Seasonal changes in plasma membrane glucose transporters enhance cryoprotectant distribution in the freeze-tolerant wood frog

Patricia A. King, Mary N. Rosholt, and Kenneth B. Storey

Abstract: One of the critical adaptations for freeze tolerance by the wood frog, *Rana sylvatica*, is the production of large quantities of glucose as an organ cryoprotectant during freezing exposures. Glucose export from the liver, where it is synthesized, and its uptake by other organs is dependent upon carrier-mediated transport across plasma membranes by glucose-transporter proteins. Seasonal changes in the capacity to transport glucose across plasma membranes were assessed in liver and skeletal muscle of wood frogs; summer-collected (June) frogs were compared with autumn-collected (September) cold-acclimated (5°C for 3–4 weeks) frogs. Plasma membrane vesicles prepared from liver of autumn-collected frogs showed 6-fold higher rates of carrier-mediated glucose transport than vesicles from summer-collected frogs, maximal velocity ($V_{max}$) values for transport being $72 \pm 14$ and $12.0 \pm 2.9$ nmol·mg protein$^{-1}$·s$^{-1}$, respectively (at 10°C). However, substrate affinity constants for carrier-mediated glucose transport ($K_{i/2}$) did not change seasonally. The difference in transport rates was due to greater numbers of glucose transporters in liver plasma membranes from autumn-collected frogs. The total number of transporter sites, as determined by cytochalasin B binding, was 8.5-fold higher in autumn than in summer. Glucose transporters in wood frog liver membranes cross-reacted with antibodies to the rat GluT-2 glucose transporter (the mammalian liver isoform), and Western blots further confirmed a large increase in transporter numbers in liver membranes from autumn- versus summer-collected frogs. By contrast with the liver, however, there were no seasonal changes in glucose-transporter activity or numbers in plasma membranes isolated from skeletal muscle. We conclude that an enhanced capacity for glucose transport across liver, but not muscle, plasma membranes during autumn cold-hardening is an important adaptation that anticipates the need for rapid export of cryoprotectant from liver during natural freezing episodes.

Résumé : L'une des adaptations critiques de la Grenouille des bois (*Rana sylvatica*), une espèce tolérant au gel, est la production de grandes quantités de glucose qui sert de cryoprotecteur des organes en cas d'exposition au gel. Le transport du glucose du foie, où il est synthétisé, vers les autres organes, où il est absorbé, nécessite des agents de transport à travers les membranes plasmatiques, en l'occurrence des protéines de transport du glucose. Les changements saisonniers de la capacité de transport du glucose à travers les membranes plasmatiques ont été évalués dans le foie et les muscles squelettiques de Grenouilles des bois capturées en été (juin) et d'autres capturées à l'automne (septembre) et aclimatées au froid (5°C pendant 3–4 semaines). Dans les vésicules des membranes plasmatiques prélevées dans le foie des grenouilles d'automne, les taux de transport du glucose par les agents de transport étaient six fois plus élevés que ceux enregistrés dans les vésicules des grenouilles d'été ($72 \pm 14$ nmol·mg protéine$^{-1}$·s$^{-1}$ comparativement à $12.0 \pm 2.9$ nmol·mg protéine$^{-1}$·s$^{-1}$) à 10°C. Cependant, les valeurs de $K_{i/2}$ ne variaient pas en fonction de la saison. La variation saisonnière des taux de transport du glucose résultait de l'abondance plus grande des agents de transport du glucose dans les membranes plasmatiques du foie des grenouilles d'automne. Le nombre total des sites de transport, déterminé par les taux de liaison à la cytochalasine B, était 8,5 fois plus élevé en automne qu'en été. Les transporteurs du glucose dans les membranes du foie des grenouilles ont réagi aux anticorps du transporteur du glucose GluT-2 de rat (l'isoforme du foie d'un mammifère) et des transferts Western ont également mis en lumière une importante augmentation des transporteurs du glucose dans les membranes du foie des grenouilles de...
l’été à l’automne. Contrairement à la situation dans le foie cependant, l’activité des transporteurs du glucose ou leur nombre dans les membranes plasmatiques du muscle squelettique ne changeaient pas en fonction de la saison. Nous croyons que la capacité plus grande de transport du glucose dans les membranes plasmatiques du foie, mais pas dans celles du muscle, au cours de l’accuitation au froid à l’automne, constitue une importante adaptation destinée à assurer le transport rapide des cryoprotecteurs du foie vers les organes durant les périodes éventuelles de gel.

