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Biochimica et Biophysica Acta 1414 (1998) 217–230

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Characterization of glucose transport activity reconstituted from heart and other tissues

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Received 24 July 1998; accepted 3 September 1998

Abstract

We examined several aspects of glucose transport reconstituted in liposomes, with emphasis on transporters of rat heart (mostly GLUT4) compared to those of human erythrocytes (GLUT1), and on effects of agents that modulate transport in intact cells. Several types of samples gave higher reconstituted activity using liposomes of egg lipids rather than soybean lipids. Diacylglycerol, proposed to activate transporters directly as part of the mechanism of insulin action, increased the intrinsic activity of heart transporters by only 25%, but increased the size of the reconstituted liposomes by 90%. The dipeptide Cbz-Gly-Phe-NH_2 inhibited GLUT4 with a K_i of 0.2 mM, compared to 2.5 mM for GLUT1, which explains its preferential inhibition of insulin-stimulated glucose transport in adipocytes. Verapamil, which inhibits insulin- and hypoxia-stimulated glucose transport in muscle, had no effect on reconstituted transporters. Heart transporters had a higher K_m for glucose uptake (13.4) than did GLUT1 (1.6 mM), in agreement with a recent study of GLUT1 and GLUT4 expressed in yeast and reconstituted in liposomes. Transporters reconstituted from heart and adipocytes were 40–70% inactivated by external trypsin, suggesting the presence of trypsin-sensitive sites on the cytoplasmic domain of GLUT4. NaCl and KCl both reduced reconstituted transport activity, but KCl had a much smaller effect on the size of the liposomes.

Keywords: Glucose transport; GLUT1; GLUT4; Diacylglycerol; Reconstitution; Liposome

1. Introduction

Reconstitution of transport proteins in liposomes is a useful technique for studying certain aspects of their kinetics and regulation apart from possible complicating factors found in intact cells. Previously we have used reconstitution by the freeze-thaw procedure [1] to study kinetics of the human erythrocyte glucose transporter [2,3]. We have also used the technique to examine compounds proposed to interact directly with glucose transporters of erythrocytes [4] and rat adipocytes [5]. In the experiments described here, we examined several aspects of the kinetics and regulation of glucose transporters, focusing on insulin-sensitive tissues (especially the heart). The major transporter isoform of these tissues is GLUT4. The results are compared to those obtained with the erythrocyte transporter (GLUT1).

The effects of two variables (lipids and salts) on the freeze-thaw reconstitution process were also examined. We show how a combination of measure-

Abbreviations: Cbz, carbobenzoxy; DAG, diacylglycerol; DMG, dimyristoylglycerol; HSP, high-speed pellet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00170-9

ments can help distinguish direct effects on transporters from effects on the reconstitution, a method which may be of general applicability.

In these experiments, we have employed an approach in which liposomes are reconstituted at low ratios of protein to lipid, such that reconstituted liposomes have at most one transporter. This approach was discussed by Gorga and Lienhard [6], and used in our previous studies [3–5,7]. As described in [6], 'Second, inasmuch as each vesicle contains either only one or no functional transporters, the fraction of the total intravesicular volume that rapidly equilibrates with substrate in the medium provides a measure of the number of functional transporter molecules. Third, because each rapidly equilibrating vesicle contains only one transporter molecule, the rate of substrate equilibration provides a measure of the intrinsic activity of the transporter'.

Thus, a change in the intrinsic activity of reconstituted transporters alters the stereospecific glucose uptake at early times, but not the total stereospecific uptake measured at later times, which depends only on the number of functional transporters (Fig. 2A of [6]). Conversely, a change in the number of reconstituted transporters affects the maximum stereospecific uptake, but not the fraction of this uptake which is achieved at early times (Fig. 2B of [6]).

Because we were interested in possible effects on the size distributions of the liposomes, we also measured the total internal volume. For this purpose we assayed the uptake of L-glucose in 24 h, whereas Gorga and Lienhard [6] measured xylitol uptake in 1 h.

In many of the experiments described below, we measured the zero-*trans* uptake of 0.2 mM glucose, a concentration well below the K_m. Under these conditions, the rate of equilibration is proportional to V_{max}/K_m. Thus, changes in this rate reflect changes in the catalytic efficiency (defined as $V_{max}/(K_m \times$ number of transporters)) rather than measuring directly the turnover number (V_{max}/number of transporters). Determination of V_{max} is technically much more difficult because as the concentration of glucose is increased, the ratio of non-specific permeation to stereospecific transport increases. While the measurements at 0.2 mM glucose in themselves do not allow us to distinguish effects on V_{max} from those on K_m, this can be determined by additional measurements at various glucose concentrations (e.g., Fig. 3 of [5]). Moreover, changes in the catalytic efficiency of the transporter in response to physiological events would affect glucose transport at subsaturating glucose levels, and thus be of interest regardless of whether they arise from changes in K_m or V_{max}.

2. Materials and methods

2.1. Materials

D-[^{14}C]Glucose was obtained from ICN and L-[1-^{3}H]glucose from Dupont NEN. Phospholipids, diacylglycerols, TPCK-treated trypsin, Cbz-Gly-Phe-NH_2, and verapamil were from Sigma. Human erythrocyte membranes were prepared by the method of Dodge et al. [8] using outdated erythrocytes donated by the American Red Cross (Louisville, KY). Alkaline-washed vesicles [9], Triton extracts [9] and purified transporters [2,9] were prepared from these membranes as described. Membrane fractions from rat adipocytes were a gift of Dr. Samuel Cushman. Bovine heart plasma membranes [7] and rat heart high-speed pellet membranes [10] were prepared as described.

2.2. Reconstitution and assay of glucose transport activity

Liposomes were prepared from soybean phosphatidylcholine (Sigma type II-S) or from egg PC (type IX-E or XV-E). The lipids were suspended in 10 mM Tris-HCl, pH 7.4, at 50 or 150 mg/ml and sonicated in a bath-type sonicator until an apparent maximum clarity had been achieved. In experiments with diacylglycerols, the DAG was included along with the phospholipids in the preparation of the liposomes.

Membrane proteins were reconstituted without prior detergent treatment, after dispersal with 0.3% cholate or solubilization with 1.5% cholate [7], or after solubilization with 0.5% Triton X-100 [9]. Following centrifugation of detergent-treated samples, the cholate and Triton were removed by gel filtration or by Bio-Beads SM-2, respectively.

