GLUT-1 mediation of rapid glucose transport in dolphin (Tursiops truncatus) red blood cells

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Chemistry Department, Bishop's University, Lennoxville, Quebec J1M 1Z7, and Membrane Transport Group, Physiology Department, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Craik, James D., James D. Young, and Christopher I. Cheeseman. GLUT-1 mediation of rapid glucose transport in dolphin (Tursiops truncatus) red blood cells. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R112–R119, 1998.—d-Glucose entry into erythrocytes from adult dolphins (Tursiops truncatus) was rapid, showed saturation at high substrate concentrations, and demonstrated a marked stimulation by intracellular d-glucose. Kinetic parameters were estimated from the concentration dependence of initial rates of tracer entry at 6°C: for zero-trans entry, Michaelis constant ($K_m$) was 0.78 ± 0.10 mM and maximal velocity ($V_{max}$) was $300 ± 9$ µmol·l cell water$^{-1}$·min$^{-1}$; for equilibrium exchange entry, $K_m$ was 17.5 ± 0.6 mM and $V_{max}$ was 8,675 ± 96 µmol·l cell water$^{-1}$·min$^{-1}$. Glucose entry was inhibited by cytochalasin B, and mass law analysis of reversible, d-glucose-displaceable, cytochalasin B binding gave values of $0.37 ± 0.03$ nmol/mg membrane protein for maximal binding and $0.48 ± 0.10$ µM for the dissociation constant. Dolphin glucose transporter polypeptides were identified on sodium-dodecyl sulfate-polyacrylamide gel electrophoresis immunoblots [using antibodies that recognized human glucose transporter isoform (GLUT-1)] as two molecular species, apparent molecular weights of 53,000 and 47,000. Identity of these polypeptides was confirmed by d-glucose-sensitive photoaffinity labeling of membranes with $[3H]$cytochalasin B. Digestion of both dolphin and human red blood cell membranes with glycoproteinase F led to the generation of a sharp band of relative molecular weight 46,000 derived from GLUT-1. Trypsin treatment of human and dolphin erythrocyte membranes generated fragmentation patterns consistent with similar polypeptide structures for GLUT-1 in human and dolphin red blood cells.

Higher primates differ from most other mammals in that red blood cells from adult individuals show a very high capacity for equilibrative glucose transport, a feature typical of fetal and neonatal erythrocytes in many mammalian species (12, 30). Glucose transport in human red blood cells has been shown to be mediated by the GLUT-1 glucose transporter isoform (nomenclature from Ref. 14) of the equilibrative hexose transporter family of integral membrane glycoproteins. Glucose transporter polypeptides are abundant (~250,000–500,000 copies per cell (1, 17, 25)) in the plasma membrane of adult human red blood cells. The GLUT-1 transporter isoform is widely expressed in mammalian cells and tissues and shows strong conservation of sequence in diverse species (for review see Refs. 3 and 13). The functional characteristics of the GLUT-1-mediated transport pathway in human erythrocytes have been the subject of intensive investigation; however, a definitive kinetic characterization of this transport system remains elusive (reviewed by Carruthers in Ref. 4). This uncertainty may be due, in part, to technical difficulties in the accurate estimation of very rapid initial rates of d-glucose transport in human red blood cells. Even at sub-physiological temperatures, transmembrane glucose fluxes are very rapid, with a half time for d-glucose entry at low substrate concentrations of less than 1 s at 20°C (20, 21). However, kinetic complexity of glucose transport in mammalian red blood cells may also reflect physical complexities of the transport pathway, for example, the influence of cytosolic hexose binding sites on sugar transport (6). Study of glucose transport in a nonprimate species showing rapid glucose permeation into adult red blood cells could assist in the identification of structural and functional features of the transporter that may be important for physiological activity and could clarify biological properties of the glucose transport pathway in human erythrocytes.

