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Bioelectrochemistry 62 (2004) 195–198
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Effects of anti-GLUT antibodies on glucose transport into human erythrocyte ghosts

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Received 6 June 2003; received in revised form 20 July 2003; accepted 22 July 2003

Abstract

We have studied the effects of anti-GLUT1 antibodies on the uptake of glucose into erythrocytes. Glucose transport into human erythrocyte ghosts was measured directly using ^{3}H-2-deoxy-glucose, or indirectly by monitoring associated volume changes using light scattering. The uptake of glucose was significantly inhibited in ghosts resealed in solutions containing specific antibodies against GLUT1. Such an effect was not observed when an antibody against the oestrogen receptor, lacking specificity towards GLUT1, was employed instead. The antibodies were also without effect on the efflux of preloaded glucose from erythrocyte ghosts. The demonstration that anti-GLUT antibodies can inhibit glucose uptake is support for the hypothesis that they exaggerate the cytoplasmic barrier to glucose uptake created by endofacial segments of GLUT1.

Keywords: GLUT1; Erythrocyte; Ghost; Antibody

1. Introduction

Resealed erythrocyte ghosts have often been used to monitor the effects of altered intracellular ligands on transport function. These studies are the foundation of our current understanding of the kinetics of a variety of cation transport systems, perhaps most notably the Na^+–K^+ ATPase (reviewed by Glynn [1]). Surprisingly little use, however, has been made of erythrocyte ghosts to study sugar transporter function, perhaps because of the ease with which intracellular sugars can be replaced or substituted in whole cells by nonmetabolized sugars. In the context of studies of sugar transport, ghost preparations have largely been employed to examine the binding of labelled ligands to specific sites within the transporter. However, Carruthers et al. (for example, Refs. [2,3]) showed that ATP interacts with binding sites on the cytoplasmic surface of GLUT1 to act as a modulator of glucose transport. Raised intracellular ATP levels above 1 mM retard glucose uptake into the human erythrocytes and ghosts by binding to specific ATP binding sites which have been located on the cytoplasmic surface of the transporter [3].

Since the glucose transporter has been purified and its primary sequence determined [4], antibodies have been raised against specific epitopes within the transporter [5]. ELISA binding assays show that only antibodies raised against epitopes facing the cytoplasmic surface of the transporter bind effectively: The extracellular facing loops of the glucose transporter are nonantigenic [6]. Antibodies raised against different GLUT isoforms are currently employed to determine distribution and levels of expression in cell membranes which have been fixed and permeabilized. Usually, commercially available antibodies are uncharacterised for the specific binding epitopes within the transporter. However, specific binding information is available in the case of two anti-GLUT1 monoclonal antibodies raised against the cytoplasmic linker segment between the transmembrane segments 6 and 7 of GLUT1 (amino acids 213–269), and against the C-terminal peptide which is also endofacial (amino acids 457–492 [7]). The

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doi:10.1016/j.bioelechem.2003.07.007

first of these antibodies has been shown to prevent cytochalasin B binding to GLUT1, whereas the antibody to the C-terminal peptide does not.

In this paper, we have estimated glucose transport in human erythrocyte ghosts using radiotracer and optical methods, and assessed the effects of incorporation of these two anti-GLUT1 antibodies, together with a third commercially available. All three antibodies, binding to different epitopes, strongly inhibit glucose uptake, while other antibodies lacking specificity for GLUT have no effect on glucose transport.

2. Methods

Unless otherwise stated, all reagents were obtained from Sigma, Poole, UK. The suspension medium was isotonic phosphate-buffered saline (PBS), adjusted to pH 7.4. Fresh human erythrocytes were obtained by venipuncture, and washed three times in isotonic saline by repeated centrifugation and resuspension.

2.1. Ghost preparation

The procedure for preparation of resealed ghosts follows the methodology of Afzal et al. [8]. Fresh human erythrocytes were washed three times in PBS by repeated centrifugation and resuspension. The cells were lysed by injection of the cells into ice-cold haemolysing medium (pH 3.6), centrifuged at 1300 rpm and loaded with reconstitution medium containing KCl (150 mM), ATP (2 mM) and Tris (25 mM) at pH 10.5. At this stage, the appropriate antibody was added at a 1:50 dilution to the ghosts. They were then resealed by incubation at 38 °C for 45 min. Finally, the resealed ghosts were washed twice in wash medium containing NaCl (150 mM) and ATP (2 mM) at pH7.4. Two antibodies raised against amino acid sequences of 213–269 and 457–492 of the human GLUT1 glucose carrier were the kind gifts of Professor Steve Baldwin, Leeds, UK [7,9]. An additional anti-rabbit GLUT1 antibody was obtained from Biogenesis, Poole, UK. An antibody raised against the regulator portion of the human oestrogen receptor was the gift of Professor Clive Coen, London, UK, and was employed as a negative control [10].

