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Kinetic characterization and radiation-target sizing of the glucose transporter in cardiac sarcolemmal vesicles

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Stereospecific glucose transport was assayed and characterized in bovine cardiac sarcolemmal vesicles. Sarcolemmal vesicles were incubated with D-[^{3}H]glucose or L-[^{3}H]glucose at 25 °C. The reaction was terminated by rapid addition of 4 mM $HgCl_2$ and vesicles were immediately collected on glass fiber filters for quantification of accumulated [^{3}H]glucose. Non-specific diffusion of L-[^{3}H]glucose was never more than 11% of total D-[^{3}H]glucose transport into the vesicles. Stereospecific uptake of D-[^{3}H]glucose reached a maximum level by 20 s. Cytochalasin B (50 μM) inhibited specific transport of D-[^{3}H]glucose to the level of that for non-specific diffusion. The vesicles exhibited saturable transport (K_m = 9.3 mM; V_{max} = 2.6 nmol/mg per s) and the transporter turnover number was 197 glucose molecules per transporter per s. The molecular sizes of the cytochalasin B binding protein and the D-glucose transport protein in sarcolemmal vesicles were estimated by radiation inactivation. These values were 77 and 101 kDa, respectively, and by the Wilcoxen Rank Sum Test were not significantly different from each other.

Introduction

The glucose transporter is an integral membrane protein which catalyzes stereospecific facilitated diffusion of D-glucose from the extracellular fluid to the cytosol. Attempts to characterize the membrane components responsible for hexose transport have been extensive (see Refs. 1 and 2 for reviews) and have focused primarily on membrane proteins from either the human erythrocyte or rat adipocyte. The glucose transporter from the erythrocyte has been purified and its size estimated [3,4]. Furthermore, the erythrocyte glucose transporter was reconstituted successfully into artificial liposomes which afforded an analysis of its kinetic features [5]. Some progress also has been made in the purification and reconstitution of glucose transporters from rat adipocytes [6], bovine thymocytes [7] and Ehrlich ascites cells [8].

In contrast, relatively little has been reported on the glucose transporter from myocardium. Kono and co-workers [9] reconstituted the glucose transport activity from subcellular fractions of rat heart into egg phosphatidylcholine liposomes in order to study the action of insulin on cardiac muscle. However, this report did not contain any

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information regarding kinetic parameters of the glucose transporter. Subsequently, Wheeler and Hauck [10] demonstrated D-glucose-reversible cytochalasin B binding in bovine myocardial sarcolemmal membranes. They also reconstituted sarcolemmal membrane proteins into soybean phosphatidylcholine liposomes and found that stereospecific D-glucose concentration in these vesicles reached a maximum level in 1 to 2 minutes. Furthermore, the initial rate of equilibration was reduced at higher concentrations of glucose indicating that the transport system was saturable. The authors emphasized that they did not attempt kinetic studies in detail because of the high proportion (66%) of non-specific (L-glucose) uptake.

As a first step in characterizing the glucose transporter and its regulation in cardiac tissue we have utilized isolated sarcolemmal vesicles from bovine heart to determine kinetic parameters. Additionally, we assessed the apparent molecular size of D-glucose-sensitive cytochalasin B binding protein and D-glucose transport protein in these vesicles by applying classical target theory to irradiation-inactivation data.

Materials and Methods

Preparation of cardiac sarcolemmal vesicles. Cardiac sarcolemmal membrane vesicles were prepared by the procedure of Kuwayama and Kanazawa [11], as modified by Slaughter et al. [12], from trimmed bovine ventricular tissue obtained fresh from a local abattoir. Vesicles thus prepared were suspended in 160 mM NaCl, 20 mM 4-morpholinepropanesulfonic acid (Mops) and tris(hydroxymethyl)aminomethane (Tris) (pH 7.4). A sample was removed for determination of protein concentration by the method of Lowry et al. [13]. The remaining vesicles were maintained at −80°C prior to use. Glucose transport assays were performed only on vesicles subjected to one freeze-thaw cycle. Results from preliminary experiments indicate that at a glucose concentration of 5 mM no transport activity was lost following a single freeze-thaw cycle.