Erythrocytes from an adult beluga whale (Delphinapterus leucas; see Ref. 9) and bottle-nosed dolphins (Tursiops truncatus; see Ref. 7) show a high permeability toward d-glucose, supporting a conclusion originally drawn from measurements of sugars in a blood sample taken from a dying porpoise (2). Small odontocetes present an opportunity to examine rapid glucose transport in red blood cells from adult mammals other than the higher primates. Paleontological evidence suggests that cetacean lineages diverged from terrestrial mammals ~55 million years ago and that the artiodacyls (pigs, camels, ruminants) represent the closest modern terrestrial relatives of the whales (27). Erythrocytes from adults of these terrestrial species exhibit low permeability toward d-glucose (reviewed by Whittam in Ref. 28), an exception to this general finding being red blood cells from the pig, which are unable to utilize extracellular glucose (18) because they possess no mediated pathway for d-glucose entry.

The biological advantages of retention of a very high glucose transport capacity in erythrocytes of adult humans and dolphins have not been clearly ascertained. This physiological phenotype is probably not determined by metabolic requirements of the red blood cells themselves, since in humans the hexose transport capacity appears to be well in excess of any possible metabolic demands of the erythrocyte (29). Dolphin red blood cells incubated in vitro have been shown to catabolize d-glucose more slowly than human red blood cells (15). One rationalization for a high glucose transport capacity is that there is a requirement to maximize glucose delivery to specific regions of the brain under conditions of physiological stress (for example under

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hypoxic conditions in aquatic mammals), and calculations of rates of glucose consumption by the brain and rates of glucose delivery to the central nervous system support this conjecture (5). Both primates and odontocetes are notable for large and complex central nervous systems, with high brain mass/body mass ratios (23).

In this study, d-glucose entry into dolphin erythrocytes was examined at a low temperature so that initial rates of entry could be estimated directly from progress curves for uptake. A simple kinetic analysis of the functional properties of the d-glucose transport pathway was undertaken. Zero-trans and equilibrium exchange measurements [nomenclature of Stein (26)] showed that, at 6°C, the dolphin erythrocyte glucose transport pathway displays marked trans-stimulation effects, similar to those observed for glucose transport in human red blood cells. Transport site density in dolphin red blood cell membranes, estimated from analysis of d-glucose-displaceable cytochalasin B binding, appears to be lower than that found in human erythrocyte membranes. Molecular studies demonstrated the presence of GLUT-1, an equilibrative glucose transporter polypeptide in dolphin red blood cell membranes. This protein was readily fragmented by exposure to trypsin and showed a markedly greater mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than human red cell GLUT-1, probably because of differences in glycosylation.

MATERIALS AND METHODS

Reagents and Solutions

D-[U-3H]glucose (10.8 GBq/mmol), D-[14C]fructose (10.2 GBq/mmol), and [3H]cytochalasin B (644 GBq/mmol) were from ICN Biomedicals Products, DuPont Canada (Markham, Ontario, Canada). L-[3H]glucose (9,573.5 GBq/mmol), [3H]H2O (37 MBq/ml), and 3-O-[methyl-3H]glucose (3.2 GBq/mmol) were from NEN Research Products, DuPont Canada (Markham, Ontario, Canada). L-[U-14C]glucose (13.4 GBq/mmol) was from ICN Biomedicals Canada (Mississauga, Ontario, Canada).

Luminescent detection of antibodies on immunoblots was accomplished using an ECL kit purchased from Amersham Canada. Glycophorin F, Ponceau S dye, and Tween 20 were from Sigma Chemical (St. Louis, MO). Trypsin (from bovine pancreas; 40 U/mg) was from Boehringer Mannheim Canada (Laval, Quebec, Canada).

Ascites fluid (stored at ~70°C) was used as a source of monoclonal antibody 65D4. This antibody has been shown to recognize GLUT-1 polypeptides in erythrocyte membranes from adult humans, neonatal pigs, and adult mice (8). A monoclonal monospecific antiserum, raised against a 12-amino acid COOH-terminal peptide sequence for human GLUT-1, was purchased from East Acres Biologicals (Southbridge, MA). The composition of phosphate-buffered saline (PBS) was (in mM) 136.8 NaCl, 2.68 KCl, 4.29 Na2HPO4, and 1.47 KH2PO4, pH 7.4, and red blood cell lysis buffer was ice-cold 5 mM phosphate buffer, pH 8.0, including 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical) as added as a stock solution of PMSF in anhydrous isopropanol immediately before use.

Venous blood samples used in this study, obtained from captive dolphins without the use of physical restraint, were excess samples left over after blood had been taken for routine veterinary tests.

