

Lack of Aquaporin 3 in bovine erythrocyte membranes correlates with low glycerol permeation

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Running Title: Bovine erythrocytes lack AQP3

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ABSTRACT

In general, erythrocytes are highly permeable to water, urea and glycerol. However, expression of aquaporin isoforms in erythrocytes appears to be species characteristic. In the present study, human (hRBC) and bovine (bRBC) erythrocytes were chosen for comparative studies due to their significant difference in membrane glycerol permeability.

Osmotic water permeability (P_f) at 23 °C was $(2.89 \pm 0.37) \times 10^{-2}$ and $(5.12 \pm 0.61) \times 10^{-2}$ cm s⁻¹ for human and bovine cells respectively, with similar activation energies for water transport. Glycerol permeability (P_{gly}) for human ($(1.37 \pm 0.26) \times 10^{-5}$ cm s⁻¹) differed in three orders of magnitude from bovine erythrocytes ($(5.82 \pm 0.37) \times 10^{-8}$ cm s⁻¹) that also showed higher activation energy for glycerol transport. When compared to human, bovine erythrocytes showed a similar expression pattern of AQP1 glycosylated forms on immunoblot analysis, though in slight higher levels, which could be correlated with the 1.5-fold larger P_f found. However, AQP3 expression was not detectable. Immunofluorescence analysis confirmed the absence of AQP3 expression in bovine erythrocyte membranes.

In conclusion, lack of AQP3 in bovine erythrocytes points to the lipid pathway as responsible for glycerol permeation and explains the low glycerol permeability and high E_a for transport observed in ruminants.

Keywords:

Aquaporin 3; glycerol permeability; water transport; erythrocyte; bovine

INTRODUCTION

Aquaporins belong to a highly conserved group of membrane proteins called the major intrinsic proteins (MIPs) that are involved in the transport of water and small solutes such as glycerol, nitrate and urea [1,2].

The first studies on water transport started in the late fifties on mammalian erythrocytes [3,4]. Evidence for the existence of water channels was based mainly on measures of high osmotic water permeability (inhibited by mercurials) [4,5], on low activation energy for transport [6] and on the ratio of osmotic to diffusion water permeabilities [7]. Only in the nineties the first water channel protein known now as aquaporin 1 (AQP1) was identified in erythrocytes [8] and nowadays many other aquaporins have been recognized in nearly all-living organisms [9].

In mammals, 13 isoforms (named AQP0 to AQP12) have been identified so far. They are differentially expressed in many types of cells and tissues in the body and can be divided into two major groups: those selective for water and functioning as water channels (called orthodox aquaporins) and those permeable to small solutes including glycerol (called “aquaglyceroporins”), emphasizing the essential nature of response to osmolarity and the need for conductance of glycerol. Glycerol, a three-carbon backbone tri-alcohol, is a key component of the majority of phospholipids and an important metabolite. Inside the cell, glycerol is immediately phosphorylated by glycerol kinase, maintaining the inward gradient that drives inward glycerol flux.

AQP1 is mainly found in erythrocytes and renal proximal tubules [10,11]. AQP1 water channels allow water to move freely and bidirectionally across the cell membrane, but exclude all ions including hydroxide, hydronium ions and protons [12], the later being essential to preserve the electrochemical potential across the

membrane. Compared with other mammalian orthodox aquaporins (namely with AQP1), AQP3 is moderately permeable to water, but highly permeable to glycerol and possibly to urea [1]. AQP3 expression has been reported in several mammalian tissues including kidney, epidermis, urinary, respiratory and digestive tracts [11] and in human erythrocytes [13].

Within the animal scale, haemorheological parameters and the composition of the erythrocyte membrane differ among species; for instance, decreased membrane fluidity due to higher sphingomyelin and lower phosphatidylcholine content has been demonstrated in bovine erythrocytes (bRBC) [14] showing remarkable organizational features deviations from humans. Likewise, expression and function of the glycerol channel AQP3 has been detected in human and rat [13] but not in mouse erythrocytes [15]. As for bovines, despite the *AQP3* gene has been identified in the bovine genome [16], very low bRBC glycerol permeability has been reported [17]. Therefore, expression, localization and functional analysis of bRBC AQP3 is still ambiguous.

In the present study, hRBC and bRBC were chosen for comparative studies due to their significant difference in membrane glycerol permeability. Permeabilities to water and glycerol were determined and were used to calculate the activation energy of transport, a valuable parameter indicating the main pathway of permeation, lipid or protein. AQP1 and AQP3 expression were assessed and correlated with the determined permeabilities. The contribution of AQP3 expression to glycerol permeation was further confirmed by immunofluorescence for the human and bovine species.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), anti-AQP1 and anti-AQP3 were from Sigma-Aldrich (St. Louis, MO). Peptide-*N*-glycosidase F (PNGase F) was from Roche (Mannheim, Germany), NP-40 from New England Biolabs (Ipswich, MA). Anti-rabbit IgG-horseradish peroxidase and Alexa Fluor 568 goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and from Molecular Probes (Invitrogen, Carlsbad, CA), Mowiol 4-88 from Calbiochem (Darmstadt, Germany). All reagents were of analytical grade.