Assay of glucose transport activity in cardiac sarcolemmal vesicles. Cardiac sarcolemmal vesicles (1–4 mg protein/ml) were thawed 30 min prior to the assay. Stock solutions of D-[^{3}H]glucose or L-[^{3}H]glucose were dried with a stream of nitrogen gas in a polystyrene test tube and were subsequently dissolved in appropriate buffers at the indicated D- or L-glucose concentrations (0.15 mCi/ml). For each assay point, 50 μl of [^{3}H]glucose solution were transferred to a 12 × 75 mm polystyrene test tube. Twenty microliters of vesicle suspension (20 to 80 μg sarcolemmal protein) were placed on the side of the tube above the level of the [^{3}H]glucose solution. At time zero, the tube contents were mixed on a vibrating platform. The reaction was terminated by rapid addition of 4 ml of ice-cold stop solution consisting of 160 mM NaCl, 20 mM Mops-Tris, 4 mM $HgCl_2$ (pH 7.4). Vesicles were immediately collected on glass fiber filters (GF/A; Whatman) which had been wetted previously in 1.5 M D-glucose. This was followed by seven 4-ml washes of the filter with stop solution. Tubes for non-specific filter binding were processed identically except that vesicles were not included in the tube contents. Values for non-specific filter binding were not different from time-zero values obtained when vesicles were present and the tube contents were mixed by addition of stop solution. The blank values comprised less than 10% of the lowest ^{3}H counts associated with glucose transport into vesicles. Initial rate estimates were obtained from regression line slopes between 0 and 5, or 6 s. All transport values were obtained from vesicles incubated at 25°C. The [^{3}H]glucose in vesicles was quantified by liquid scintillation spectroscopy.

Cytochalasin B binding assay. Cytochalasin B binding to cardiac sarcolemmal vesicles was measured using the technique described by Sergeant and Kim [14]. Cytochalasin B binding was measured by incubating vesicles with a concentration range of unlabeled cytochalasin B (0–10 μM) and 0.007 μCi of [^{3}H]cytochalasin B in the presence of either 0.5 M D- or L-glucose. All incubations were done in buffer (160 mM NaCl, 20 mM Mops-Tris (pH 7.4)) containing 5 μM cytochalasin E. D-Glucose-displaceable cytochalasin B binding was determined as the difference between cytochalasin B binding in the presence of L- and D-glucose. The number of cytochalasin B-binding sites was estimated by non-linear regression analysis using the BDATA computer program (EMF Software, Knoxville, TN).

Radiation-target sizing of cytochalasin B binding protein and D-glucose transport protein. The cardiac sarcolemmal vesicle samples for irradiation were spread as a thin film in aluminum planchets. 500 μl of vesicle suspension (0.5–3.0 mg protein) were used for each sample. The vesicle sample in each planchet was quick-frozen in liquid nitrogen, packed on dry ice, and shipped to Buffalo, New York for irradiation-inactivation. At no time during shipment to or from Buffalo, or during radiation treatment, were vesicle samples allowed to thaw. Radiation treatment was as described by Jung et al. [4]. Briefly, a Van de Graaff accelerator was used to generate a fast (1.5 MeV) electron beam. The still-frozen vesicle samples were placed in an aluminum chamber on a chain conveyer which passed under the electron beam with a uniform geometry and frequency. During irradiation, the samples in the chamber (−20 psi) were maintained at −45 to −50°C. Radiation doses were measured at the sample irradiation temperature using blue cellophane film (DuPont MSC-300) as described previously [4]. Following irradiation, frozen vesicle samples were shipped on dry ice back to Columbia, Missouri for determination of cytochalasin B binding and D-glucose transport activity. Radiation-inactivation data were analyzed using classical target theory as described by Kepner and Macey [15].