[Traduit par la Rédaction]

Introduction

The wood frog, Rana sylvatica, is one of a small group of amphibians and reptiles that tolerate the freezing of extra-cellular body fluids during winter hibernation on the forest floor (for a review see Storey and Storey 1992). One of the adaptations that supports freezing survival in many freeze-tolerant species, both vertebrate and invertebrate, is the accumulation of high concentrations of low molecular weight cryoprotectants (often sugars or polyhydric alcohols). The colligative properties of these compounds help to prevent cell water from freezing by lowering the freezing point and by raising the osmotic pressure of extracellular fluid (for a review see Storey and Storey 1988). For the wood frog and two other anurans the cryoprotectant is glucose (Storey and Storey 1992). Ice formation on peripheral skin sites triggers signals that activate glycogen synthesis in liver, and less than 5 min after freezing begins, blood glucose levels start to rise rapidly (Storey and Storey 1985). Within a few hours glucose levels in core organs can rise by 100-fold or more to reach 150–300 μmol/g wet mass (Storey 1987; Storey and Storey 1986). Synthesis of this sugar is supported by huge reserves of glycogen (up to 180 mg/g wet mass) and high activities of glycogen phosphorylase in wood frog liver and a freezing-induced mechanism for the rapid activation of phosphorylase (Storey and Storey 1988). Glucose is then moved out of the liver and distributed to other organs.

The primary mode of glucose movement across animal cell membranes is facilitated diffusion via glucose-transporter proteins (Pessin and Bell 1992). These highly conserved proteins facilitate hexose movements down concentration gradients. We predicted, therefore, that a critical element in the cryoprotectant response to freezing by frogs would be changes to the glucose-transporter system of cell membranes to greatly increase the rate at which glucose could be moved out of the liver and into the cells of other organs. In a recent study we compared glucose flux across membrane vesicles, and glucose-transporter numbers (determined by cytochalasin B binding) and kinetic properties in liver and skeletal muscle membranes from the wood frog with those from the leopard frog, Rana pipiens, a freeze-intolerant species (King et al. 1993). The results showed that R. sylvatica had a much higher capacity for glucose transport across liver plasma membranes than R. pipiens. Maximal rates of carrier-mediated glucose influx into liver plasma membrane vesicles were 8-fold higher in wood frogs than in leopard frogs, and the total number of transporter sites in liver membranes was 4.7-fold greater in the freeze-tolerant species (King et al. 1993). However, Kₘ for glucose transport and Kᵦ for cytochalasin B binding did not differ between species, indicating

that the freeze-tolerant species has greatly increased the numbers of glucose transporters in liver plasma membranes but has not modified their properties. The high glucose concentrations and high rates of glucose transport for cryoprotectant distribution are a phenomenon needed only for winter hibernation of the wood frog. During the rest of the year glucose metabolism must be regulated as in all vertebrates and blood glucose levels are typically maintained at less than 5 μmol/mL. We wondered, then, whether the capacity for glucose transport across plasma membranes would vary on a seasonal basis in the wood frog, serving normal glucose homeostasis in the summer months when the animals are feeding but changing during the autumn cold-hardening period to support the high rates of cryoprotectant flux during winter freezing and thawing. The present study analyses seasonal changes in glucose transport by membrane vesicles, and the numbers and properties of glucose transporters in R. sylvatica liver and skeletal muscle membranes.