Monosaccharide Flux Measurements

Zero-trans entry. d-Glucose uptake was determined from measurements of intracellular radioactivity after exposure of intact erythrocytes to solutions containing predetermined concentrations of d-glucose and tracers amounts (~37 kBq/ml) of tritiated d-glucose. Samples of whole blood in heparinized tubes (Vacutainer) were subjected to gentle centrifugation (300 g, 5 min, 6°C) to pellet the erythrocytes. Blood plasma and white blood cells were removed by aspiration, and the erythrocytes were resuspended in ~10 volumes of PBS, pH 7.4, at room temperature. This centrifugation and washing process was repeated four times to remove plasma and platelets and to deplete the cells of intracellular glucose. Entry of glucose into washed red blood cells was assayed by a conventional isotopic tracer protocol (7). Uptake of isotope was initiated by rapid addition of 100 µl of labeled glucose solution containing tritiated tracer to 100 µl of red blood cell suspension in PBS (~20% hematocrit) layered over an immiscible butyl pthalate oil layer in a 1.5-ml microcentrifuge tube. Influx of glucose was terminated after a predetermined incubation time by rapid addition of 200 µl of ice-cold 200 µM phloretin in PBS solution and immediate centrifugation in a benchtop microcentrifuge (Fisher model 235A, 12,000 g, 10 s). Red blood cells sedimented to give an erythrocyte pellet separated by an oil layer from aqueous extracellular medium. The supernatant aqueous layer was removed by aspiration, and the microcentrifuge tube was washed by careful addition of 1 ml distilled water above the oil layer. The washing fluid was removed by aspiration. The walls of the tube were wiped dry, and most of the oil layer was removed with the use of absorbent cotton buds. The erythrocyte pellet was dispersed, and the cells were disrupted by addition of 0.5 ml 0.5% (wt/vol) trichloroacetic acid solution, and the suspension was mixed on a vortex mixer. Protein was pelleted by a brief centrifugation in a microcentrifuge (12,000 g, 30 s), and 0.5 ml of the clear supernatant fluid was taken for scintillation counting after addition of 4 ml Ecolight (ICN Biochemicals) scintillation fluid. Water spaces in the pellet were determined from separate incubations of erythrocytes in which H2O (for total water space) or [14C]glucose (extracellular water space) replaced tritiated glucose tracer. Quench corrections were performed using an external standard method (Beckman LS-6500 scintillation counter). Initial rates of glucose entry were estimated from progress curves with the use of a polynomial curve fit procedure (CA-CricketGraph III, Computer Associates). Transport parameters were estimated from the initial rate data using Enzfitter nonlinear regression data analysis program (BioSoft, Cambridge, UK).

Equilibrium exchange entry. Red blood cells were isolated from whole blood as described in the zero-trans entry protocol and then washed with ~10 volumes of a solution containing the desired concentration of d-glucose in phosphate saline. Cells were incubated at room temperature for 15-45 min (longer times for higher glucose concentrations) and then washed again with a fresh portion of the appropriate glucose solution. After this washing step, the cell suspension (at ~20% hematocrit) was subjected to the temperature appropriate for the transport assay. An experimental protocol identical to that described for zero-trans entry measurements was then followed. Preliminary experiments showed that at 6°C equilibrium exchange fluxes of d-glucose were much more rapid than zero-trans entry fluxes, so shorter incubation times were used to define progress curves for equilibrium
exchange parameters were estimated as described for zero-trans entry.

Preparation of erythrocyte plasma membranes (ghost membranes) from human, pig, and dolphin erythrocytes. Erythrocytes were collected into 7-ml evacuated sample tubes containing 100 USP units of lithium heparin (Vacutainer, Becton Dickinson, Rutherford, NJ), mixed by inversion, and chilled on ice. Cells were stored at 6°C until used; preparation of red blood cell membranes was completed within 24 h of the blood samples being taken. Blood samples were subjected to gentle centrifugation (300 g, 5 min, 6°C) to pellet the erythrocytes. Blood plasma and white blood cells were removed by aspiration, and the red blood cells were resuspended in 10 volumes of PBS, pH 7.4. This centrifugation and washing procedure was repeated twice to remove blood plasma proteins and platelets. The loose red blood cell pellet was added to 100 volumes of ice-cold lysis buffer and gently stirred on ice for ~3 min. The lysed cell suspension was subjected to centrifugation (10,000 revolutions per minute, 15 min, 6°C, Sorvall SA-600 rotor), and supernatant fluid was removed by aspiration. The loose red blood cell membrane pellet was resuspended in ice-cold 5 mM phosphate buffer, and the small dense “button” pellet was discarded. The centrifugation and washing procedure was repeated (three or four times) to give an off-white membrane pellet that was resuspended in 5 mM phosphate buffer, pH 8.0, and frozen in small portions for storage at -70°C.