Statistics. To determine if there were differences in target size estimates of molecular weights for cytochalasin B binding protein and glucose transport protein a regression line was fit to the data from each irradiated sample and the slopes of these lines were used as data points. The Wilcoxen Rank Sum Test was then applied to the slopes to compare the target size estimates for the cytochalasin B binding and glucose transport proteins. The estimated variability of the mean target size is expressed as the standard deviation obtained directly from the estimates of the standard deviation of the slopes of the regression lines. All other values are reported as means ± S.E.

Chemicals. D-[^{3}H]Glucose and L-[^{3}H]glucose were obtained from American Radiolabeled Chemicals Inc., St. Louis, MO. D-Glucose and L-glucose were obtained from Sigma Chemical Co., St. Louis, MO. [^{3}H]Cytochalasin B was obtained from New England Nuclear, Boston, MA. Cytochalasin B was obtained from Aldrich Chemical Company, Milwaukee, WI.

Results

Time-course of glucose transport and effect of vesicle quantity

The time-course of stereospecific glucose transport into bovine myocardial sarcolemmal vesicles is illustrated in Fig. 1. In these experiments, nonspecific diffusion (represented by influx of 10 μM L-[^{3}H]glucose) was never more than 11% of total D-[^{3}H]glucose transport into the vesicles. Intravesicular D-glucose concentration approached a maximum value by 20 s.

The effects of vesicle quantity (represented by vesicular protein) on stereospecific [^{3}H]glucose uptake are illustrated in Fig. 2. In this experiment increasing amounts of a vesicle preparation were incubated in either D-[^{3}H]glucose or L-[^{3}H]glucose (both 20 μM) for 5 s. Linear regression analysis of D-[^{3}H]glucose influx and vesicle protein produced an r^2 value of 0.99. Non-specific diffusion of L-[^{3}H]glucose into the vesicles was negligible at all vesicle quantities.

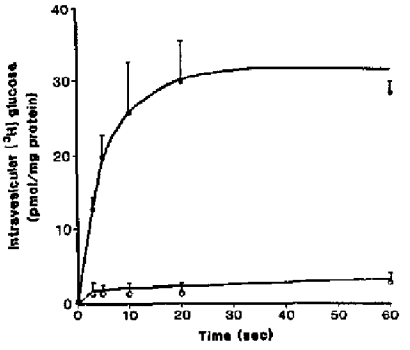

Fig. 1. Time-course of stereospecific glucose transport into bovine myocardial sarcolemmal vesicles. Vesicles were rapidly mixed with 10 μM D-[^{3}H]glucose or -[^{3}H]glucose for times indicated and collected on glass filters as described in Materials and Methods. Data points are the means ± S.E. for at least three separate vesicle preparations. D-[^{3}H]Glucose (●); L-[^{3}H]glucose (○).

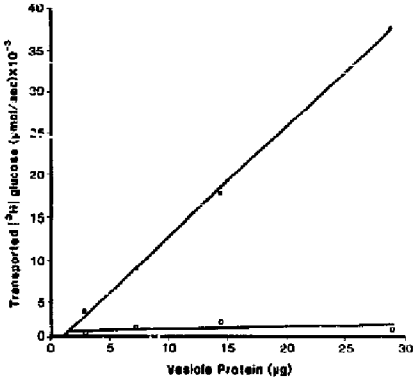

Fig. 2. Effect of vesicle quantity on [^{3}H]glucose uptake. Glucose uptake was measured in the presence of 20 μM glucose as described in Materials and Methods and with different amounts of vesicles (represented by vesicle protein). $r^2 = 0.99$ for linear regression of D-[^{3}H]glucose uptake. D-[^{3}H]Glucose (●); L-[^{3}H]glucose (○).

Inhibition by cytochalasin B

Inhibition of D-[^{3}H]glucose uptake by cytochalasin B is illustrated in Fig. 3. Cytochalasin B has been shown to be a potent, competitive inhibitor of carrier-mediated glucose transport in a number of preparations including isolated cardiomyocytes [16]. In cardiac sarcolemmal vesicles, cytochalasin B (50 μM) inhibited specific transport of D-[^{3}H]glucose and decreased its initial uptake to the level of that for non-specific (L-[^{3}H]glucose) diffusion.