Materials and methods

Animals

Male wood frogs, R. sylvatica (6–9 g), were collected in the Ottawa area in late June (summer frogs) and mid-September (autumn frogs). Animals were washed in a tetracycline bath. Summer frogs were held at 15°C and used within 24 h. Autumn frogs were placed in plastic boxes containing damp sphagnum moss and held at 5 ± 1°C for 3–4 weeks before use. For tissue sampling, frogs were double pithed, then liver and thigh muscles were rapidly dissected out. Tissues were immediately frozen in liquid nitrogen and then stored at −80°C until membranes were prepared.

Muscle membrane preparation

The protocol for preparing plasma membranes was derived from the methods of Bers (1979) and Grindem (1988), as previously described (King et al. 1993). Approximately 6 g of tissue representing muscle from 6–10 frogs was used for each membrane preparation. As the tissue thawed, it was minced and then homogenized in 250 mM sucrose, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, at 4°C. Homogenization was performed with a polytron (Kinetica, Switzerland) and a Potter–Elvemjem homogenizer. Through this procedure and the subsequent steps, the tissue was maintained at approximately 4°C. Potassium chloride and sodium pyrophosphate (final concentration 300 and 25 mM, respectively) were added to the homogenate and mixed well, and the sample was centrifuged for 1 h at 227 000 × g. The pellet was resuspended in 34% w/v sucrose and included in a discontinuous sucrose gradient (45, 34, 32, 30, 27, 12% w/v sucrose) and then centrifuged for
16 h in a Beckman swinging bucket SW 28 rotor at 68 000 × g. Fractions were collected from the gradient at the 12/27 and 27/30 interface, diluted with water, and centrifuged at 331 000 × g for 60 min. The resulting pellets were resuspended in approximately 1 mL of the sucrose buffer and stored in liquid nitrogen until assayed for transport activity and cytochalasin B binding.

Liver membrane preparation

The method of Sulakhe (1986) and Sulakhe and Lautt (1987) was used to prepare the liver membranes, as previously described (King et al. 1993). Approximately 2–3 g of liver representing 10–12 frogs was used for each membrane preparation. The frozen liver was thawed and minced in 4 volumes of sucrose buffer (300 mM sucrose, 0.5 mM dithiothreitol (DTT), 0.5 mM CaCl₂, and 10 mM Tris-HCl, pH 7.4, at 4°C) and then homogenized in a Dounce homogenizer with an A pestle. All steps were carried out at 4°C. The homogenate was filtered through cheesecloth and centrifuged at 10 000 × g for 12 min. The pellet was resuspended in the homogenization buffer (1 mL/g original tissue) using the Dounce homogenizer. This suspension was then brought to 46.5% sucrose by addition of 69% sucrose (w/w) in 10 mM Tris-HCl and 0.5 mM DTT, pH 7.4. The concentration was confirmed using a refractometer. This mixture was overlaid by an equal volume of 41% sucrose and then centrifuged at 110 000 × g for 75 min in a swinging bucket rotor. The plasma membranes collected at the interface were removed, diluted in 10 mM Tris-HCl, 0.5 mM DTT, pH 7.4, and centrifuged at 40 000 × g for 40 min. The pelleted plasma membranes were suspended in approximately 1.5 mL of the Tris–DTT buffer using a ground-glass homogenizer.

Enzymes

Samples of the original homogenate and the final membranes were taken for the measurement of protein and the membrane marker enzyme, 5′-nucleotidase. Proteins were measured by the Coomassie brilliant blue method (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, Calif.). The activity of 5′-nucleotidase was measured by the method of Avruch and Wallach (1971), as previously described (King et al. 1993).