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Protein content of the suspension was estimated by a dye-binding method using protocols and reagents supplied by Bio-Rad.

Cytochalasin B binding assays. Reversible binding of tritiated cytochalasin B to dolphin erythrocyte membranes was assayed in the presence of 400 mM D-glucose or the same concentration of sorbose (a sugar known to show little interaction with erythrocyte glucose transport systems). Portions (40 µl) of erythrocyte suspension (0.93 mg protein/ml) were placed in 5 mm × 20 mm polyallomer ultra centrifuge tubes (Beckman Airfuge, Beckman Instruments, Palo Alto, CA) together with 140 µl 1 M D-glucose or L-glucose solution. Graded amounts of tritiated cytochalasin B were added in 2 µl of ethanol. The mixture was incubated for 40 min at room temperature before centrifugation (Beckman Airfuge, 28 lbs/ in.2 at gauge, 15 min). Supernatant fluids were sampled and the concentration of free tritiated cytochalasin B estimated by scintillation counting (Ecolite scintillation fluid, ICN Biochemicals). Quench corrections were performed using an external standard method. The membrane pellet was dispersed by addition of 50 µl of 0.5% (vol/vol) Triton X-100 detergent in distilled water, and the amount of bound tritiated cytochalasin B was estimated by scintillation counting. Nonspecific binding of tritiated cytochalasin B was estimated from tubes in which 68 µM unlabeled cytochalasin B was included in addition to the tritiated ligand. Binding data were analyzed using Enzfitter nonlinear regression data analysis program ( Biosoft, Cambridge, UK).

Photolabeling of erythrocyte membranes with tritiated cytochalasin B. Red blood cell membrane suspensions (final concn 0.75–1 mg protein/ml) in medium containing 2.5 mM phosphate, pH 8.0, 10 mM cytochalasin E, 500 mM D-glucose or L-glucose, and 0.5–1 µM tritiated cytochalasin B were incubated on ice for 2 h. Samples were briefly purged with nitrogen and were then irradiated in 1 mm light path quartz cuvettes at a distance of 7 cm from a water-jacketed 450 W mercury lamp (Conrad Hanovia) for 20 s. Following irradiation, dithiothreitol solution (0.5 M) was added to give a final concentration of 10 mM, and the red blood cell membranes was recovered by centrifugation (90 s, microcentrifuge), solubilized in SDS-PAGE sample buffer (incubation at 37°C for 10 min), and then subjected to electrophoresis (9% polyacrylamide gel). For fluorography, gels were treated with Enhance fluorography cocktail (NEW Research Products, DuPont Canada) according to the manufacturer’s directions. Exposure times of the treated and dried gels were performed at ~70°C, using Kodak XAR-5 X-ray film.

SDS-PAGE and immunoblot protocols. Polyacrylamideslab gels (8, 9, or 10% acrylamide) were prepared and run using standard protocols and reagents supplied by Bio-Rad (Mississauga, Ontario, Canada) using the Laemmli buffer system. Prestained molecular weight standards (Rainbow Markers, Amersham Canada) and unstained molecular weight standards (high molecular weight, Sigma Chemical) were included in separate lanes.

For immunoblot analysis, proteins were transferred to nitrocellulose (0.45-µm pore size, Bio-Rad) or polyvinylidene difluoride (Immobilon, Millipore Canada, Napean, Ontario, Canada) membranes using the Towbin buffer system [25 mM tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine, pH 8.8], with 5% (vol/vol) methanol. When appropriate, transferred proteins were visualized using Ponceau S or AmidoBlack stains. In immunodetection protocols, blots were treated with PBS containing 0.05% (vol/vol) polyoxylene sorbitan monolaurate (Tween 20) and 3% (wt/vol) dried nonfat milk powder to block nonspecific antibody binding. After incubation with primary and secondary antibodies (diluted in blocking solution) and appropriate washing steps (PBS containing 0.05% Tween 20), following the protocols given by the manufacturer of the ECL Western blot kit (with the substitution of PBS for Tris-buffered saline), antibody binding was determined by exposure of X-ray film (Fuji RX) to luminescence generated by peroxidase activity bound to the blot (exposure times 5 to 8 min).