Mixtures of liposomes (25 mg lipid/ml) and membrane proteins (0.1 or 0.2 mg protein/ml) were frozen

in liquid nitrogen and allowed to thaw at room temperature. The samples were then subjected to sonication using either a bath sonicator (for 5 s) or a probe sonicator (Branson model 200 with microtip). For the probe sonicator, the optimum sonication power and time were found to vary with the particular microtip and how long it had been in use; typically, settings of 3.0 or 3.5 and times of 4–8 s were used.

Glucose uptake into the liposomes was measured using filtration assays as described [7], with D-[^{14}C]glucose used to measure total glucose uptake and tracer L-[^{3}H]glucose used to correct for non-specific uptake. For most experiments, the zero-*trans* uptake of 0.2 mM D-glucose was measured. In some cases, zero-*trans* uptake of glucose at various other concentrations was measured. For measurement of equilibrium exchange, liposomes were reconstituted in the absence of glucose, then equilibrated with non-radioactive glucose. Assays were initiated by the addition of radioactive glucose at the same concentration [2]. For experiments with salts, the salts were included at the indicated concentrations during the initial preparation of the liposomes, the reconstitution, and the assays. For experiments with Cbz-Gly-Phe-NH_2 and verapamil, the compounds were added at the initiation of the assays. For experiments with trypsin, the enzyme was added after reconstitution but at least 20 min before the assays. For all experiments, the osmolarity of the stopping solution (containing 0.5 mM $HgCl_2$, and kept on ice) was adjusted with NaCl or KCl to equal the osmolarity of the assay solutions.

Previously we [7] and others [11] noted that protein-free liposomes of egg lipids took up a small excess of D-glucose over L-glucose. This excess D-glucose uptake was subtracted before calculation of stereospecific uptake due to reconstituted glucose transporters. Control experiments were performed to determine the appropriate corrections for the range of experimental conditions encountered in this study: for various time points up to 30 min; for liposomes of soybean as well as of egg lipids; for various salt concentrations; and for two different preparations of D-[^{14}C]glucose (catalog Nos. 11047 and 11050, having radiochemical purities of 99.5% and 96%, respectively).

2.3. *Calculation of effects of diacylglycerol and salts on intrinsic activity, reconstitution efficiency, and liposome volume*

In order to assess the effects of agents which were present throughout the reconstitution and assays, the following model was employed. An agent might affect the intrinsic activity of reconstituted transporters, the efficiency with which transporters are reconstituted in an active state, and the average internal volume of reconstituted liposomes. The effects of the agent on these parameters, relative to control liposomes, are designated *a*, *b*, and *c*, respectively. As noted in Section 1, at the low glucose concentration employed (0.2 mM), changes in the intrinsic activity could arise from changes in V_{max} and/or K_m.

Experimentally, the effects of the agents on the initial rate of stereospecific uptake (estimated at 30 s), the maximum stereospecific uptake (estimated at 30 min), and the total internal liposome volume (estimated from the uptake of L-glucose in 24 h) were measured. The effect on the L-glucose uptake was used as the value of *c*, based on the assumption that the effect on the sizes of liposomes containing transporters is similar to the effect on the total population of liposomes. The effect on the initial rate of uptake was assumed to be the product of the effects on the intrinsic activity and reconstitution efficiency, or *ab*. The effect on the maximum stereospecific uptake (when liposomes containing transporters have equilibrated external and internal D-glucose, but have taken up relatively little L-glucose) was assumed to be the product of the effects on the reconstitution efficiency and liposome volume, or *bc*.

The values of *ab*, *bc*, and *c* were averaged over all experiments. The average value of *bc* was divided by the average value of *c* to yield *b*, the effect on the reconstitution efficiency. For the experiments with diacylglycerol, the ratio of the 30 s uptake to 30 min uptake (which gives *ab*/*bc*, or *a*/*c*) was also determined in each experiment, and then averaged over all experiments. This ratio was then multiplied by the average value of *c* to yield *a*, the effect on the intrinsic activity. For experiments with salts, in which there were fewer experiments in which both the 30 s and 30 min uptakes were measured, *b* was determined as described, and the average value of *ab* was divided by this to yield *a*.

Table 1
Reconstitution of glucose transporters in liposomes of egg or soybean lipids

Sample	Stereospecific uptake (μl/(mg×2 min))		Relative uptake (egg lipids/soybean lipids)	
	Egg lipids	Soybean lipids	Stereospecific	Non-specific
Rat heart HSP				
Untreated (7)	3.7 (27)	7.6 (41)	0.49 ± 0.10	0.64 ± 0.19
Cholate-dispersed (7)	15.1 (53)	8.5 (40)	1.87 ± 0.17	1.03 ± 0.06
Bovine heart PM				
Untreated (3)	8.6 (55)	8.3 (57)	1.01 ± 0.23	0.80 ± 0.28
Cholate-dispersed (4)	12.7 (63)	8.8 (26)	1.66 ± 0.40	0.62 ± 0.35
Cholate-solubilized (8)	10.9 (42)	5.5 (22)	2.10 ± 0.20	0.70 ± 0.13
Rat adipocyte HSP				
Untreated (3)	8.7 (30)	15.1 (34)	0.59 ± 0.13	0.62 ± 0.02
Cholate-dispersed (3)	13.3 (47)	14.0 (26)	1.00 ± 0.19	0.27 ± 0.11
Human erythrocytes				
Ghosts, untreated (5)	144 (87)	101 (73)	1.38 ± 0.11	0.70 ± 0.25
Alkaline-washed vesicles, untreated (3)	405 (88)	315 (77)	1.38 ± 0.08	0.72 ± 0.10
Alkaline-washed vesicles, Triton-solubilized (4)	160 (84)	80 (50)	2.06 ± 0.37	0.44 ± 0.04
Purified transporter (3)	435 (85)	392 (85)	1.08 ± 0.09	1.10 ± 0.13

Liposomes were prepared from either egg lipids (Sigma phosphatidylcholine type IX-E) or soybean lipids (Sigma phosphatidylcholine type II-S), and reconstituted with the indicated type of sample. Total (D-glucose), non-specific (L-glucose), and stereospecific (D- minus L-glucose) uptake of 0.2 mM glucose in 2 min were measured. Results are expressed as absolute uptakes (in μl equilibrated with the external solution) in the first two columns, and as relative uptakes (± S.E.) in the last two columns. Figures in parentheses next to the samples indicate the number of experiments. Figures in parentheses in the first two columns indicate the percentage of total uptake which was stereospecific.

