Vol. 103, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1981 Pages 759-765

MEMBRANE VESICLES FROM NEWBORN RAT SKELETAL MUSCLE RETAIN STEREOSPECIFIC D-GLUCOSE TRANSPORT PROPERTIES

Janet Stiernberg and Edward F. LaBelle

The University of Texas Medical Branch
Department of Human Biological Chemistry & Genetics
Galveston, Texas 77550

Received October 14,1981

SUMMARY: A novel procedure has been developed to prepare membrane vesicles from newborn rat skeletal muscle which retain a stereospecific D-glucose transport system characteristic of intact muscle. The glucose transport system found in these rat muscle membrane vesicles exhibits counterflow, inhibition by cytochalasin B and phloridzin, and kinetics consistent with a carrier-mediated process. We conclude that this procedure will allow the rapid preparation of membrane vesicles retaining a stable glucose transport activity essential for eventual purification of the transporter.

In order to better understand the mechanism by which insulin exerts its effect on either the adipocyte or the muscle cell, an improved understanding of glucose transport into these cells is essential. One of the most important physiological functions of insulin is the stimulation of glucose transport across the surface membranes of such cells. Although the system required for glucose transport across the plasma membrane of the muscle cell has been characterized by workers using either intact tissue (1) or isolated myocytes (2), little work has been done with subcellular fractions from such tissue. The specific uptake of D-glucose into sarcolemmal vesicles has been demonstrated by Cheng, et al. (3). However, the method used to obtain these vesicles required that the tissue be exposed to high ionic strength for a long period at slightly alkaline pH to extract contractile proteins from the myotubes (4). Such harsh treatment may alter or destroy the activity of membrane-bound proteins rendering it unsuitable for further purification. Indeed, we have shown that high salt concentrations will destroy the activity of the mitochondrial enzyme succinate dehydrogenase.

In these studies we have developed a novel procedure for preparing a

ABBREVIATIONS: PMSF - phenylmethylsulfonylfluoride

0006-291X/81/220759-07$01.00/0

stable glucose transport system from skeletal muscle to allow for the eventual purification of this transporter. Portions of this study have been previously reported (5).

METHODS: For vesicle preparation, muscle was dissected from the hind legs of 4-5 day old rats, collected in 9-10 volumes ice-cold homogenization medium containing sucrose (0.25 M), histidine (30 mM, pH 7.4), Na_2-EDTA (1 mM), and PMSF (100 μM), and then incubated at 22°C for 60 min. The muscle was then minced with dissection scissors and homogenized at 4°C with 5 strokes of a glass-glass Dounce tissue grinder (Pestle B). The homogenate was centrifuged for 10 min at 2500xg and the pellet discarded. The low speed supernatant was centrifuged for 30 min at 150,000xg to pellet the membrane vesicles, which were then resuspended in homogenization medium and washed once by centrifugation. The final pellet was suspended in medium containing sucrose (0.25 M) and histidine (30 mM, pH 7.4) at a protein concentration between 6 and 9 mg/ml and stored at -70°C. Protein was determined using established techniques (6).

The accumulation of D-[^{3}H]-glucose by the membrane vesicles was determined by a modification of the method of Penefsky (7). A 1-ml syringe barrel (5 mm, i.d.) was plugged with glass wool and filled with Sephadex G-50 (40-80 mesh) previously swollen in NaCl (150 mM), $MgSO_4$(2 mM), and Tris-HCl (10 mM, pH 7.4). The syringe barrel was placed in a test tube and centrifuged 2 min at 250xg. An aliquot (100 μl) of reaction mixture at 22°C containing membrane vesicles in NaCl (126 mM), $MgSO_4$ (1.7 mM), Tris-HCl (8.4 mM, pH 7.4), and either D-[^{3}H]-glucose (4 μCi, 2.4 mM) or L-[^{3}H]-glucose (4 μCi, 2.4 mM) was applied to the top of the Sephadex in the syringe barrel. Centrifugation was repeated as above, and the [^{3}H]-content of the effluent determined. With this technique greater than 99.9% of the free extravesicular [^{3}H]-glucose was retained by the Sephadex in the syringe barrel.

Specific binding of [^{3}H]-ouabain to membrane vesicles was measured by a modification of the method of Lin and Akera (8). After separating bound and unbound ouabain, the pellet (with bound ouabain) was suspended in 0.5 ml H_2O and the radioactivity determined. Specific ouabain binding was defined as the total ouabain binding observed in the absence of excess unlabeled ouabain minus nonspecific ouabain binding observed in the presence of excess unlabeled ouabain (1 mM).