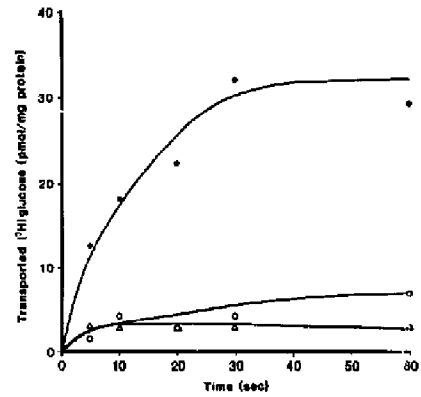

Fig. 3. Cytochalasin B inhibition of stereospecific glucose uptake. Glucose concentrations were 10 μM and cytochalasin B concentration was 50 μM. D-[^{3}H]Glucose (●); L-[^{3}H]glucose (○); D-[^{3}H]glucose plus cytochalasin B (△).

Kinetic parameters of the glucose transporter

The kinetic parameters of specific D-glucose transport into vesicles were estimated from the initial D-glucose and L-glucose transport rates determined over a broad range of substrate concentrations (Fig. 4). The 0, 3, and 5 s ($N = 4$), or 0, 3 and 6 s ($N = 6$), uptakes at each glucose concentration were used as a measure of initial rates. Influx of L-glucose was subtracted from that for D-glucose to obtain stereospecific transport rates. Eadie-Hofstee analyses of the data yielded a K_m for the glucose transport of 9.3 ± 0.9 mM and a V_{max} of 2.6 ± 0.6 nmol/mg per s (mean of ten separate vesicle preparations). The K_m and V_{max} values obtained by non-linear regression analysis were 11.7 ± 1.9 mM and 2.8 ± 0.7 nmol/mg per s,

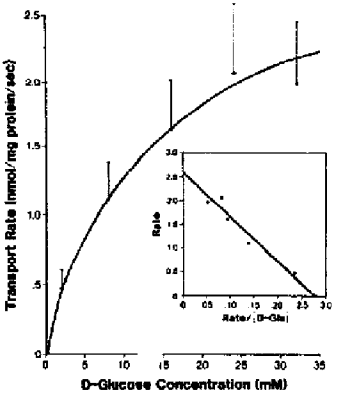

Fig. 4. Effect of D-glucose concentration on transport rates in sarcolemmal vesicle preparations. The stereospecific D-glucose transport was determined by subtracting L-glucose influx from that for D-glucose. The calculated K_m and V_{max} values from non-linear regression analysis were 11.7 ± 1.9 mM and 2.8 ± 0.7 nmol/mg per s, respectively (means ± S.E. of ten separate vesicle preparations). Inset: Eadie-Hofstee plot of the same data. Rate = D-glucose transport rate (nmol/mg per s) and [D-Glu] = D-glucose concentration (mM). The kinetic parameters were $K_m = 9.3 \pm 0.9$ mM and $V_{max} = 2.6 \pm 0.6$ nmol/mg per s for the Eadie-Hofstee plot.

respectively. The transporter turnover number calculated from a V_{max} of 2.6 nmol/mg per s and a B_{max} of 13.2 pmol/mg for cytochalasin B binding was 197 glucose molecules per transporter per s.

Radiation-target sizing of cytochalasin B binding protein and glucose transport protein

Specific binding of cytochalasin B to vesicles was progressively reduced with an increasing dose of radiation (Fig. 5a). The target size of the cytochalasin B binding protein in sarcolemmal vesicles determined from three independent experiments was 77 ± 5 kDa ($\pm$S.D.). Fig. 6a illustrates D-[^{3}H]glucose-specific transport into vesicles as a function of radiation dose. Transport decreased with increasing amounts of radiation and the molecular weight estimate of the D-glucose transport protein was 101 ± 5 kDa ($\pm$S.D.) ($N = 10$ independent experiments). Statistical analysis showed there was no difference between these two molecular weight estimates ($P > 0.1$). Finally, to determine if irradiation altered either the transport K_m or cytochalasin B binding K_d these