3. Results

3.1. Comparison of reconstitutions using two types of lipids

In a study of reconstituted adipocyte glucose transporters, Robinson et al. [11] found a 2-fold higher activity using liposomes of egg lipids (phosphatidylcholine type IX-E from Sigma) rather than soybean lipids (PC type II-S). Since our previous reconstitution studies [2–4,7,12] had used soybean lipids, we tested for a similar enhancement of activity with egg lipids. In the experiments summarized in Table 1, samples were reconstituted into liposomes of the two types; the relative uptake (egg/soybean) was determined for samples reconstituted in the same experiment. The stereospecific uptake of 0.2 mM glucose was measured in 2 min. At this time it has reached 50–100% of its maximum value, and changes only slowly with time (Fig. 1 [3] and Fig. 2 [7]). Thus, changes in the intrinsic activity of the transporter will have relatively little effect on the uptake. However, the uptake will be directly proportional to the reconstitution efficiency, and nearly proportional to the internal volume of the liposomes.

Previously we [3,7,12] and others [13,14] had shown that transport can be reconstituted using membranes without prior detergent treatment, because the freeze-thaw procedure [1] results in fusion of protein-lipid vesicles with liposomes. Table 1 includes results obtained for untreated erythrocyte ghosts, rat heart and adipocyte HSP membranes, and bovine heart plasma membranes. It also includes results with membranes subjected to detergent treatment. For heart and adipocyte samples, 'cholate-dispersed' membranes were treated with low concentrations of cholate and the supernatants from 15 000 × *g* centrifugations were reconstituted [7,11], while 'cholate-solubilized' membranes were treated with higher concentrations of cholate and the supernatants from 100 000 × *g* centrifugations were reconstituted [7]. In both cases cholate was removed by gel filtration before reconstitution. Finally, Table 1 includes results from stages of the purification of the human erythrocyte transporter [2]: alkaline-washed vesicles, a Triton extract of the vesicles (with Triton removed

by Bio-Beads prior to reconstitution), and the purified transporter.

Depending upon the type of sample, sometimes egg lipids and sometimes soybean lipids gave higher activity. However, for each type of membrane, the ratio of stereospecific uptake using egg lipids to that using soybean lipids (third column) was higher for detergent-treated samples than for untreated membranes. In addition, the non-specific uptake was, for most samples, significantly lower for egg lipids (fourth column).

For cholate-treated heart membranes, both bovine and rat, the egg lipids gave about 2-fold higher activity. This enhancement was explored further using cholate-solubilized bovine heart membrane proteins. When the time courses of stereospecific uptake were compared, it was found that the ratio was initially low (1.25 at 30 s) but increased with time (to 2.5 at 20 min). These experiments were performed at low ratios of protein to lipid, such that most liposomes should have at most one transporter [3,7]. Under these conditions the initial rate of uptake is proportional to the product of the number of transporters reconstituted and their intrinsic activity, while the final extent of uptake is determined by the number of transporters reconstituted and the internal volumes of liposomes containing transporters. The larger effect on the final than on the initial uptake suggests that the major effect was on the liposome size, with about 2-fold larger volumes being produced with egg lipids. However, in two experiments using reconstituted erythrocyte ghost proteins, the initial rate of glucose uptake was 2-fold higher using egg lipids. This indicates an effect on the intrinsic activity and/or reconstitution efficiency. Since the uptake in 2 min (which is relatively insensitive to changes in the intrinsic activity) is also higher in egg lipids (Table 1), the latter appears more likely.

In most of the experiments described below, we employed cholate-dispersed heart membrane proteins reconstituted using egg lipids. In a few of these experiments, the lipids were a different catalog listing (Sigma type XV-E) which replaced that used for the experiments of Table 1 (type IX-E). The particular lot (038F71651) of type XV-E used in these experiments gave activities about 20–30% lower than the type IX-E when the two were compared using rat heart HSP membranes. However, two other lots (80H7125 and 90H7115) gave unacceptably low activity with reconstituted heart transporters. Previously we had reported variations among activities obtained using different lots of soybean lipids (type II-S) [7]. The comparisons in Table 1 were made using the lots of both egg and soybean lipids which yielded the highest transport activity.

3.2. *Effects of diacylglycerol*

An early event in insulin action on muscle and adipose tissue is an increase in diacylglycerol. Strålfors [15] showed that exogenous diacylglycerols could stimulate glucose uptake 6-fold in rat adipocytes. The stimulation appeared to be due neither to translocation of glucose transporters nor to activation of protein kinase C. This suggested a direct effect of DAG on the intrinsic activity of glucose transporters. A direct effect was also suggested as an explanation of the results of Standaert et al. [16], who observed stimulation of deoxyglucose uptake in BC3H-1 myocytes by exogenous dioctanoylglycerol.

If an effect of DAG on the intrinsic activity of glucose transporters is important in insulin action, it would have to be on GLUT4, the major isoform in plasma membranes of insulin-stimulated cells. We tested the effects of DAG on the activity of reconstituted rat heart HSP glucose transporters (also mostly GLUT4). Most of these experiments employed dimyristoylglycerol (ditetradecanoylglycerol), the most stimulatory of the diacylglycerols tested by Strålfors [15]; as in that study, a racemic mixture of *sn*-1,2 and *sn*-2,3 enantiomers was used.

We incorporated diacylglycerols into the liposomes by adding them to the phospholipids during the initial sonication step, and observed a stimulation of initial rates. However, this could result not only from an increase in the intrinsic activity of the reconstituted transporters, but also from an increase in the number of transporters reconstituted (reconstitution efficiency). We resolved these by measuring the effects of DAG on not only the initial rate of uptake (estimated from the uptake in 30 s), but also on the maximum stereospecific volume (estimated from the uptake in 30 min) and on the total liposome volume (estimated from the L-glucose uptake in 24 h).

Results obtained using dimyristoylglycerol and egg lipids are shown in Fig. 1A. For all three determina-

tions the effect reached a maximum at about 4% DMG. The initial rate of uptake (filled circles) was increased by about 30% at 1 or 2% DMG and by an average of 43% at 4–8% DMG. There was a much larger effect on the stereospecific volume (open circles), which was increased an average of about 90% at 4–8% DMG. The total liposome volume (triangles) was also increased, by an average of about 65% for 4–8% DMG.

These data were analyzed as described in Section 2 with the results shown in Fig. 1B. The major effect of DMG appears to be on the liposome volume, which increased as much as 65%. The reconstitution efficiency was affected very little (an average increase of 15% for 4–8% DMG). Finally, the intrinsic activity was increased slightly; this effect appeared maximal at 1% DMG, and averaged 27% over the range of 1–8% DMG.