RESULTS AND DISCUSSION: In these studies we have developed a novel procedure to prepare membrane vesicles from skeletal muscle which retain the stereospecific D-glucose transport properties of intact muscle. This procedure avoided the use of high salt concentrations that might damage membrane proteins, and the vesicles could be stored at -70°C for 10-12 days without loss of glucose transport activity. Even after 6 months at -70°C the vesicles retained 50-60% of their initial activity. The membrane vesicles were capable of specific D-glucose transport (Fig. 1). The specific uptake process, defined as D-glucose uptake minus L-glucose uptake, reaches a maximum within 2-5 min. The rapid nature of this D-glucose accumulation process was consistent with what

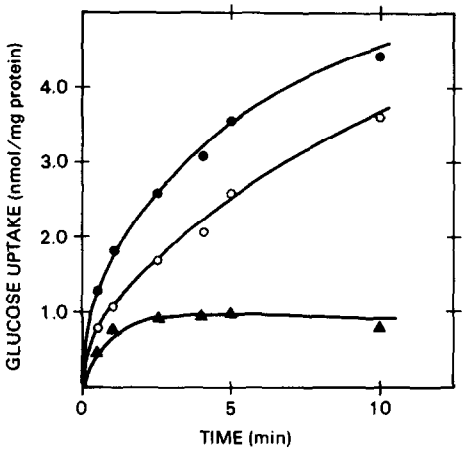


Figure 1. Effect of Time on Glucose Accumulation by Rat Muscle Membrane Vesicles.

The accumulation of either D-[^{3}H]-glucose (●——●) or L-[^{3}H]-glucose (o——o) by aliquots of rat muscle membrane vesicles (127 µg protein) was determined for the times indicated as described in Methods. The difference between D-glucose and L-glucose accumulation (stereospecific D-glucose accumulation) is also shown (▲——▲).

other investigators have observed for D-glucose transport into small vesicles (3,9,10). Intact cells do not take up L-glucose (2,11); however, this process has been observed in numerous subcellular fractions (3,9,12). Therefore, L-glucose uptake, which most likely results from a nonspecific leak introduced during homogenization, was measured as a control for either artifactual glucose binding or non-carrier mediated glucose leakage into vesicles. This nonspecific glucose uptake was 60% of the total D-glucose uptake at 5 min and continued to increase for 5 hrs, at which time the stereospecific D-glucose accumulation declined to zero. Paper chromatography was used to prove that the [^{3}H]-compound accumulated by the vesicles was indeed glucose. Following paper chromatography with methanol/ethanol/water (45/45/10, v/v/v), the [^{3}H]-labeled compound accumulated by the vesicles migrated the same distance (R_f = 0.49) as either standard unlabeled D-glucose or the D-[^{3}H]-glucose obtained commercially. (Data not shown).

In order to assess whether or not the vesicles contained sarcolemmal membrane, specific [^{3}H]-ouabain binding activity was measured. Ouabain is

TABLE I

LABELED GLUCOSE REMAINING IN THE VESICLES

(nmol/mg protein ± S.D., n = 3)

Sample	D-Glucose	L-Glucose
Control Vesicles	81.7 ± 6	49.6 ± 3
Diluted Vesicles	49.2 ± 4	40.0 ± 1
Glucose Leaving the Vesicles in 1 Min.	32.5 ± 6	9.6 ± 3

EFFLUX OF GLUCOSE FROM RAT MUSCLE MEMBRANE

After 30 min of uptake at 22°C of either labeled D-glucose or L-glucose (2 μCi, 3.85 mM in medium containing 103 mM NaCl, 1.4 mM $MgSO_4$, and 7 mM Tris-HCl, pH 7.4) the membrane vesicles were either diluted 5-fold with nonradioactive medium lacking glucose or not diluted at all. An aliquot of the control vesicles (those which were not diluted) was applied to a Sephadex column and centrifuged as described in Methods. An aliquot of the diluted vesicles was incubated for 1 min to allow release of intravesicular glucose before applying to the Sephadex column.

known to bind the (Na^+,K^+)-ATPase, a marker enzyme for plasma membrane. The vesicles bound 6.21 ± 2.2 pmol ouabain specifically per mg vesicle protein. The specific binding of ouabain indicates that the membrane vesicles contain sarcolemmal membrane.