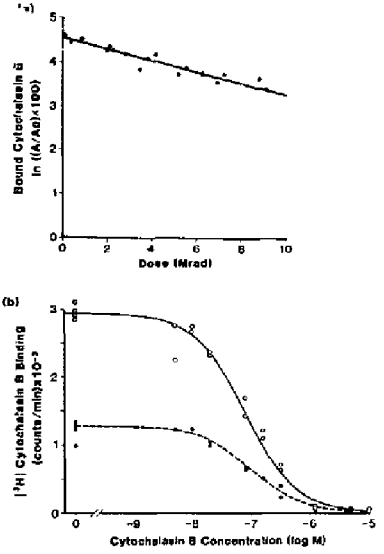

Fig. 5. Radiation-inactivation of cytochalasin B binding protein in bovine cardiac sarcolemmal vesicles: (a) Semi-log plot of survival dose relationship for glucose-sensitive cytochalasin B binding. Each data point represents bound cytochalasin B (A) normalized relative to that of nonirradiated controls (A_0). Single point estimates were determined in the presence of 23 nM cytochalasin B. The data are from three mutually independent experiments. (b) Semi-log plot of cytochalasin B binding to sarcolemmal vesicles before (○) and after (●) a radiation dose of 3.3 Mrad. Before irradiation $K_d = 67.0$ nM and $B_{max} = 13.2$ pmol/mg. Following irradiation $K_d = 83.0$ nM and $B_{max} = 7.2$ pmol/mg.

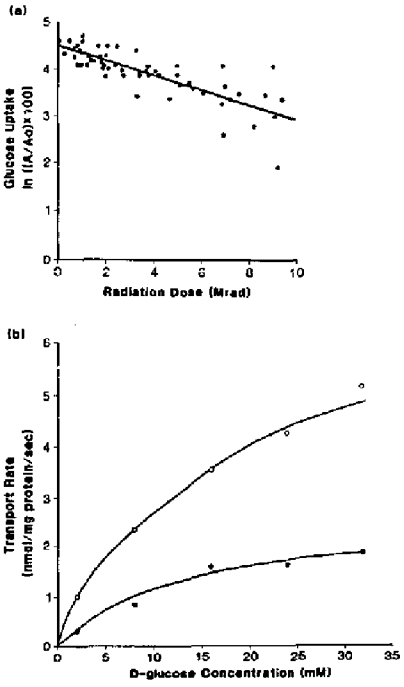

Fig. 6. Radiation-inactivation of glucose transport protein in bovine cardiac sarcolemmal vesicles: (a) Semi-log plot of survival dose relationship for stereospecific D-glucose transport. Each data point represents the initial rate of transport of 1 mM D-glucose (A) normalized relative to that of nonirradiated controls (A_0). Initial D-glucose transport rates were determined as described in Materials and Methods. The data are from ten mutually independent experiments. (b) Effect of D-glucose concentration on initial transport rates into sarcolemmal vesicles before (○) and after (●) a radiation dose of 3.3 Mrad. Before irradiation $K_m = 11.1$ mM and $V_{max} = 6.2$ nmol/mg per s. Following irradiation $K_m = 14.2$ mm and $V_{max} = 2.8$ nmol/mg per s.

parameters were determined for a single sarcolemmal vesicle preparation prior to and after irradiation. The effects of irradiation on cytochalasin B binding and glucose uptake are shown in Figs. 5b and 6b, respectively. Irradiation appeared to have no major effect on K_d or K_m values. The before and after irradiation values (respectively) for K_d were 67.0 and 83.0 nM and for K_m were 11.1 and 14.2 mM.

Discussion

The experiments reported here show that bovine cardiac sarcolemmal vesicles contain the D-glucose transporter. The transporter is functional in that it shows stereospecificity to substrates and is sensitive to inhibition by cytochalasin B. Most importantly, the vesicles are composed of native membranes. These data are therefore an accurate reflection of glucose transport behavior in intact myocardial sarcolemma without complications which may be caused by cytoplasmic components.