Erythrocyte sampling and preparation

Venous blood samples, collected in citrate anticoagulant (2.7 % citric acid, 4.5 % trisodium citrate and 2% glucose), were obtained from healthy human volunteers (Faculdade de Farmácia, Universidade de Lisboa) and from adult bovine animals (INIA, Santarém, Portugal). Animal protocols were approved by the Animal Care Committee of the National Veterinary Authority, following European Union guidelines (N. 86/609/EEC). Erythrocytes' mean volume in isotonic solution was determined using a CASY-1 Cell Counter (Schärfe System GmbH, Reutlingen, Germany) and were 82 and 45 fL for hRBC and bRBC respectively.

Fresh blood was centrifuged at 750 $\times g$ for 5 min at 4°C and plasma and buffy coat were discarded. Packed erythrocytes were washed three times in PBS (KCl 2.7 mM, KH₂PO₄ 1.76 mM, Na₂HPO₄ 10.1 mM, NaCl 137 mM, pH 7.4), diluted to 0.5% haematocrit and immediately used for experiments.

Stopped-flow light scattering experiments

Stopped-flow experiments were performed on a HI-TECH Scientific PQ/SF-53 apparatus, which has a 2 ms dead time, temperature controlled and interfaced with a microcomputer. Experiments were performed at temperatures from 10 °C to 37 °C. For each experimental condition, 11-15 replicates were analysed. For the measurement of the osmotic water permeability (P_f), 100 μ L of 0.5% erythrocyte suspension was mixed with an equal volume of PBS containing 200 mM sucrose as a non-permeable osmolyte to produce a 100 mM inwardly directed sucrose gradient. The kinetics of cell shrinkage was measured from the time course of 90° scattered light intensity at 400 nm until a stable light scatter signal was attained. P_f was estimated by $P_f = k (V_o/A)(1/V_w(osm_{out})_\infty)$, where V_w is the molar volume of water, V_o/A is the initial cell volume to area ratio and $(osm_{out})_\infty$ is the final medium osmolarity after the applied osmotic gradient and k is the single exponential time constant fitted to the light scattering signal [18].

For glycerol permeability (P_{gly}), 100 μ L of 0.5% erythrocyte suspension was mixed with an equal volume of hyperosmotic PBS containing 200 mM glycerol creating a 100 mM inwardly directed glycerol gradient. After the first fast cell shrinkage due to water outflow, glycerol influx in response to its chemical gradient was followed by water influx with subsequent cell reswelling. P_{gly} was calculated as $P_{gly} = k (V_o/A)$, where k is the single exponential time constant fitted to the light scattering signal of glycerol influx [19].

The activation energy (E_a) of water and glycerol transport was calculated from the slope of the Arrhenius plot ($\ln P_f$ or $\ln P_{gly}$ as a function of $1/T$) multiplied by the gas constant R .

All solution osmolarities were determined from freezing point depression on a semi-micro osmometer (Knauer GmbH, Berlin, Germany) using standards of 100 and 400 mOsM.

Osmotic lysis

Bovine P_{gly} was studied by osmotic fragility, using a 0.5% erythrocyte suspension in a glycerol lysis solution (300 mM glycerol, 0.3×PBS, pH 7.4) [20]. Experiments were performed at temperatures ranging from 15 °C to 37 °C. Glycerol influx causes cell lysis that can be monitored as a decrease in light absorption. After the fast initial shrinkage due to water efflux, cells swell and eventually haemolyse as glycerol enters. A 200 µL-aliquot of erythrocyte suspension in glycerol lysis solution was harvested at predetermined times and absorbance at 625 nm was immediately measured on a microplate spectrophotometer (SpectraMax 340, Molecular Devices Corp., Sunnyvale, CA). P_{gly} was determined from the rate constant of haemolysis obtained by a single exponential fit to the absorbance data [20].

Ghosts Preparation

Washed erythrocytes were resuspended in chilled lysis buffer (11 mM Tris-HCl, pH 7.6, containing 1 mM PMSF) at ~1:10 (v:v) ratio as previously described [21]. After centrifugation at 30000 xg for 20 min at 4 °C the opaque buttons of residual leukocyte debris were removed from translucent erythrocyte membranes. Erythrocyte ghosts were kept frozen at -80 °C until use. Protein content was determined by the Bradford method [22].