We also varied the liposome lipids and the acyl chain of the DAG. The effects of DMG on the 30 s and 30 min stereospecific uptake were determined using soybean rather than egg lipids. In two experiments each at 2% and 3% DMG, the effects were similar to those obtained with egg lipids (data not shown). Effects of dipalmitoylglycerol, which was much less stimulatory than DMG in the study of Strålfors [15], were tested using egg lipids. Two experiments each determined the effects of 1% and 2% dipalmitoylglycerol on the 30 s and 30 min uptake; these were similar to the effects of DMG (data not shown). While these two sets of experiments did not address whether the variations in liposome composition or acyl chain affected the liposome volume (the parameter most affected by DMG), they suggest that neither variation had a large effect on the intrinsic activity.

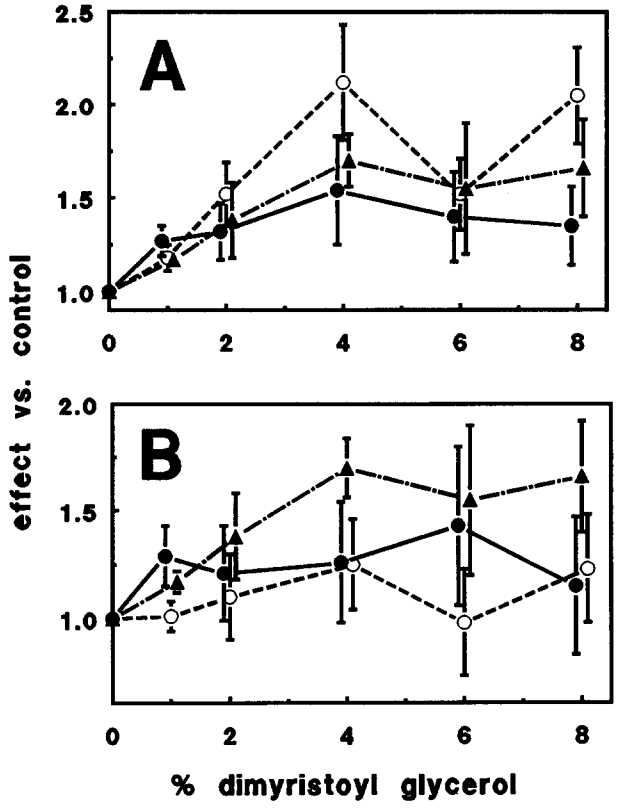

Fig. 1. Effects of dimyristoylglycerol on reconstituted rat heart HSP glucose transporters. Liposomes were prepared from egg lipids containing the indicated concentration of dimyristoylglycerol, and reconstituted with cholate-dispersed rat heart HSP membrane proteins. (A) Observed effects: ●, initial rate of stereospecific uptake of 0.2 mM glucose, estimated from uptake at 30 s; ○, total stereospecific uptake, estimated from uptake at 30 min; ▲, total liposome volume, estimated from uptake of L-glucose in 24 h. Results were normalized to control liposomes in the same experiments, and are means ± S.E. from three to five experiments. (B) Calculated effects: ●, intrinsic activity; ○, reconstitution efficiency; ▲, liposome volume (same data as in A). Some points have been offset with respect to the x-axis for clarity.

3.3. *Effects of dipeptide*

Aiello et al. [17] reported that the dipeptide Cbz-Gly-Phe-NH_2 inhibited insulin-stimulated glucose transport more strongly than basal transport in adipocytes. We found that the dipeptide inhibited adipocyte glucose transporters after reconstitution, indicating that it acts directly on the transporter [5]. Reconstituted human erythrocyte transporters were also inhibited, with 50% inhibition seen between 1.5 and 2 mM ([5], Fig. 2). The inhibition was not reduced by increasing concentrations of glucose ([5], Fig. 3).

We tested the effects of the dipeptide on glucose transport by reconstituted heart HSP membrane proteins; results are shown in Fig. 2 (solid circles). A fit of these data gave a maximum inhibition of 71 ± 6% and a K_i of 0.20 ± 0.05 mM (solid line). The broken line in Fig. 2 is a fit to data for reconstituted erythrocyte membrane proteins, using both the results shown in Fig. 2 of [5] and additional determinations.

The parameters of this fit are 123 ± 24% for the maximum inhibition and 2.5 ± 0.8 mM for K_i.

We considered the possibility that the heart data arose from a major component (GLUT4) with a low K_i and a minor component (GLUT1) with a higher K_i. Assuming that both components could be inhibited 100% and that GLUT1 had a K_i of 2.5 mM, as observed for erythrocyte transporters, a fit to the data gave similar amounts of the two isoforms and a K_i of 0.12 ± 0.05 mM for GLUT4. Alternatively, assuming that 79% of the transporters are GLUT4 [18] and that GLUT1 had a maximum inhibition of 100% and a K_i of 2.5 mM, a fit gave a maximum inhibition of 63% and a K_i of 0.17 ± 0.06 mM for GLUT4. Both of these fits gave curves very similar to the solid line in Fig. 2.

3.4. Effects of verapamil

Cartee et al. [19] observed that verapamil at 10–200 μM inhibited insulin- or hypoxia-stimulated 3-*O*-methylglucose transport by rat skeletal muscle. Both (+)- and (−)-verapamil were equally effective, indicating that the effect was not due to blocking of Ca^{2+} channels, and suggesting that it could be a direct inhibition of GLUT4. We tested the effects of verapamil on the initial rate of glucose transport by reconstituted rat heart HSP membrane proteins. In seven determinations at 300 or 1000 μM verapamil, we observed insignificant (5 ± 10%) inhibition. Glucose transport by reconstituted erythrocyte membrane proteins also was not significantly inhibited by 200–1000 μM verapamil (9 ± 4% inhibition, $n = 4$).

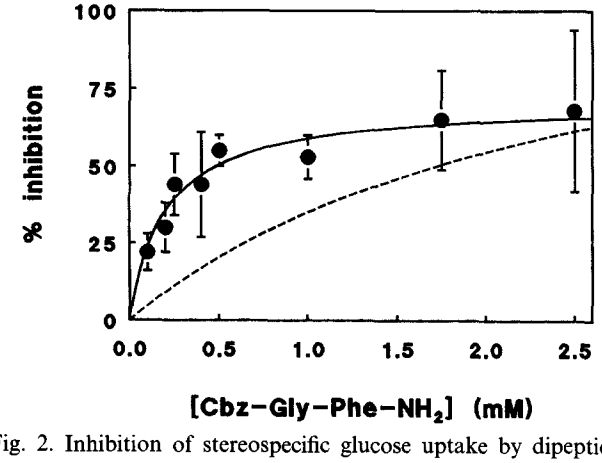

Fig. 2. Inhibition of stereospecific glucose uptake by dipeptide. Liposomes were reconstituted with cholate-dispersed rat heart HSP membrane proteins, or with human erythrocyte membrane proteins. Stereospecific uptake of 0.2 mM glucose in 30 s was determined in the presence of the indicated concentrations of Cbz-Gly-Phe-NH_2. ●, heart HSP proteins. Results are means ± S.E. for three to eight determinations. The solid line indicates a fit of these data (maximum inhibition = 71%, K_i = 0.20 mM). The dashed line is a fit to erythrocyte membrane protein data from [5], plus additional determinations (maximum inhibition = 123%, K_i = 2.5 mM).