Cytochalasin B binding

Equilibrium d-glucose inhibitable [3H]cytochalasin B (CB) binding was measured, and the concentration of glucose transporters was calculated as described by Wardzala et al. (1978). Scatchard plots were generated from binding studies in which membranes were incubated with varying concentrations of CB in the presence or absence of excess d-glucose. Cytochalasin E was used to decrease nonspecific binding. The total number of transporters (R₀) and the dissociation constant (Kₐ) were determined from a linear plot derived by subtracting along the radial axes of binding curves generated in the presence of d-glucose from those in the absence of d-glucose.

Western blotting

Samples from frog liver were subjected to electrophoresis in an 8% SDS–PAGE gel along with samples of rat liver homogenate and plasma membranes (Hirshman et al. 1988); 20 mg of protein per lane was used for plasma membrane preparations, and 100 mg of protein for the whole homogenates that were used to prepare those membranes. Separated proteins were electrophoretically transferred to a nitrocellulose membrane, incubated at room temperature for 1 h in a blocking solution with 0.2% Tween (Bio-Rad Laboratories) and 3% fatty-acid-free bovine serum albumin (Sigma Chemical Corp., St. Louis, Mo.), and incubated overnight at 4°C with a polyclonal antibody to GluT-2, which was provided by Dr. Sam Cushman. Antibody binding was assessed with autoradiography after incubation with [125I]labeled protein A (Amersham Corp., Arlington Heights, Ill.) by exposing the nitrocellulose sheet to Kodak XAR-5 film at −80°C for 12–16 h. The autoradiographic bands were analyzed by video densitometry (Gel/Image Technology systems, Technology Resources, Nashville, Tenn.).

Transport measurements

Measurements of d-[14C]glucose and l-[3H]glucose uptake into membrane vesicles were made under conditions of equilibrium exchange, using a rapid-filtration technique as previously described by King et al. (1989). The transport medium contained 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, and 20 mM HEPES, pH 7.6, at 22°C, or pH 7.77 at 10°C (the temperature coefficient for HEPES is 0.014 pH unit/°C) and equal concentrations of l- and d-glucose (1–40 mM). The incubation media included 6 mM/l 80 mM l-[3H]glucose (20 Ci/mmol Du Pont Canada Inc., Mississauga, Ont.) and 1.6 mM/l 80 mM d-[U-14C]glucose (265 mCi/mmol, Du Pont). Uptake was initiated by combining the vesicles and incubation medium. Routinely, uptake was measured at four time points between 0 and 2 s for frog liver and between 0 and 5 s for frog muscle. The uptake of both d- and l-glucose was linear over these time periods. The equilibrium uptake was measured by incubating the reaction mixture for 1 h. All reactions were stopped by addition of 1 mL of ice-cold stop solution (250 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 20 mM HEPES, pH 7.8, at 4°C) containing 0.2 mM phloretin, and the membranes were quickly transferred to a nitrocellulose filter (Millipore, HA 0.45 mm) under vacuum. The filter was washed with stop solution and analysed by liquid scintillation counting, using quench correction for a dual label.

The initial rates of l- and d-glucose uptake were obtained from the linear portion of a graph of influx (in nanomoles per milligram of protein) versus time, and the carrier-mediated or facilitated transport was obtained by subtracting the initial rate of l-glucose influx from that of d-glucose. The carrier-mediated flux was plotted against glucose concentration. The kinetic constants for transport, maximal velocity (Vmax) and the glucose concentration producing the half-maximal transport rate (K½), were derived both from a nonlinear least-squares fit of the data (Wilkinson 1961) and from the x and y intercepts of a double reciprocal transformation of the data (Lineweaver–Burk plot).

Statistical analysis

Data are expressed as means ± SE and were analyzed using Student’s t test (two-tailed).
Fig. 1. Glucose influx versus glucose concentration for liver plasma membrane vesicles from summer-collected wood frogs. Values are rates of D-glucose uptake minus rates of L-glucose uptake at each glucose concentration, i.e., carrier-mediated transport. For the summer frogs, data were collected from three separate membrane preparations, each representing the livers of 10–12 frogs. The line is a nonlinear least-squares fit of the data. The inset is a double reciprocal plot of the data.