2.2. Photometric monitoring of glucose entry

A method adapted from that introduced by Sen and Widdas [11] and previously described [12] was employed. Aliquots of pre-warmed suspensions of ghosts (7.5 μl) were added to a 1-cm^2 fluorescence cuvette containing 3 ml of PBS supplemented with 50 mM glucose, which had been pre-warmed to the required temperature (35 °C). The suspensions were mixed vigorously and the rate of entry of D-glucose into cells was monitored photometrically, starting within 5 s of mixing, using a Hitachi F2000 fluorescence spectrometer ($E_{ex} = E_{em} = 650$ nm) with a temperature-controlled cuvette. After the initial 2–3 s period of cell shrinkage due to osmotic equilibration, the rate of swelling was a linear function of the rate of glucose uptake into the ghosts. When glucose transport was abolished with high concentrations of cytochalasin B or phloretin, no change in light scattering after the initial osmotic equilibration occurred. The time courses of D-glucose entry were fitted to monoexponential curves of the form $y_t = A(1 - B\exp(Ct))$, where the emission, y_t, is recorded at time, t; the coefficient, A, is a scaling factor that fits the curves to the emission, B and C are the exponential coefficients and t is the time in seconds at which the observed data y_t were obtained.

2.3. Radioisotope fluxes

Samples were added to tubes containing 5 mM unlabelled 2-deoxyglucose and 0.2 μCi ml^{-1} ^{3}H-2-deoxyglucose and incubated in the absence or presence of cytochalasin B (83 μM) at room temperature for 10 min. They were then centrifuged and washed three times in ice-cold $MgCl_2$ wash solution containing $MgCl_2$ (107 mM), MOPS (10 mM) at pH 7.4. Radioactivity was measured by scintillation counting.

3. Statistics

Mean data are presented as the mean ± S.E.M. from n individual determinations, each performed in triplicate. Significant differences were determined using Student's unpaired t test.

4. Results

Glucose uptake into human red cell ghosts was monitored at 35 °C using light scattering. Glucose uptake is much slower than exit therefore such that it can be readily followed at this temperature. Fig. 1 shows the effects of the two specific anti-GLUT1 antibodies on the time course of uptake of glucose from isotonic PBS which had been supplemented with 50 mM glucose. Given that the K_m for glucose uptake into red cells at 35 °C has been reported to be approximately 3 mM, the initial rate of uptake in this experiment reflects near-maximal net transport since at the concentrations employed here, the GLUT1 transporter will be approximately 96% saturated. Fig. 2 indicates that both anti-GLUT1 antibodies could significantly inhibit glucose uptake, and that the magnitude of the inhibition (80%) was roughly similar. In contrast, in resealed ghosts containing the non-specific anti-oestrogen receptor antibody glucose uptake was not altered, precluding a non-specific effect of antibody inclusion in the resealing media on glucose transport. In separate series of experiments (data not shown), neither anti-GLUT antibody was able to inhibit net glucose

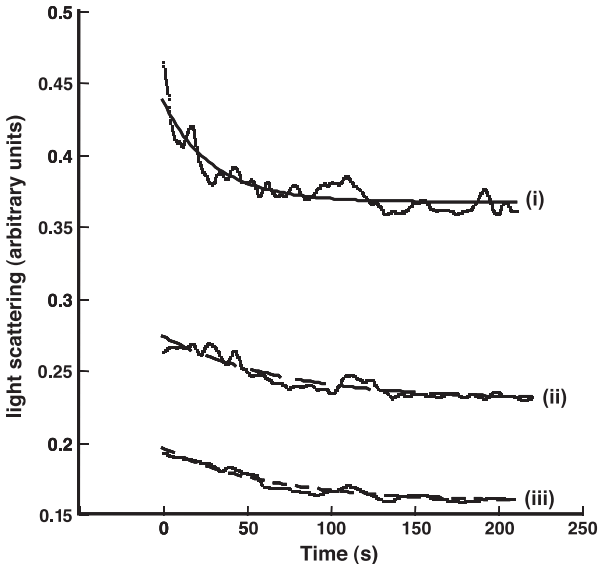

Fig. 1. Volume changes of erythrocyte ghosts associated with glucose uptake, assessed by light scattering. Traces represent ghosts resealed in media (i) lacking antibody; (ii) supplemented with an antibody raised against the TM6-7 linker peptide of GLUT1 (amino acids 213–269); (iii) supplemented with an antibody raised against the C-terminus of GLUT1 (amino acids 457–492). Monoexponential fits of the data yielded k values of 0.033 ± 0.001, 0.014 ± 0.001 and $0.015 \pm 0.001 s^{-1}$, respectively.

efflux from cells preloaded with 100 mM glucose. Fig. 3 presents results from experiments in which the rate of uptake of glucose was assessed by radioisotopic flux methodology. Experiments performed in the absence of cytochalasin B showed no effect of a commercially available antibody on glucose uptake (data not shown). In the presence of cytochalasin B, however, the antibody was

