glucose transporters in response to insulin. This fraction was highly enriched with GLUT4 glucose transporters but did not contain marker for the sarcolemma or T-tubule. It did contain, but was not enriched with, sarcoplasmic reticulum marker enzymes, suggesting that the intracellular compartment was not in the sarcoplasmic reticulum.

Rodnick et al. [10] isolated the intracellular vesicles containing GLUT4 from rat skeletal muscles using immunoabsorption chromatography. Insulin treatment resulted in a 40% decrease in GLUT4 levels in these vesicles, confirming that they contained an intracellular pool of glucose transporters. The intracellular vesicle contained GLUT4 as its major component and was not enriched in markers specific for the sarcolemma, transverse tubules, sarcoplasmic reticulum or mitochondria. This study and that of Douen et al. [13] demonstrate that the glucose transporter resides in the cell in a vesicle that is not associated with any previously described membrane compartment.

Role of T-tubules in Glucose Transport

Although we feel that the significance of GLUT4 labeling on the surface sarcolemma remains an open question, there is general agreement among studies that GLUT4 is present in T-tubule membranes. It is necessary to consider, therefore, whether glucose transport may be occurring across the T-tubule membrane and what metabolic significance this might have. As stated previously, the strongest evidence that glucose transport does occur across the T-tubule membrane is that there are transporters located on the T-tubule membrane and that there are GLUT-4 containing vesicles in contact with or near the membrane. It seems unlikely that GLUT4 containing vesicles would be translocated from a peripherally located Golgi complex deep into the muscle fiber except for the purpose of glucose transport. The other argument for the "T-tubule glucose transport hypothesis" is that such transport presents unique advantages for the muscle cell. As discussed above, the T-tubules are continuous with the surface sarcolemma and form channels into the interior of the muscle fiber. Glucose could diffuse or be moved by the pumping action of contraction into the transverse tubules and then be transported across the membrane deep into the interior of the muscle fiber. This mode of transport directly into the interior of the cell would be advantageous over transport across the sarcolemma and subsequent diffusion around the myofibrils to reach the interior of the muscle. Thus, the T-tubules present a pathway for the delivery of substrates into the center of the muscle cell where many glycolytic enzymes and glycogen deposits are located. It is generally believed that glucose is immediately phosphorylated when it enters muscle and the localization of hexokinase between myofibrils [14] would further augment the efficacy of the transport of glucose across the T-tubule membrane.

It has been argued: (1) that the T-tubule in skeletal muscle is too narrow and long to allow efficient diffusion of glucose into the center of the muscle, and (2) that there is no evidence for translocation of glucose transporters to the T-tubule membrane in response to insulin or exercise. Several lines of evidence counter these arguments. Using stereological analysis of rat extensor digitorum longus and soleus muscles, Cullen et al. [15] reported that the transverse tubule in cross section is elliptical in shape with a minor axis of approximately 30 nm and a major axis of 120 nm. They also reported that the surface area density of the T-tubule system is approximately 30 $\mu m^2/100\ \mu m^3$, which is 2 to 3 times that of the sarcolemma. Although the diffusion distance down a T-tubule is difficult to determine because of the branched nature of the tubule system, the average length of a T-tubule between nodes (branch points) is 0.9 μm, with an average of 5.4 nodes around each myofibril [16]. By contrast, the sarcomere length is 2.4 μm [15].

The presence of serum albumin in the T-tubule [17] establishes that the fluid inside the T-tubule is in contact with extracellular fluid. The rate of exchange between the extracellular fluid and the T-tubule contents remains to be determined. In this regard, however, it is reasonable that, in addition to the movement of substances by simple diffusion, the contraction and relaxation of muscle fibers could have a pumping action forcing extracellular fluid in and out of the T-tubule.

The other important question to be addressed is whether translocation of glucose transporters to the T-tubule membrane occurs in response to insulin and/or muscle contraction. Using a novel membrane isolation technique, Marette et al. [18] recently reported in a preliminary communication that insulin stimulated the translocation of GLUT4 glucose transporters to the T-tubules from an intracellular organelle of rat skeletal muscle. The best immunocytochemical evidence to favor translocation comes from a study with heart muscle in which Slot et al. [12] found that glucose transporters moved to all areas of the plasmalemma (including the T-tubule) in response to insulin and exercise. We have not been able to demonstrate movement of glucose transporters to the T-tubule in skeletal muscle by electron microscopy. However, in our electron micrographs we find vesicles in close proximity to the T-tubule membrane that may be involved in translocation. We have considered the possibility that it may be beyond the resolution of our techniques to quantify translocation if this process occurs over these very small distances.