Results

Plasma membrane marker enzyme activities and enrichments

The activity of 5'-nucleotidase was used as a marker for the plasma membrane. Table 1 shows that 5'-nucleotidase activities in R. sylvatica liver homogenates were significantly higher in autumn frogs than in summer frogs (P < 0.005), but because of high variation between preparations, the difference was not sustained in the isolated membranes. When compared with activities in the homogenates, enzyme activity in the plasma membrane fraction showed similar enrichment for autumn and summer frogs, 7.9- and 8.0-fold, respectively. Thus, the liver plasma membrane preparations were enriched in a canalicular (5'-nucleotidase) marker. We have previously found that frog liver membrane preparations were also enriched in potassium-dependent P-nitrophenol phosphatase, which is a marker of the sinusoidal (basolateral) membrane (King et al. 1993). Activities of this enzyme were also measured in the present study, but the absolute activities in both homogenate and membrane samples were too low to give reliable results. The 5'-nucleotidase activities for muscle homogenates and membranes are also shown in Table 1. Skeletal muscle plasma membranes from R. sylvatica showed similar activities in both summer and

Table 1. Activity of the marker enzyme, 5'-nucleotidase, and enrichment of plasma membrane vesicles isolated from wood frog liver and muscle.

<table>
<thead>
<tr>
<th></th>
<th>Enzyme activity (nmol·mg⁻¹·min⁻¹)</th>
<th>-fold enrichment</th>
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<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>12.8±0.7</td>
<td>102±36</td>
</tr>
<tr>
<td>Summer</td>
<td>5.4±0.8</td>
<td>45±17</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>1.8±0.1</td>
<td>44±9</td>
</tr>
<tr>
<td>Summer</td>
<td>1.8±0.1</td>
<td>71±2</td>
</tr>
</tbody>
</table>

NOTE: Values are given as means ± SE for n = 3 liver preparations and n = 2 muscle preparations. Enzyme activity was measured at 37°C.
Fig. 2. Glucose influx versus glucose concentration for liver plasma membrane vesicles from autumn-collected wood frogs. Values are rates of D-glucose uptake minus rates of L-glucose uptake at each glucose concentration, i.e., carrier-mediated transport. For the autumn frogs, data were collected from the pooled plasma membranes from three preparations. The solid line is a nonlinear least-squares fit of the data. The broken line represents the data for liver membranes of summer frogs presented in Fig. 1 and shown here for comparison on the same scale.

Table 2. Characteristics of the glucose-transporter system in plasma membranes from liver and muscle of wood frogs R. sylvatica.

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$ (nmol · mg$^{-1}$ · s$^{-1}$)</th>
<th>$K_{1/2}$ (mM)</th>
<th>$R_o$ (pmol · mg$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
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<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Autumn</td>
<td>72±14</td>
<td>63±18</td>
<td>70</td>
<td>212</td>
</tr>
<tr>
<td>Summer</td>
<td>12±2.9</td>
<td>32±16</td>
<td>8.2±0.8</td>
<td>237±13</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>6.3±0.5</td>
<td>18.5±3</td>
<td>3.5</td>
<td>37</td>
</tr>
<tr>
<td>Summer</td>
<td>5.1±1.4</td>
<td>13.8±11</td>
<td>4.0</td>
<td>32</td>
</tr>
</tbody>
</table>

Note: Values are given as means ± SE; $n = 2$ for muscle and $n = 3$ for liver. Single values for cytochalasin B binding studies represent instances when preparations with low protein recovery had to be pooled before use. $V_{max}$ (maximal velocity) and $K_{1/2}$ (substrate-affinity constant for carrier-mediated glucose transport) were determined by nonlinear least-squares fit (± SE) of the data in Figs. 1, 2, and 5. $R_o$ is the number of transporters determined from cytochalasin B binding assays; $K_d$ is the dissociation constant for cytochalasin B binding.