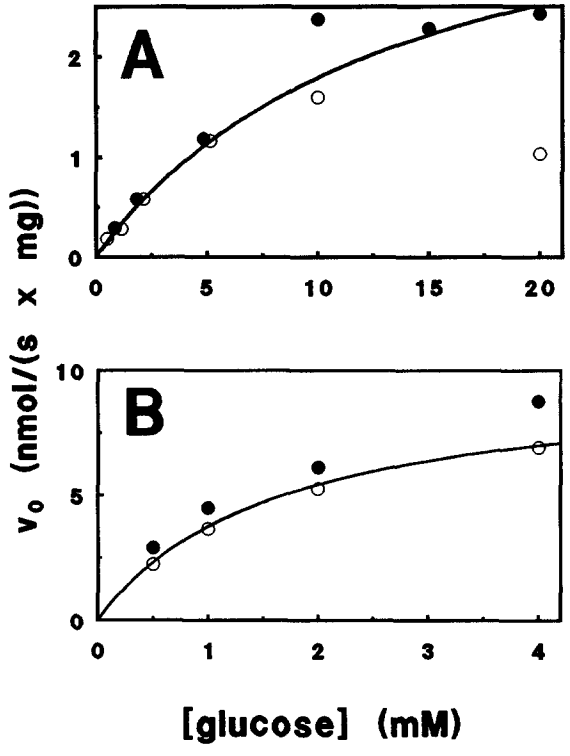

Fig. 3. Zero-*trans* uptake by reconstituted heart and erythrocyte transporters. Cholate-dispersed rat heart HSP membrane proteins (A) or untreated human erythrocyte membrane proteins (B) were reconstituted into liposomes of egg lipids. Initial rates of stereospecific glucose uptake at the indicated glucose concentrations were estimated from the uptake in 30 s (A) or 20 s (B). Data are from two experiments for each type of sample, which are indicated by filled or open circles. Some points in A have been offset with respect to the x-axis for clarity. The curves are drawn to non-linear regression fits of the data to the Michaelis-Menten equation: (A) K_m = 13.4 ± 1.8 mM, V_{max} = 4.2 ± 0.6 nmol/(s × mg); (B) K_m = 1.6 ± 0.3 mM, V_{max} = 9.8 ± 1.1 nmol/(s × mg).

3.5. Net and exchange uptake by reconstituted heart transporters

It was of interest to compare the kinetic features of the reconstituted rat heart HSP transporters (mostly GLUT4) to those of the reconstituted human eryth-

rocyte transporter (GLUT1), which we had previously characterized [2]. Fig. 3A shows initial rates of transport under zero-*trans* conditions for heart transporters reconstituted using egg lipids. A fit to the data gave a K_m of 13.4 ± 1.8 mM, much higher than the value of 1.2 mM observed for the purified erythrocyte transporter [2]. To ensure that this difference did not arise from the use of egg lipids rather than soybean lipids (used in [2]) we also tested erythrocyte membrane proteins reconstituted using egg lipids (Fig. 3B). These data gave a K_m of 1.6 ± 0.3 mM, in good agreement with the earlier result.

Fig. 4 shows the rates of glucose transport catalyzed by reconstituted heart proteins under equilibrium exchange conditions. The K_m was 62 ± 23 mM, much higher than for zero-*trans* uptake. Previously we determined an exchange K_m of 35 mM for the reconstituted erythrocyte transporter [2].

The results shown in Figs. 3A and 4 indicate that at high glucose concentrations, the reconstituted heart transporters have higher rates for exchange than for net flux (exchange acceleration). In addition, two experiments with reconstituted adipocyte HSP transporters (also mostly GLUT4) yielded 2.4- and 1.9-fold higher initial rates for exchange than for net uptake of 20 mM glucose. This is in contrast to 3-*O*-methylglucose transport in insulin-stimulated adipocytes, where zero-*trans* uptake and equilibrium exchange have similar kinetic parameters (e.g., [20]), and zero-*trans* efflux is faster than exchange [21,22]. Previously we noted that the reconstituted erythrocyte transporter displayed more exchange acceleration than intact erythrocytes [2]. Thus, reconstituted GLUT1 and GLUT4 both had greater than expected exchange acceleration. We were concerned that this might be an artifact of osmotic effects, since at the beginning of the zero-*trans* uptake assays the liposomes encounter an osmotic gradient which should make them shrink.

We therefore tested the effects of osmolarity on the exchange acceleration. Liposomes were prepared and assayed in either the usual buffer (10 mM Tris-HCl) or in buffer to which salt was added, reducing the osmotic gradient present at the beginning of zero-*trans* assays. For the rat heart transporters, the exchange acceleration was measured at 20 mM glucose with no salt, or with 5 or 10 mM NaCl or 20 mM KCl added. The average ratio of initial rates (exchange/net) decreased from 2.58 ± 0.28 (mean ± S.E., $n = 9$) with no added salt to 2.31 ± 0.28 ($n = 4$) with 10 mM NaCl added and 1.54 ± 0.22 ($n = 3$) with 20 mM KCl added. These data were extrapolated to infinite osmolarity by plotting the ratios vs. the reciprocals of the osmolarities. A linear regression fit gave an y-intercept of 1.44, suggesting that much of the accelerated exchange observed in the absence of salt is due to osmotic effects.