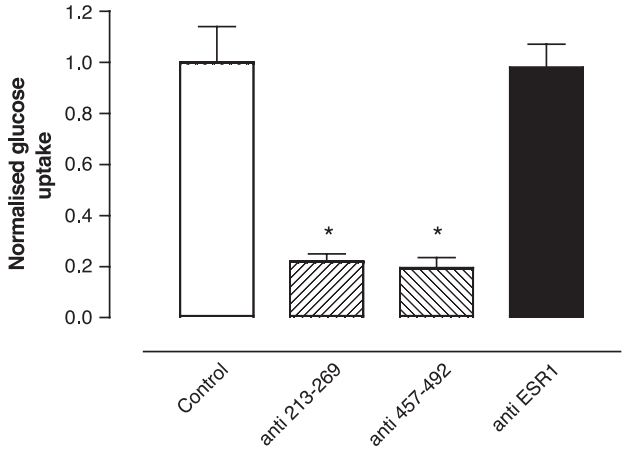

Fig. 2. Rates of glucose entry into erythrocyte ghosts assessed using light scattering measurements of cell volume changes. Rates of glucose entry were normalized to that recorded in ghosts resealed in the absence of antibody. Data are presented for the ghosts containing endofacial anti-GLUT1 antibodies raised against the TM6-7 linker peptide (anti 213–269), or the C-terminus (anti 457–492) and for ghosts containing an antibody against the regulator portion of the human oestrogen receptor (anti ESR1). *$P < 0.001$.

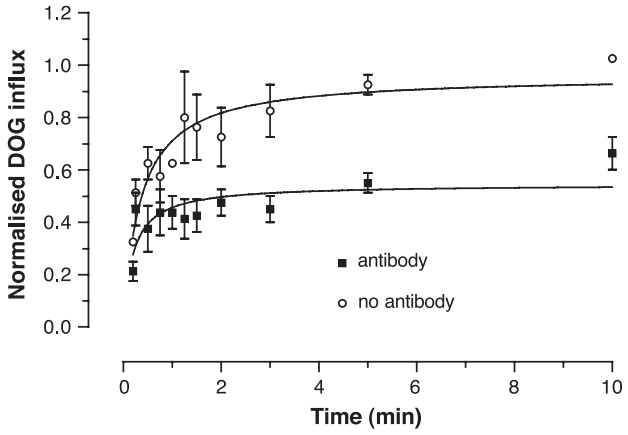

Fig. 3. ^{3}H-2-Deoxyglucose uptake into erythrocyte ghosts resealed in the absence or presence of an anti-GLUT1 antibody. Experiments were performed in solutions supplemented with 83 μM cytochalasin B to retard uptake. Data are the means ± S.E.M. of three separate determinations; results are normalised to fluxes at 10 min recorded in the absence of antibody.

similarly able to reduce ^{3}H-glucose uptake, although the magnitude of inhibition was somewhat smaller (approximately 50%) than that observed with the antibodies raised against specific endofacial peptides. These results indicate that anti-GLUT1 antibodies raised against cytoplasmic regions of the carrier protein have strong inhibitory actions on the net entry of glucose into resealed ghosts.

5. Discussion

It is well-established that the erythrocyte GLUT1 glucose transporter is an asymmetric transporter [13–15]. Recent work by Carruthers et al. [3] has shown that this asymmetry is controlled by the binding of ATP to the GLUT1 protein. This binding results in conformational changes, which induce glucose to accumulate in a vestibular region of the transporter situated within the cytoplasmic domain of GLUT. This leads to the observed asymmetry of glucose transport, with a reduction in both the apparent V_{max} and K_m of net influx, but is without significant effects on glucose exit kinetics or exchange kinetics. Since anti-GLUT antibodies inhibit glucose entry without significantly affecting glucose exit, it can be inferred that they exert their effects by exaggerating the accumulation of glucose within the vestibular domain.

The putative binding site at which ATP exerts inhibitory actions on glucose uptake is reported to comprise a number of endofacial residues. It is of note that the avian erythrocyte glucose carrier in the presence of ATP mediates virtually no net glucose transport, with the carrier effectively behaving as an obligatory exchange transporter [16,17]. However, if ATP is absent from the resealing media of ghosts or ATP-depleted avian erythrocytes are employed, net glucose transport is observed [17,18]. Moreover, the observation

that GLUT4, in which the C-terminal portion of the carrier protein is abridged, fails to demonstrate accelerated glucose transport when operating in exchange mode [19,20] may be of significance in the present context. The finding that both the antibody directed against the C-terminal of GLUT1 and that against the TM6-7 linker peptide can inhibit net glucose efflux is in keeping with previous reports that the C-terminus of GLUT1 is a major determinant of glucose translocation and affinity [21,22] and suggests that these regions of the protein, possibly along with the N-terminal peptide, all contribute to the walls of the cytoplasmic vestibule which constitutes a barrier to glucose equilibration with the cytosolic compartment.

Acknowledgements

This work was supported by The Wellcome Trust, UK, and Arthritis Research Campaign, UK. We thank Professor Stephen Baldwin, University of Leeds, UK, and Professor Clive Coen, King's College, London, UK, for provision of antibodies.

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