Bovine sarcolemmal vesicles are more suitable for transport studies on the myocardial glucose carrier than the vesicle preparation reported previously by Wheeler and Hauck [10]. These authors reconstituted the glucose transporter from bovine heart into artificial liposomes and found significant nonspecific diffusion of L-[^{3}H]glucose which amounted to about 66% of total flux. The high nonspecific uptake is indicative of the leakiness of the vesicles in their preparation, and therefore, the preparation reported here is the first description of a non-leaky vesicle system which includes the myocardial glucose carrier. As an example of the utility of this membrane system we were able to use it to measure transporter number (cytochalasin B binding) and transport rate (V_{max}) and calculate a turnover number for the transporter.

Previously, reliable estimates of kinetic constants of the cardiac glucose transporter have not been reported in isolated membrane preparations because of leakiness. As an alternative, isolated cardiac myocytes have been used in other studies to examine kinetics of the heart glucose transporter. The K_m values (approx. 2–7 mM) reported for the glucose carrier in isolated cardiocytes [17–20] are slightly lower than the value reported here (approx. 9 mM). This discrepancy might reflect species differences or inherent difficulties such as metabolic processing of glucose or viability when using cells. Alternatively, alterations in kinetic constants could result from either changes in bioactivity during vesicle preparation or inefficient reconstitution of the transport system. Indeed, the K_m value reported for the purified human erythrocyte glucose transporter reconstituted into liposomes was lower (0.7 to 1.2 mM) [5] than the range of values (4.7 to 12.5 mM) reported for intact erythrocytes [14,21]. Despite these differences, the range of K_m values reported for the rat adipocyte (6.3 to 11.7) [22,23] is similar to those reported for the adipocyte glucose carrier in plasma membrane vesicles or egg phosphatidylcholine liposomes (9 to 15 mM) [6,24].

Radiation inactivation target size measurement has been used successfully to study the multimolecular assembly states of numerous membrane proteins [25]. Kempner and Schlegel [26] stated that target-sizing is the only method which directly relates molecular structure with function. However, Kempner and Schlegel also noted that there are limitations to the method and these include an average inherent error of 14%. We concur with Jacobs and co-workers [27] who stressed that measured target sizes at the molecular level should be considered tentative because of the incomplete understanding of the nature of energy transfer in protein quaternary structure and supramolecular assemblies. These limitations are to be kept in mind with the following discussion and conclusions regarding the target-sizing data.

The radiation target-size estimates for the glucose transporter using either cytochalasin B binding (77 kDa) or glucose transport (101 kDa) were not different statistically and the average of the two estimates was about 90 kDa. This size is approximately double that estimated using SDS-polyacrylamide gel electrophoresis, electrophoretic protein transfer, and labeling with antisera prepared against the human erythrocyte glucose transporter [10]. These methods yielded a single band with a molecular size of 45 kDa from membranes of dog and bovine myocardium. Thus, it is possible that in order to transport glucose the transporter must be in the dimeric form. This idea is not unprecedented and Jarvis and co-workers

[28] reached a similar conclusion for the human erythrocyte glucose transporter. Their radiation inactivation analysis of lyophilized erythrocyte membranes indicated an apparent M_r of 124 ± 11 kDa while previous studies using SDS-polyacrylamide gels identified the glucose carrier as band 4.5 peptide (apparent M_r 45–66 kDa). Consequently, Jarvis proposed that the glucose transporter exists in erythrocyte membranes as a dimer. Alternatively, the 90 kDa value found in the present study might reflect a complex of the monomer and a protein of equal size. In fact, Jacobs et al. [27] have speculated that the rat adipocyte glucose transporter could exist as a monomer in the plasma membrane and as a complex with another protein of equal size in the intracellular storage pool. Whether this transporter/protein arrangement is a common feature of glucose transporting cells, including cardiac myocytes, remains to be determined.

Acknowledgements

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