Kinetic constants for glucose uptake and quantification of CB binding in frog liver plasma membrane vesicles

Carrier-mediated glucose influx versus glucose concentration for liver plasma membrane vesicles at 10°C is shown in Figs. 1 and 2. The kinetic constants for transport, derived from nonlinear least-squares fits of these data, are shown in Table 2. For membranes from summer frog liver, $V_{max}$ for carrier-mediated transport was $12.0 ± 2.9$ nmol · mg$^{-1}$ · s$^{-1}$, and $K_{1/2}$ was $32 ± 16$ mM (Fig. 1 and Table 2). When transport kinetic constants were estimated from the $x$ and $y$ intercepts of a double reciprocal plot (Fig. 1 inset), similar values were obtained: $14.0$ nmol · mg$^{-1}$ · s$^{-1}$ for $V_{max}$ and $40$ mM for $K_{1/2}$. Figure 2 shows the data for carrier-mediated glucose transport for liver membrane vesicles from autumn frogs; for comparison, the broken line shows the summer frog data on the same scale. The liver membranes from the autumn frogs displayed a much higher $V_{max}$ of $72 ± 14$ nmol · mg$^{-1}$ · s$^{-1}$ than that of the summer frog membranes ($P < 0.025$), and a $K_{1/2}$ value of $63 ± 18$ mM (Fig. 2 and Table 2).

The decrease in transport capacity observed in the membranes from the summer frogs was largely due to differences in the number of glucose-transporter proteins. Using the same membrane preparations as for transport kinetics, the number of transporter sites per milligram of protein ($R_o$) was determined by cytochalasin B binding (Table 2). The number of membrane transporters in the liver membranes of summer frogs was much lower than in autumn frogs; $R_o$ autumn frogs. The membranes were highly enriched in activity compared with the homogenates, 24- and 39-fold for autumn and summer frogs, respectively. These results are similar to previously reported enzyme values for R. sylvatica and R. pipiens liver and muscle plasma membrane vesicles (King et al. 1993).
Fig. 3. Western blots of plasma membrane preparations from the livers of wood frogs. Samples containing 20 μg of protein per lane were separated by SDS-PAGE and immunoblotted with polyclonal antibodies to GluT-2, the major glucose transporter found in livers. The first lane shows a pooled sample from several autumn frogs and the next three lanes show samples from three individual summer-collected frogs. Increasing amounts of a rat liver membrane preparation were used to test linearity of binding: 10, 20, and 40 μg of protein were added to the last three lanes shown \((r = 0.99)\).

<table>
<thead>
<tr>
<th>pool</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>10</th>
<th>20</th>
<th>40 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>aut. summer frog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frog liver PM</td>
<td></td>
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<td>PM</td>
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</table>

values of 8.2 ± 0.8 pmol · mg⁻¹ for summer frogs were only 12% of the values for autumn frogs. However, the \(K_d\) values for cytochalasin B binding were not significantly different between the two groups of animals.

The results of Western blotting using antibodies to GluT-2, the isoform of the glucose-transporter protein present in mammalian liver, are shown in Figs. 3 and 4. The blots of plasma membrane preparations are shown in Fig. 3, with increasing amounts of rat plasma membrane protein used to test linearity of binding. Densitometry analysis (given in relative dark score after subtraction of background) resulted in 805 units for the single pooled autumn frog liver membrane preparation and 266 ± 170 units for the summer frog liver preparations \((n = 3)\). The rat membrane preparations showed a linear increase in absorbance (correlation coefficient \(r = 0.99\)) with increasing protein concentration. Figure 4 shows the results for whole-liver homogenates from frogs, with an equivalent amount of protein from rat liver shown in the first lane. Densitometry analysis gave 484 ± 3 units for autumn frog liver and 130 ± 91 for summer frog liver \((n = 3\) for each group). This difference was significant at \(P < 0.06\) when variances were assumed to be unequal and \(P < 0.02\) when equal variance was assumed. That is, the analysis of autumn frogs, which were adapted to a uniform low temperature \((5°C \text{ for } 3-4 \text{ weeks})\) after collection, produced very consistent amounts of GluT-2 binding to the whole-liver homogenate samples (i.e., low standard error). In contrast, the summer frogs, which were not acclimated before use, showed much wider variability in the quantities of GluT-2, indicating heterogeneity in glucose-transporter protein levels.