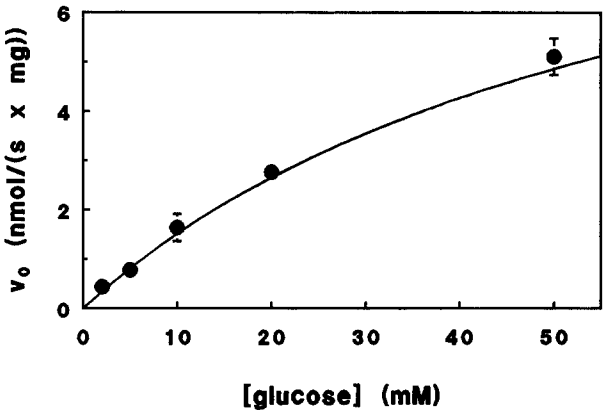

Fig. 4. Equilibrium exchange uptake by reconstituted rat heart transporters. Samples were reconstituted and assayed as in Fig. 3A, except that the liposomes were preincubated with the indicated concentrations of non-radioactive D-glucose. Three experiments were performed. In one the reconstituted activity was higher than in the other two, so the data were normalized to the mean rate obtained at 2 mM glucose, and the resulting means (± S.E.) are plotted. For the 5 and 20 mM points, the error bars lie within the diameters of the symbols. The data were then fit as for Fig. 2, with the line drawn to the resulting parameters (K_m = 62 ± 23 mM, V_{max} = 10.9 ± 3.4 nmol/(s × mg)).

For erythrocyte transporters, two experiments were performed in which the exchange acceleration was measured at 5 mM glucose (since the K_m for net flux is much lower than for the heart transporters, exchange acceleration can be observed at lower glucose concentrations). The ratio of exchange to net uptake was actually higher in the presence (3.3- and 3.9-fold) than in the absence (2.9- and 2.8-fold) of 20 mM KCl, although the differences were not statistically significant. Thus, in this case it appears that osmotic effects are not responsible for the higher than expected degree of exchange acceleration.

3.6. *Effects of trypsin*

Addition of trypsin after reconstitution decreases

the activity of the human erythrocyte transporter by about 40–50% [2,3]. This was consistent with inactivation at the cytoplasmic domain [23] and a scrambling of orientations in the reconstituted liposomes, such that about half of the transporters had this domain exposed on the external surface. We measured the effects of trypsin treatment on reconstituted transporters from HSP membranes of rat heart and adipocytes (Table 2). For both cell types the uptake was reduced by trypsin treatment, with the adipocyte transporters being more sensitive.

3.7. Effects of NaCl and KCl

Because addition of high concentrations of sugars to reconstituted liposomes could cause problems due to osmotic shrinking, we explored carrying out the reconstitution and assays in the presence of added salt to minimize osmotic effects. Fig. 5 shows the effects of NaCl and KCl on reconstituted heart and erythrocyte transporters. Effects on the initial rates, the maximum stereospecific uptakes, and the liposome volumes were determined. For heart transporters reconstituted using egg lipids, all three of these measures decreased with increasing salt concentration; however, the decreases were much larger for added NaCl than for added KCl. We resolved these effects into effects on the intrinsic activity, reconstitution efficiency, and liposome volume as for the experiments with DAG (Fig. 1B). The results (not shown) indicated that neither salt had a significant effect on the intrinsic activity. The two salts had similar effects on the reconstitution efficiency, which was decreased by about 15%, 30%, and 45% at 5, 10, and 20 mM salt, respectively. However, they differed significantly in their effects on the liposome size, as indicated by the L-glucose uptake (Fig. 5, squares). At 20 mM salt, the liposomes prepared in KCl had a 3-fold higher volume than those prepared in NaCl.

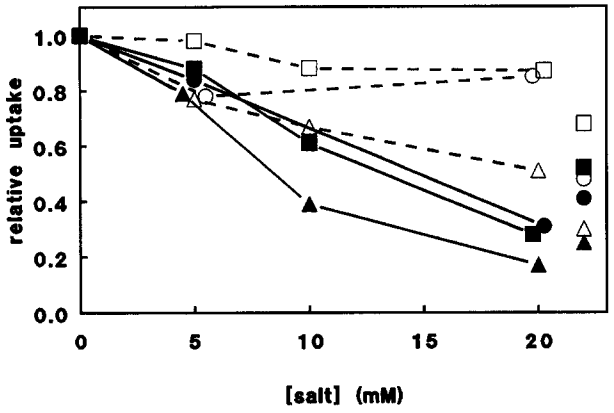

Fig. 5. Effects of NaCl and KCl on reconstituted transporters. Cholate-dispersed rat heart HSP membrane proteins were reconstituted into liposomes of egg lipids; human erythrocyte membrane proteins were reconstituted into liposomes of soybean lipids. Liposomes were prepared, reconstituted, and assayed in 10 mM Tris buffer plus the indicated concentration of salt. Circles, initial rate of stereospecific uptake of 0.2 mM glucose, estimated from uptake at 30 s; triangles, total stereospecific uptake, estimated from uptake at 30 min; squares, total liposome volume, estimated from uptake of L-glucose in 24 h. Filled symbols and solid lines, NaCl; open symbols and broken lines, KCl. Data plotted at 5, 10, and 20 mM and connected by lines are for heart transporters (some points have been offset with respect to the x-axis for clarity). The six data points plotted at the far right are for erythrocyte transporters at 20 mM salt. Results were normalized to liposomes without added salt in the same experiments, and are means from two or three experiments.

Similar experiments were carried out using erythrocyte transporters reconstituted using soybean lipids

Table 2
Effects of trypsin on reconstituted glucose transporters

Sample	Uptake vs. control			
	30 s	2 min	5 min	20 min
Heart	0.68 ± 0.12 (n = 5)	0.54 (0.54, 0.53)	0.69 ± 0.12 (n = 3)	
Basal adipocytes	0.29 (0.41, 0.17)			0.33 (0.34, 0.32)
Insulin-treated adipocytes		0.46 (0.35, 0.57)		

Rat heart HSP membrane proteins were reconstituted into liposomes of egg lipids after cholate dispersal. HSP membrane proteins from basal or insulin-treated rat adipocytes were reconstituted without detergent treatment. After reconstitution, liposomes were incubated with TPCK-treated trypsin (0.02 mg/ml) for 20 min or more before assays were begun. Stereospecific uptake of 0.2 mM glucose in the indicated time was measured, and compared to that for liposomes not treated with trypsin. Results are means ± S.E. (n = 3 or 5), or means with individual values in parentheses (n = 2).

in the presence or absence of 20 mM salt (Fig. 5, data at far right). As in the heart experiments, the intrinsic activity was not affected by the added salt, and both salts decreased the reconstitution efficiency by about 50%. However, the decrease in volume with added NaCl (48%) was only 1.5-fold larger than that with added KCl (32%) (squares). A single experiment for heart transporters reconstituted using soybean lipids and tested at 20 mM salt also showed smaller differences between NaCl and KCl than those shown for heart transporters in Fig. 5. This suggests that the lipid type rather than the source of transporters was more important in determining the relative effects of NaCl and KCl.