In order to rule out the possibility that the stereospecific D-glucose accumulation represented binding rather than transport, efflux of glucose from the vesicles was analyzed. For these experiments vesicles were preloaded with either labeled D- or L-glucose, diluted with nonradioactive medium, and incubated to determine the rate of glucose release. As shown in Table I, D-glucose could leave vesicles preloaded with the labeled compound faster than could L-glucose. As pointed out by Cheng, et al. (3), high affinity binding of D-glucose to sarcolemma could not account for both the preferential uptake of D-glucose and the preferential release of D-glucose.

The stereospecific uptake of D-glucose was directly proportional to the membrane vesicle protein concentration (data not shown). Glucose uptake also increased as a function of glucose concentration (data now shown), but no K_m or V values could be determined since the stereospecific D-glucose uptake was

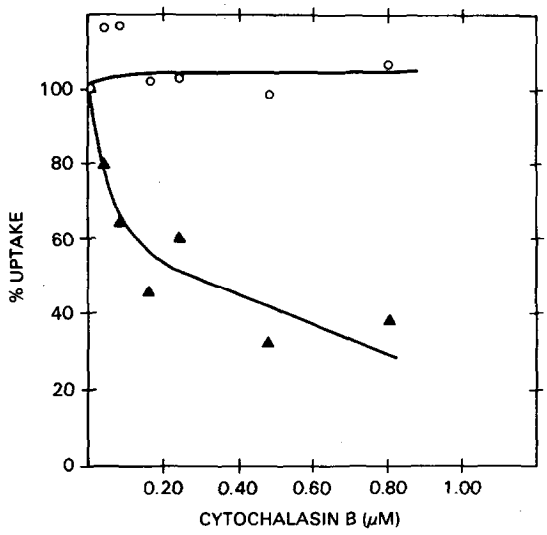
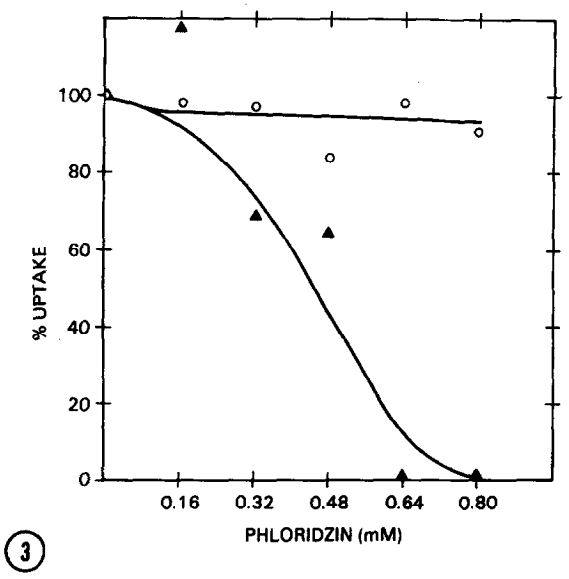


Figure 2. Effect of Increasing Concentrations of Cytochalasin B on Glucose Accumulation by Rat Muscle Membrane Vesicles.

Aliquots of rat muscle membrane vesicles (36 μg protein) were incubated for 5 min as described in Methods with increasing amounts of Cytochalasin B. The L-glucose uptake (o——o) and the stereospecific D-glucose uptake (▲——▲) are expressed as percent of control values.

too rapid to permit determination of an initial rate. Although Cheng, et al. (3) do not demonstrate that they are measuring an initial rate of D-glucose transport, their data show specific D-glucose uptake is a saturable function of glucose concentration. While transport would show an initial rate saturable with substrate, the extent of D-glucose accumulation should be directly proportional to glucose concentration.

As shown in Figures 2 and 3, cytochalasin B and phloridzin, well known inhibitors of glucose transport into intact cells (11,13-15), inhibited stereo specific D-glucose uptake into membrane vesicles. They had no inhibitory effect on the uptake of L-glucose which ruled out non-specific damage to the vesicles. The concentrations of cytochalasin B (0.28 μM) and phloridzin (0.45 mM) required for 50% inhibition of specific D-glucose transport into rat muscle membrane vesicles were similar to the concentrations found effective by other investigators (3,9,13,14). Complete inhibition of the stereo-