When it was necessary to reprobe blots (with a different antibody preparation), the membranes were first washed twice with 100-ml volumes of PBS-Tween solution (10 min with gentle agitation). The blot was then placed in stripping buffer (100 mM 2-mercaptoethanol, 2% wt/vol SDS, 62.5 mM Tris-HCl, pH 6.8) warmed to 50°C and incubated for 30 min with occasional gentle agitation. This procedure removed both primary and secondary antibodies. Blots were washed three times at room temperature (100 ml PBS-Tween, 10 min, gentle agitation) to remove stripping buffer and then incubated for 1 h in blocking medium, before the immunodetection protocol was repeated.

Digestion of erythrocyte membrane polypeptides with trypsin. Digestions were performed by incubation of 30 µl dolphin or human erythrocyte membranes (1.5 mg/ml protein concentration) suspended in 5 mM phosphate buffer, pH 8.0, on ice for 30 min in the presence of 0.25 or 0.025 mg/ml trypsin. The digestion was terminated by addition of 2 µl 100 mM PMSF (freshly dissolved in dimethyl sulfoxide) and incubation at room temperature for 5 min. Samples were dissolved by addition of 35 µl electrophoresis sample buffer, with incubation at room temperature for 5 min before SDS-PAGE analysis.

Digestion of erythrocyte membrane glycoproteins with glycopeptidase F. Digestions were performed by solubilization of 30 µg membrane protein in a volume of 10 µl of 1% SDS. The solubilized sample was prepared for digestion by addition of 30 µl digestion buffer (2.6% vol/vol Nonident P-40; 40 mM Tris buffer, pH 8.0; 5 mM 1,10-phenanthroline; and 33 mM 2-mercaptoethanol). Glycopeptidase F (0.5 U) was added, and the sample was incubated at 35°C for 6 h, when additional glycopeptidase F (0.5 U) was added. The incubation was continued for a further 16 h at 35°C. The digestion was terminated by addition of 5 µl 10% (wt/vol) SDS and subse-
sequent addition of 45 µl SDS-PAGE sample buffer. The mixture was incubated at room temperature for 10 min before samples were applied to the polyacrylamide gel and subjected to SDS-PAGE.

RESULTS

Kinetic Results

Zero trans-entry of d-glucose into dolphin red blood cells at 6°C is markedly slower than d-glucose entry at room temperature (7), making possible the estimation of initial rates of transport using simple manual assay methods. Figure 1 shows a progress curve for d-glucose entry under zero-trans conditions. Isotopic entry of d-glucose into dolphin red blood cells was found to be very much faster under equilibrium exchange conditions than for entry into glucose-depleted cells. Michaelis-Menten parameters for zero-trans entry were estimated as Michaelis constant ($K_m$) = 0.78 ± 0.10 mM and maximal velocity ($V_{max}$) = 300 ± 9 µmol·l cell water$^{-1}$·min$^{-1}$ (SE, unweighted data) and for equilibrium exchange entry as $K_m$ = 17.5 ± 0.6 mM and $V_{max}$ = 8,675 ± 96 µmol·l cell water$^{-1}$·min$^{-1}$ (Fig. 2).

Inhibition of d-Glucose Entry by Cytochalasin B

D-Glucose entry into dolphin red blood cells is acutely sensitive to inhibition by micromolar concentrations of cytochalasin B. At 21°C and 12 mM extracellular d-glucose, zero-trans entry in the presence of 40 µM cytochalasin B was less than 2% of the uninhibited transport rate. At 37°C, a slow entry of d-glucose in the presence of 40 µM cytochalasin B was substantially reduced by the presence of 2 mM HgCl$_2$ to give a rate only slightly higher than that observed for L-glucose entry (data not shown).