Kinetic characteristics of glucose transport and quantification of transporter numbers in plasma membrane vesicles isolated from frog skeletal muscle

The transport characteristics of skeletal muscle membranes isolated from summer and autumn \(R. \text{ sylvatica}\) were also examined (Fig. 5). Glucose uptake was slower in the muscle membrane vesicles than in liver. As a result, transport by the muscle membranes was measured at a higher temperature, 22°C, over 5 s, as previously described (King et al. 1993). The transport \(V_{\text{max}}\) for membranes from summer frogs was 5.1 ± 1.4 nmol · mg⁻¹ · s⁻¹. \(V_{\text{max}}\) for membranes from autumn \(R. \text{ sylvatica}\) was similar, 6.3 ± 0.5 nmol · mg⁻¹ · s⁻¹ (Table 2), and both values were similar to those reported previously (King et al. 1993). The \(K_{1/2}\) values did not differ significantly between autumn and summer frogs. When estimated from the \(x\) and \(y\) intercepts of a double reciprocal plot, similar \(V_{\text{max}}\) and \(K_{1/2}\) values were obtained \((6.7 \text{ nmol · mg}^{-1} · \text{s}^{-1} \text{ and } 20 \text{ mM}, \text{ respectively}, \text{ for}
Fig. 4. Western blots of the whole-liver homogenates from which plasma membranes were prepared. Samples containing 100 μg of protein per lane were separated by SDS–PAGE and immunoblotted with polyclonal antibodies to GluT-2. The first lane shows rat liver homogenate, followed by three autumn and three summer frog liver homogenates.

Discussion

The present data show that the glucose-transport capacity of wood frog liver plasma membranes varies seasonally, being lower in the summer months and high in the autumn, when frogs are prepared to enter winter hibernation. Carrier-mediated transport activity was 6-fold higher in liver membranes from cold-acclimated frogs than in vesicles from frogs collected in June. This difference could be attributed to greater numbers of glucose-transporter proteins in the membranes of autumn than in summer frogs. Thus, cytochalasin B binding studies showed 8.5-fold greater numbers of glucose transporters in liver membranes from autumn than in those from summer frogs. Densitometry analysis of the immunoblots gave a similar result: the amount of material cross-reacting with antibodies to the rat liver glucose transporter, GluT-2, was 3-fold greater for the liver vesicle preparations and 4-fold greater for whole-liver homogenates from autumn versus summer frogs. However, whereas transporter numbers varied seasonally, the kinetic properties of the liver glucose transporters did not change significantly between the two seasons. $K_{1/2}$ values for glucose transport, measuring transporter affinity for glucose, and $K_d$ values for cytochalasin B binding were not significantly different between autumn and summer animals. Furthermore, calculated average carrier turnover numbers ($V_{max}/R_0$), a measure of transporter activity in the membranes, were very similar, 1029/s for autumn and 1463/s for summer preparations (small sample numbers did not allow statistical comparisons). Thus, it is apparent that the capacity for cryoprotectant synthesis during winter hibernation is served by large increases in the numbers of glucose transporters in wood frog liver plasma membranes without apparent changes in the properties of the transporters.