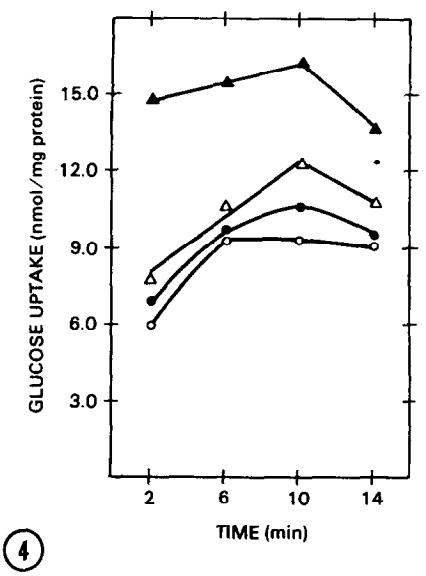


Figure 3. Effect of Increasing Concentrations of Phloridzin on Glucose Accumulation by Rat Muscle Membrane Vesicles.

Aliquots of rat muscle membrane vesicles (83 µg protein) were incubated for 5 min as described in Methods with increasing amounts of phloridzin. The L-glucose uptake (o——o) and the stereospecific D-glucose uptake (▲——▲) are expressed as percent of control values.

Figure 4. Demonstration of Counterflow in Rat Muscle Membrane Vesicles.

Aliquots of rat muscle membrane vesicles (176 µg protein) were preincubated with unlabeled D- or L-glucose (either 5 mM (open symbols) or 50 mM (Closed symbols)) in medium containing NaCl (75 mM), $MgSO_4$ (1 mM), and Tris-HCl (5 mM, pH 7.4) for 20 min at 22°C. These mixtures were then diluted 10-fold with labeled and unlabeled D-or L-glucose to give a final extravesicular glucose concentration of 5 mM. At the indicated times, the incubations were terminated by the Sephadex centrifugation procedure, and the radioactivity in the recovered vesicles was determined as described in Methods. Circles show L-glucose uptake. Triangles show D-glucose uptake.

specific glucose transport was also possible with both cytochalasin B and phloridzin.

The membrane vesicles were shown to be capable of uphill counterflow when a concentration gradient was induced across the vesicle membrane (Fig. 4). This phenomenon has also been observed with intact muscle (1) and other cellular systems (16). When the ratio of internal D-glucose to external D-glucose was 10:1, the uptake of D-[^{3}H]-glucose was dramatically enhanced, while a

similar gradient of L-glucose failed to effect either L-[^{3}H]-glucose (Fig. 4) or D-[^{3}H]-glucose uptake (data not shown).

The glucose transport system found in these rat muscle membrane vesicles exhibits counterflow, inhibition by cytochalasin B and phloridzin, and kinetics consistent with a carrier-mediated process. We conclude that this glucose transport system remains intact and is identical with the one in intact muscle. The rat muscle membrane vesicles are reasonably stable and such stability is essential if a transport protein is to be purified.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Irma Mendoza for secretarial assistance. This work was supported by grants from the National Institutes of Health (AM-25244) and the Muscular Dystrophy Association.

REFERENCES

1. Morgan, H.E., Regen, D.M. and Park, C.R. (1964) J. Biol. Chem. 239, 369-374.
2. Schudt, C., Gaertner, U. and Pette, D. (1976) Eur. J. Biochem. 68, 103-111.
3. Cheng, L.C., Rogus, E.M. and Zierler, K. (1978) Biochim. Biophys. Acta 513, 141-155.
4. Cheng, L.C., Rogus, E.M., Zierler, K. (1977) Biochim. Biophys. Acta 464, 338-346.
5. Stiernberg, J. and LaBelle, E.F. (1981) Federation Proc. 40, 1982.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891-2899.
8. Lin, M.H. and Akera, T. (1978) J. Biol. Chem. 253, 723-726.
9. Zala, C. A. and Perdue, J.F. (1980) Biochim. Biophys. Acta 600, 157-172.
10. Hilden, S.A. and Sacktor, B. (1979) J. Biol. Chem. 254, 7090-7096.
11. LeFevre, P.G. (1961) Pharmacol. Rev. 13, 39-70.
12. Paris, S. and Ailhaud, G. (1980) Biochim. Biophys. Acta 601, 630-639.
13. Kohn P.G. and Clausen, T. (1971) Biochim. Biophys. Acta 225, 277-290.
14. Mizel, S.B. and Wilson, L. (1972) J. Biol. Chem. 247, 4102-4105.
15. Bloch, R. (1973) Biochemistry 12, 4799-4801.
16. Stein, W.D. (1967) The Movement of Molecules Across Cell Membranes, pp. 126-176, Academic Press, New York and London.