Glucose-Displaceable Binding of Tritiated Cytochalasin B

Mass law analysis of d-glucose displaceable cytochalasin B binding to dolphin red blood cell membranes (Fig. 3) suggests the presence of saturable binding sites [maximal binding capacity ($B_{max}$) = 0.37 ± 0.03 nmol/mg protein and dissociation constant ($K_d$) of 0.48 ± 0.10 µM]. The $K_d$ value is higher than that reported for human red blood cell membranes ($K_d$ = 0.17 µM, for example see Ref. 25), and the $B_{max}$ value is lower than the value reported for glucose transport-associated sites in human red blood cell membrane preparations (up to 1.1 nmol/mg protein, see Ref. 1).

Photolabeling of Dolphin Red Blood Cell Membranes With Tritiated Cytochalasin B

Covalent photolabeling of polypeptides in dolphin red blood cell membranes with tritiated cytochalasin B is
shown in Fig. 4. D-Glucose inhibition of photolabeling of polypeptides with approximate relative molecular weight (M_r) 46,000–54,000 is apparent.

Identification of Dolphin Erythrocyte Glucose Transporter Polypeptides on Western Blots

Dolphin GLUT-1 polypeptides were identified on immunoblots using polyclonal antibodies directed against the COOH-terminal region of human GLUT-1. This antiserum stains a broad band in the band 4.5 region (M_r, 45,000–65,000) of human erythrocyte membrane proteins but does not give any signal with red blood cell membranes prepared from adult pig erythrocytes (Fig. 5). Erythrocytes from adult pigs are impermeable to d-glucose (18) and lack glucose transporter polypeptides that can be detected in the plasma membranes of erythrocytes from neonatal pigs (8). The polyclonal antiserum detected a pair of bands M_r 52,000, and 47,000 in the dolphin erythrocyte membrane preparation (Fig. 5). This pattern of two adjacent bands was observed with red blood cell membranes from all four dolphins examined in this study (2 males and 2 females; ~14 yr old). A mouse monoclonal antibody (65D4) known to recognize glucose transporter polypeptides in several different mammalian species (8) identified bands of identical mobility on immunoblots (Fig. 6).

Digestion of Erythrocyte Membrane Polypeptides With Trypsin

Dolphin GLUT-1 differed from human GLUT-1 in its electrophoretic mobility on SDS-PAGE (Fig. 5). Limited trypsin digestion of unsealed plasma membrane preparations was performed to examine the primary structure of the dolphin protein. Blots of digested membranes were first probed with polyclonal antiserum to detect COOH-terminal fragments of the GLUT-1 protein and then stripped and reprobed with monoclonal antibody 65D4 to detect polypeptide fragments that had lost COOH-terminal epitopes. Digestion of the unsealed human red blood cell membranes under these mild conditions generated a sharp band (containing COOH-terminal epitopes) of M_r 25,000, which appears to correspond to a fragment M_r 25,500 described by Davies and co-workers (10) in a study of tryptic digestion of human red cell glucose transporter. A band of similar mobility was generated by trypsin treatment of the dolphin red blood cell membranes. Monoclonal antibody 65D4 recognized a fragment of apparent M_r 28,000 in the dolphin tryptic digest and bands of apparent M_r 48,000 (and a very faint band M_r 44,000, not visible after photography but whose position is indicated on Fig. 6), which might reflect removal of COOH-terminal peptides from dolphin GLUT-1 (which migrated with apparent M_r 53,000 and 47,000 on this gel). The monoclonal antibody detected a smear of material in the human tryptic digest that shows the production of glycosylated protein fragments of apparent M_r 25,000–45,000.

Fig. 3. D-glucose displaceable binding of tritiated cytochalasin B to isolated dolphin erythrocyte membranes under equilibrium conditions as described in MATERIALS AND METHODS. Inset: Scatchard plot of the data. Solid lines show maximal binding = 0.37 nmol/mg membrane protein and K_d = 0.48 µM.

Fig. 4. Fluorogram of SDS-polyacrylamide gel electrophoresis (PAGE) gel of dolphin red blood cell membranes subjected to photolabeling in the presence of tritiated cytochalasin B as described in MATERIALS AND METHODS. Lane 1, irradiation in the presence of 500 mM L-glucose; lane 2, irradiation in the presence of 500 mM D-glucose.
Digestion of GLUT-1 with Glycopeptidase F

Treatment of dolphin erythrocyte membranes with this enzyme, known to cleave most high mannose, hybrid, and complex N-linked carbohydrate structures from membrane glycoproteins, generated a sharp band of apparent Mr 46,000, of identical mobility to a sharp band produced by digestion of human red blood cell membranes in parallel incubations (Fig. 7).