The numbers and properties of glucose transporters in skeletal muscle plasma membranes, by contrast, showed no significant changes between summer and autumn animals. However, in our previous study we found that maximal glucose transport activity in $R. sylvestica$ muscle membranes was 8-fold higher than in equivalent preparations from $R. pipiens$ (King et al. 1993). It appears then, that different adaptive strategies are used by different organs of the wood frog. The freeze-tolerant wood frog maintains constantly higher levels of glucose transporters in skeletal muscle plasma membranes than a freeze-intolerant species, but does not appear to modify the amounts of muscle transporters seasonally. A note of caution must be added, however, because the glucose-transport capacity of mammalian and frog muscle is known to increase in response to insulin or work load, this effect resulting from a rapid increase in the numbers of the GluT-4 isoform in muscle membranes as the result of translocation.
Fig. 5. Glucose influx versus glucose concentration for autumn (●) and summer (○) frog skeletal muscle plasma membrane vesicles. Values are rates of D-glucose uptake minus rates of L-glucose uptake at each glucose concentration, i.e., carrier-mediated transport. Data were collected from two separate membrane preparations from each group. The lines are nonlinear least-squares fits of the data. The inset is a double reciprocal plot of the data.

of the transporters from an inactive intracellular pool (Holloszy and Narahara 1965; King et al. 1989; Pessin and Bell 1992; Zaninetti et al. 1988). It remains to be determined whether natural freezing exposures might stimulate the same mechanism in wood frog muscle to increase glucose-transport capacity when cryoprotectant uptake is immediately required. The strategy used by liver, however, is different. Glucose-transport activity and transporter numbers in summer *R. sylvatica* were similar to values for *R. pipiens* liver (King et al. 1993), indicating the general range for the normal functioning of the amphibian liver. However, the very great demand for glucose export from liver during the early minutes and hours of freezing in *R. sylvatica* has apparently necessitated an anticipatory increase in liver plasma membrane glucose transporters as part of the autumn cold-hardening of the species. Because the GluT-2 isoform, the major glucose transporter in liver, is not subject to rapid regulation by translocation from an inactive cytosolic pool, as is the muscle GluT-4, the anticipatory elevation of glucose transporters in liver plasma membranes during autumn cold-hardening may be the only mechanism that can raise the glucose-transport capacity to the level required for rapid cryoprotectant distribution during freezing.

The kinetic properties of liver and muscle glucose transporters in *R. sylvatica* reported here are similar to our previous report for this species (King et al. 1993) and the differences between the two transporter types reflect the general pattern of organ-specific properties seen in mammals (Pessin and Bell 1992). The liver GluT-2 isoform is a high-$K_m$, high-$V_{max}$ transporter that mediates both the uptake and release of glucose and does so under conditions that may be unique to liver, i.e., when the glucose concentration within the cell exceeds that in the circulation (Pessin and Bell 1992). The muscle GluT-4 isoform, by comparison, has lower $K_m$ and $K_d$ values and those of *R. sylvatica* agree well with values reported for rat skeletal muscle analysed under similar conditions (King et al. 1989). The results of Western blotting with antibodies to rat liver GluT-2 clearly show that this isoform is present in frog liver and has good cross-reactivity with the mammalian isoform, supporting previous reports that glucose transporters are highly conserved proteins (Pessin and Bell 1992). Furthermore, the immunoblotting experiments are specific in clearly showing that amounts of the GluT-2 isoform in *R. sylvatica* liver change seasonally, increasing in autumn animals. The results shown in Fig. 4, comparing GluT-2 levels in homogenates of summer and
autumn liver, also indicate very tight and consistent amounts of the transporter in autumn frogs acclimated to a constant low temperature, whereas transporter amounts varied quite widely in summer frogs that were not laboratory acclimated before sampling. This shows that there is considerable heterogeneity in glucose-transporter numbers during the summer months, probably related to the dietary intake of particular individuals or modified by the environmental conditions (e.g., temperature) of the habitat. However, acclimation to a constant low temperature, similar to the temperatures of the winter microhabitat, elevated liver GluT-2 to the high and consistent levels needed to support freezing-induced cryoprotectant distribution during winter hibernation.

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