4. Discussion

4.1. Egg lipids vs. soybean lipids

In the experiments summarized in Table 1, we found that the use of liposomes of egg lipids rather than soybean lipids increased the reconstituted stereospecific glucose uptake, and decreased the non-specific uptake, for many types of samples. This improved the signal to noise ratio for reconstituted transport by as much as 5-fold. In general the effects were greater for detergent-treated membranes compared to untreated membranes of the same type. Increased stereospecific uptake in 2 min with egg lipids could arise from greater reconstitution efficiency, greater liposome sizes, or a combination of both. Results with bovine heart plasma membranes indicated an effect on liposome size, while those with erythrocyte ghosts indicated an effect on reconstitution efficiency.

The fact that the egg lipids generally were more effective for detergent-treated than for untreated membranes suggests that a lipid component which assists in the reconstitution may be present in the native membranes, lost during detergent treatment, and then resupplied more readily by egg than by soybean lipids. In the case of the erythrocyte transporter, the purification procedure results in a reassociation of the protein with membrane lipids, and the reconstitution again becomes insensitive to the choice of egg or soybean lipids.

Phosphatidylethanolamine (PE), which makes up about 20% of the egg lipids (type IX-E) but only 5–10% of the soybean lipids (data provided by Greg Wall, Technical Services, Sigma), could possibly be responsible for the differences. Reconstituted glucose transporters from LM cells were more active at higher ratios of PE to PC [24]. The two types of lipids also differ in their fatty acyl groups; the egg lipids have more palmitic, stearic, and oleic residues, while the soybean lipids are more enriched in linoleic residues. The egg lipids also have a much higher content of PC (60% vs. 15%), but we previously found that less activity was obtained with soybean lipids more enriched in PC [7].

We also found, in this study, considerable variation among different lots of a single egg lipid product (type XV-E), and previously reported similar variations among lots of soybean lipids (type II-S) [7]. Thus, investigators obtaining unsatisfactory reconstitution results may wish to test multiple lots of a given lipid preparation.

The results in Table 1 include various stages in the purification of the human erythrocyte transporter. Using the results for egg liposomes, the specific activity of the reconstituted alkaline-washed vesicles (400 μl equilibrated in 2 min/mg protein) was about 2.5-fold higher than both the ghosts (140 μl/mg) and the Triton extract (160 μl/mg), and was nearly as high as the purified transporter (440 μl/mg). This suggests that a large amount of the transport activity is lost at the Triton extraction step.

4.2. Effects of diacylglycerol

The experiments shown in Fig. 1 were prompted by reports that exogenous diacylglycerol could stimulate glucose transport [15,16], and the suggestion that the direct interaction of DAG with glucose transporters could be part of the mechanism of action of insulin. Using data of Okamura et al. [25], the ratio of DAG to total lipid in rat ventricular tissue is about 0.24%. If higher levels occur in plasma membranes of cardiomyocytes, and these are increased further in response to insulin, contents on the order of several percent might be attained. While these levels of DAG produce significant effects on reconstituted glucose transport activity (Fig. 1A), the calculated increase in the intrinsic activity of the transporter (Fig. 1B) is only about 25%. Such an effect

would be relatively small compared to that of translocation.

In contrast to the original report of Strålfors [15], more recent studies from the same group [26] indicated that DAG does in fact cause translocation of GLUT4. Others (e.g., [27,28]) have also concluded that an increase in DAG in response to insulin is unlikely to activate glucose transport directly. Moreover, Eckel and Reinauer [29] observed a *decrease* in DAG in insulin-treated cardiomyocytes.

As reviewed by Zidovetski and Lester [30], long-chain (C_{12}-C_{18}) saturated diacylglycerols at levels such as we used (Fig. 1) destabilize the structure of lipid bilayers, enhancing the formation of the hexagonal phase. Such effects have been correlated with activation of phospholipases and protein kinase C, which could be analogous to effects on the intrinsic activity of the reconstituted glucose transporters (filled circles, Fig. 1B). Formation of the hexagonal phase has also been associated with increased membrane fusion (e.g., [31]), which could explain the production of larger liposomes in the freeze-thaw process (triangles, Fig. 1A,B). This effect may be of practical importance, since transport is more readily measured in reconstituted liposomes having larger internal volumes.

4.3. Effects of dipeptide

As shown in Fig. 2, concentrations of the dipeptide Cbz-Gly-Phe-NH_2 below 1 mM inhibit reconstituted transporters from heart (circles and solid line) much more strongly than those from erythrocytes (broken line). This suggests that GLUT4, the major isoform in heart, has a higher affinity for the dipeptide (K_i of 0.1–0.2 mM) than does GLUT1 (K_i of 2.5 mM). The inhibition of the heart transporters appears to reach a maximum of less than 75%, possibly because their less sensitive GLUT1 component is only partially inhibited at the concentrations studied. The transport inhibitors forskolin, dipyridamole, and isobutylmethylxanthine also have higher affinity for GLUT4 than for GLUT1 [32].

Since adipocyte plasma membranes become more enriched in GLUT4 relative to GLUT1 upon insulin stimulation, differential sensitivity of GLUT1 and GLUT4 can explain the observation of Aiello et al. [17] that insulin-stimulated glucose transport in adipocytes is more sensitive than basal transport to inhibition by the dipeptide. Using K_i values of 0.15 mM and 2.5 mM for GLUT4 and GLUT1, respectively, a maximum inhibition of 100% for both isoforms, and contents of GLUT4 of 69% in basal cells and 90% in insulin-stimulated cells [33], it can be calculated that 1 mM dipeptide would inhibit transport by 69% and 81% in basal and in insulin-stimulated cells, respectively. This agrees qualitatively with the values of 51% and 76% observed by Aiello et al. ([17], Table I).

Epand et al. [34] also observed a differential sensitivity of basal and insulin-stimulated adipocyte transport to Cbz-Gly-Phe-NH_2. However, the inhibition by the dipeptide was much more potent in this study (58% inhibition of insulin-stimulated uptake by 10 μM dipeptide) than in our studies or those of Aiello et al. [17].

In contrast to these results with mammalian glucose transporters, Booz and Bianchi, in studies of frog muscle, found that 1.5 mM Cbz-Gly-Phe-NH_2 inhibited basal, contraction-stimulated, and insulin-stimulated 3-*O*-methylglucose transport similarly [35]. Thus, GLUT1 and GLUT4 of frog may have similar affinities for the dipeptide, or the relative proportions of the two forms in the plasma membrane may not be altered by insulin or contraction.