Since current methods for membrane isolation or immunocytolocalization have not provided a definitive answer to where glucose transport is occurring we felt that an entirely different experimental approach is needed. The technique of surface labeling the GLUT4 protein with a bis-mannose photolabel [19] seems a possible solution since it would label the glucose transporter on the surface where glucose transport is occurring, whether it be the sarcolemma or the T-tubule. The radiolabeled GLUT4 could then be localized by autoradiography. We have started experiments with this technique and preliminary results [20] show that in insulin stimulated muscle the label is located on the T-tubule membrane, with very little labeling on the surface sarcolemma of the muscle fiber. This would seem to confirm our immunocytolocalization observation that the GLUT4 is predominantly on the T-tubule membrane and that transport may occur primarily across this membrane deep within the muscle fiber.

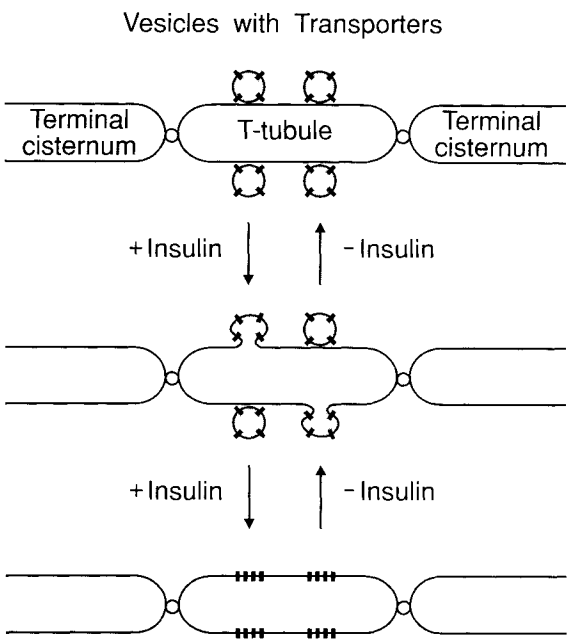

Fig. 3. A cartoon showing the proposed mechanism for increased glucose transport in response to insulin.

The T-tubule Glucose Transport Hypothesis

Based on the evidence cited above we propose the following working hypothesis that should be amenable to experimental testing. Most of the glucose transport in skeletal muscle occurs across the T-tubule membrane. In the basal state most of the glucose transporters are in intracellular vesicles near the T-tubule (Figure 3). In response to insulin the vesicles move to and fuse with the T-tubule membrane. When the insulin response is removed small vesicles bud endocytically from the T-tubule membrane. The vesicles remain attached to the T-tubule or, if detached, stay in close apposition to the T-tubule as a membranous complex, ready to re-enter the tubular membrane again upon stimulation by insulin and/or contraction of the muscle cell.

REFERENCES

1. Cushman SW, Wardzala LJ: J Biol Chem 255:4758–4762, 1980.
2. Suzuki K, Kono T: Proc Natl Acad Sci USA 77:2542–2545, 1980.
3. Fushiki T, Wells JA, Tapscott EB, Dohm GL: Am J Physiol 256:E580–E587, 1989.

4. Klip A, Ramlal T, Young DA, Holloszy JO: FEBS Lett 224:224–230, 1987.
5. Sternlicht E, Barnard RJ, Grimditch GK: Am J Physiol 254:E633–E638, 1988.
6. Hirshman MF, Goodyear LJ, Wardzala LJ, Horton ED, Horton ES: J Biol Chem 265:989–991, 1990.
7. Goodyear LJ, Hirshman MF, Smith RJ, Horton ES: Am J Physiol 261:E556–E561, 1991.
8. Burdett E, Beeler T, Klip A: Arch Biochem Bioph 253: 279–286, 1987.
9. Friedman JE, Dudek RW, Whitehead DS, Downes DL, Frisell WR, Caro JF, Dohm GL: Diabetes 40:150–154, 1991.
10. Rodnick KJ, Slot JW, Studdska DR, Hanpeter DE, Robinson LJ, Geuze HJ, James DE: J Biol Chem 267:6278–6285, 1992.
11. Bornemann A, Ploug T, Schmalbruch H: Diabetes 41: 215–221, 1992.
12. Slot JW, Geuze HJ, Gigergack S, James DE, Lienhard GE: Proc Natl Acad Sci USA 88:7815–7819, 1991.
13. Douen AG, Burdett E, Ramlal T, Rastogi S, Vranic M, Klip A: Endocrinol 128:611–616, 1991.
14. Lawrence GM, Trayer IP: Histo Chem J 17:353–371, 1985.
15. Cullen JJ, Hollingworth S, Marshall MW: J Anat 138: 297–308, 1984.
16. Peachey LD, Franzini-Armstrong C: In Handbook of Physiology, Section 10, Skeletal Muscle; edited by Peachey LD, Adrian RH, Geiger SR: American Physiology Society, Bethesda, MD, 1983, pp 23–71.
17. Knudson CM, Campbell KD: J Biol Chem 264:10795–10798, 1989.
18. Marette A, Burdett E, Douen A, Klip A: Diabetes 41: 42A, 1992.
19. Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield D, Simpson IA, Cushman SW: J Biol Chem 265:18172–18179, 1990.
20. Dudek RW, Dohm GL, Wilson CM: Diabetes 41:152A, 1992.