DISCUSSION

The pathway for rapid mediated entry of D-glucose into erythrocytes from adult dolphins shows remarkable functional similarity to the GLUT-1-mediated pathway in human red blood cells; in particular, a very marked trans-stimulation of hexose entry was observed at low temperatures. Mass law analysis of D-glucose-displaceable cytochalasin B binding to dolphin red cell membranes suggests that the site density of equilibrium glucose transporters is lower than that observed in equivalent preparations of human erythrocyte membranes, consistent with observations of D-glucose transport rates reported in this study, and previously (7), that indicated that entry of D-glucose into dolphin red blood cells was less rapid than entry into adult human red blood cells. A trans-stimulation effect (29-fold, calculated from \( V_{\text{max}} \)) is less than that reported for human red blood cells at 0°C [\(-100\) fold (21)]. However, trans-stimulation of glucose transport in human red blood cells declines with increasing temperature (19, 21), so the value estimated for dolphin red blood cells at 6°C is close to that expected for human red blood cells. \( K_m \) values for equilibrium exchange for the dolphin system are within the range of values reported for human red blood cells [13–25 mM, (20)]; however, a higher \( K_d \) value for glucose-displaceable cytochalasin B binding, suggests that GLUT-1 in T. truncatus erythrocytes may differ slightly in its functional properties from the human red cell glucose transporter. Plasma glucose levels reported for peripheral venous blood samples of T. truncatus [6.3 ± 1.3 mM (5), 7.2 ± 0.2 mM (24), 10.1 ± 0.2 mM (22)] are higher than those observed for fasting humans [3.9–5.6 mM (11)], so a
At 21°C, the rate of D-glucose entry from 12 mM presence of high (20–40 µM) concentrations of cytochalasin B were too slow for quantitative estimation over periods of up to 5 min. At higher temperatures, some influx was apparent even in the presence of high (20–40 µM) concentrations of cytochalasin B. At 21°C, the rate of D-glucose entry from 12 mM D-glucose in the extracellular medium in the presence of 40 µM cytochalasin B was ~2% of the uninhibited rate (data not shown). At 37°C, entry of D-glucose into dolphin red blood cells treated with 40 µM cytochalasin B was faster than the entry of L-glucose, and D-glucose entry was reduced by addition of 2 mM HgCl₂, suggesting that at least part of the entry flux was via a mediated pathway. This stereospecific glucose transport may be a consequence of incomplete inhibition of dolphin GLUT-1 by cytochalasin B, some of which may be sequestered within the red blood cell. However, the presence of an additional mediated hexose transport pathway, with very low activity, cannot be ruled out. Taken together, these findings point to a remarkable functional similarity in the glucose transport properties of red blood cells from adult dolphins and adult humans. The existence of this physiological phenotype in an advanced cetaceous species implies that the extraordinarily high glucose permeability of erythrocytes from adult humans and apes serves a physiological role and that it is not a quirk peculiar to the primate lineage.

Perspectives

The extraordinarily high glucose transport capacity of red blood cells from adult humans and higher primates has remained a physiological curiosity for almost a century. Demonstration of very high GLUT-1-mediated hexose permeability in erythrocytes from adult odontocetes suggests that this physiological trait has arisen at least twice in evolutionary sequences leading to advanced mammals. This could indicate that glucose delivery to the central nervous system represents a critical physiological constraint in the evolution of large and complex brains in mammals. Possibly, in vivo glucose demand by certain regions of the brain under conditions of physiological stress may be higher than anticipated from hexose clearance rates determined under experimental conditions. Dolphin red blood cells should also provide a valuable experimental system for comparative investigations of the molecular basis of kinetic peculiarities of glucose transport revealed in studies of human erythrocytes. The considerable evolutionary distance between humans and dolphins and differences in intracellular concentrations of enzymes and metabolites in red blood cells from these species should allow comparative studies to identify features of this hexose transport pathway that are important for red blood cell function in advanced mammals.

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REFERENCES