4.4. Effects of verapamil

Cartee et al. [19] found that verapamil inhibited insulin- or hypoxia-stimulated methylglucose transport in skeletal muscle by a mechanism independent of its effects on calcium channels. It was proposed that the drug was either blocking translocation or inhibiting transporters directly. We found that reconstituted glucose transporters (from either heart or erythrocytes) were not inhibited by verapamil, arguing against the latter. Montero et al. [36] observed that in chicken enterocytes, 2-deoxyglucose transport (which is Na^+-independent) was unaffected by 200 μM verapamil. On the other hand, Vera et al. [37] reported that 100 μM verapamil produced 60% inhibition of 2-deoxyglucose uptake by *Xenopus* oocytes expressing rat GLUT1. Possibly this was an effect of the drug on phosphorylation (which can be rate-determining for 2-deoxyglucose uptake [38]) rather than transport.

4.5. Net and exchange flux of GLUT1 and GLUT4

As shown in Fig. 3, reconstituted rat heart transporters (mostly GLUT4) had a higher K_m for zero-*trans* uptake (13 mM) than did human erythrocyte transporters (GLUT1) (1.6 mM). Fig. 4 shows that even higher kinetic parameters were obtained under equilibrium exchange conditions. However, experiments using variable salt concentrations showed that at least some of the differences between net and exchange flux for the reconstituted heart transporters were artifacts of the osmotic changes in the zero-*trans* experiments. Thus, the true K_m for zero-*trans* flux may be higher than 13 mM. The K_m observed for exchange (62 ± 23 mM, Fig. 4) is not significantly different than that observed for reconstituted erythrocyte transporters (35 ± 4 mM [2]).

The finding of a higher (net flux) or similar (exchange) K_m for GLUT4 compared to GLUT1 might be considered surprising in light of results using 3T3-L1 adipocytes [39] or exogenous GLUT1 and GLUT4 expressed in *Xenopus* oocytes [40,41], in which 3–12-fold lower K_m values were observed for GLUT4 than for GLUT1. However, these studies all measured 3-*O*-methylglucose transport under equilibrium exchange conditions.

In studies using glucose as a substrate [42,43], plasma membrane vesicles from insulin-stimulated or exercised skeletal muscle (in which transporters would be mostly GLUT4) had exchange K_m values of about 20 mM, much higher than the exchange K_m values for GLUT4 reported in [39–41] (2–7 mM). Another type of skeletal muscle plasma membrane vesicle preparation, containing GLUT4 but no detectable GLUT1, had a zero-*trans* uptake K_m for glucose of 16–18 mM [44]. NMR data from intact, insulin-stimulated muscle yielded an estimated K_m of 17 mM [45]. Thus, differences between our results and those of [39–41] may be due to the choice of substrate.

Recently, Kasahara and Kasahara [46] expressed rat GLUT1 and GLUT4 in *Saccharomyces cerevisiae*, reconstituted the proteins in liposomes, and assayed glucose transport. Under zero-*trans* conditions, GLUT4 had a K_m of 12 mM, in excellent agreement with our value of 13 mM (Fig. 3A). GLUT1 had a much lower K_m (3.5 mM), in qualitative agreement with our results for human GLUT1 (1.6 mM, Fig. 3B). They also measured equilibrium exchange of glucose, obtaining values of 22 and 26 mM for GLUT4 and GLUT1, respectively. These also agree qualitatively with our finding of higher K_m values under exchange conditions for both isoforms.

4.6. Effects of trypsin

As shown by the results in Table 2, reconstituted GLUT4 transporters from rat heart and adipocytes are partially resistant to trypsin treatment, as is the case for reconstituted GLUT1 [2,3]. This suggests that GLUT4, like GLUT1 [23], has trypsin-sensitive sites present at the cytoplasmic domain; reconstitution with a scrambling of orientations would make about half of the transporters susceptible to external trypsin [2,3]. Eight sites of trypsin cleavage, all cytoplasmic, have been identified for GLUT1 [47]. For six of these, arginine is either conserved in the corresponding position of GLUT4, or else lysine in GLUT1 is replaced by arginine in GLUT4. Thus, it seems likely that the cytoplasmic domain of GLUT4 should also be sensitive to trypsin.

Experiments with adipocytes demonstrated cleavage of GLUT4 by extracellular trypsin [48]. However, in this study both the trypsin concentration (1 mg/ml vs. 0.02 mg/ml) and temperature (37° vs. room temperature) for treatment were higher than in our experiments. The cleavage observed in [48] may therefore occur at sites which are less susceptible than the cytoplasmic sites.

4.7. Effects of NaCl and KCl

The experiments shown in Fig. 5 were aimed at determining levels of salt which could reduce osmotic shrinking at the initiation of transport assays, while still providing suitable reconstituted transport activity. We observed decreases in the activity when the liposomes were prepared and reconstituted with heart transporters in the presence of 5–20 mM NaCl or KCl (Fig. 5). Calculations indicated that the added salts did not reduce the intrinsic activity, which is as expected for a protein which physiologically operates at much higher ionic strength. Rather, the inhibitory effects appeared to be related to the fusion of liposomes with transporter-containing membranes and with each other. Previously, Pick

[49] reported that salts decrease the sizes of freeze-thaw liposomes, with changes similar to those we observed (Fig. 5, squares).

The data of Fig. 5 also show that for a given salt concentration, greater reconstituted transport activity was obtained using KCl (open symbols) rather than NaCl (filled symbols). This difference appeared to be due primarily to differences in liposome sizes (squares). Possibly some of the smaller liposomes are not retained on the Millipore filters, contributing to the decrease in the measured transport activity, and accentuating the differences between the effects of NaCl and KCl. In the original report of the freeze-thaw reconstitution of glucose transporters, Kasahara and Hinkle [1] observed only a 23% decrease in the initial rate of reconstituted transporters when 150 mM KCl was used, a much smaller inhibitory effect than we observed (Fig. 5).

Oka and MacDonald [50] prepared sonicated liposomes without salt, added salt prior to a freeze-thaw step, and dialyzed away the salt. This formed giant liposomes, which were much larger for KCl than for NaCl. Thus, in our experiments, an ability of KCl to promote liposome aggregation may have offset its inhibitory effects to a greater extent than for the case of NaCl. Alternatively, Oka and MacDonald [50] noted differences between hydration of Na^+ and K^+ ions as salt solutions are frozen; such differences might influence the freeze-thaw process.

Acknowledgements

We thank Celia G. Emberts for additional technical assistance. This work was supported by grants from Juvenile Diabetes Foundation, American Heart Association Kentucky Affiliate, and the University of Louisville President's Research Initiative.

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