Electrophoresis and Immunoblotting

Twenty μg of ghost proteins were solubilized in sample buffer (5% SDS (w/v), 5% 2-mercaptoethanol (v/v), 10% glycerol (v/v), 0.01% bromophenol blue (w/v) and 0.0625 M Tris-HCl, pH 6.8) containing 8 M urea, at room temperature for 1 h [23]. Electrophoresis was performed on SDS-containing polyacrylamide gels (12% acrylamide) according to the method of Laemmli [24]. The gels were stained with Coomassie Brilliant Blue R-250.

Western immuno-analysis was performed according to standard methods on a PVDF (polyvinylidene difluoride) Immobilon-P 0.45mm membrane (Millipore; Bedford, MA) using the anti-AQP1 (1:500 dilution) or the anti-AQP3 (1:200 dilution) primary antibodies and the anti-rabbit IgG-horseradish peroxidase (1:5000 dilution) as the secondary antibody. Immunoblots were developed with enhanced chemiluminescence agents (Immobilon Western Chemiluminescent HRP Substrate; Millipore, Bedford, MA) and the images were acquired in a CCD imager ChemiDoc XRS (Bio-Rad; Hercules, CA) using the Quantity One software (Bio-Rad).

N-Deglycosylation of membrane proteins

Ghost proteins were *N*-deglycosylated with PNGase F. Briefly, 20 μg of membrane proteins were mixed with 30 μL of glycoprotein denaturing buffer (0.5% SDS and 1% β -mercaptoethanol) and heated at 100 $^{\circ}\text{C}$ for 10 min. After cooling, 3 μL of 10% NP-40 and 3 μL of 0.5 M sodium phosphate buffer (pH 7.5) were added. Deglycosylation was carried out at 37 $^{\circ}\text{C}$ for 4 h in the presence of 1 U of PNGase F and PMSF (1:100). A parallel assay, without enzyme, was run as a control for possible endogenous hydrolase activities. After enzymatic digestion, proteins were

denatured in electrophoresis sample buffer containing 8 M urea at a ratio of 1:2 (v:v) as described above.

Immunofluorescence assays

Thin films of erythrocytes were air-dried, fixed in chilled methanol for 10 min at -20 °C and stored at -20 °C until use. For immunofluorescence assays, erythrocytes were washed twice with PBS for 5 min and blocked with PBS containing 1% BSA, for 1 h at room temperature. Samples were incubated with anti-AQP3 antibody (1:50 dilution in the above blocking solution) overnight at 4 °C, in a humidified chamber. After two more washes, cells were incubated for 2 h at room temperature with Alexa 568-conjugated anti-rabbit IgG goat secondary antibody (1:200 dilution in blocking solution) in a humidified chamber, were washed twice with PBS and mounted with Mowiol 4-88. Images were acquired on an inverted epifluorescence microscope (Nikon TE2000-S, Tokyo) coupled to a colour video camera. Captured digital images were processed through the image analysis software Image-Pro Plus 7.0 (MediaCybernetics, Bethesda, MD).

RESULTS

Glycerol permeability is much lower in bovine erythrocytes

Membrane permeability to water and glycerol in hRBC and bRBC was assessed by stopped flow spectroscopy or osmotic lysis. Figure 1 depicts typical stopped flow light scatter signals of cell volume changes when gradients of sucrose (water permeability, Figure 1A) and glycerol (Figure 1B) were imposed. The time courses of cell volume changes were used to calculate P_f and P_{gly} , as described in Methods. P_f values obtained at 23 °C were $(2.89 \pm 0.37) \times 10^{-2} \text{ cm s}^{-1}$ and $(5.12 \pm 0.61) \times 10^{-2} \text{ cm s}^{-1}$ (n=5), for hRBC and bRBC respectively. For hRBC, a P_{gly} of $(1.37 \pm 0.26) \times 10^{-5} \text{ cm s}^{-1}$ (n=5) was obtained using the stopped flow technique, while for bovine, a three order lower P_{gly} value was achieved ($(5.82 \pm 0.37) \times 10^{-8} \text{ cm s}^{-1}$ (n=4)) using the osmotic lysis assay (Figure 1C), as no signal of glycerol influx in bovine red cells could be detected within the stopped flow experimental time scale (Figure 1B).

Activation energy indicates different mechanisms of glycerol permeation

The E_a for water and glycerol transport was estimated from an Arrhenius plot. Similar water transport E_a values were obtained for human ($3.40 \pm 0.35 \text{ kcal mol}^{-1}$) and bovine erythrocytes ($4.12 \pm 0.98 \text{ kcal mol}^{-1}$) (Figure 2). However, the E_a for glycerol permeation was different between the human and bovine species (8.52 ± 0.81 and $19.13 \pm 1.65 \text{ kcal mol}^{-1}$). This variation corroborate with the differences in P_{gly} found and suggest that permeation occurs through different pathways: while in human

AQP3 plays an important role, in bovine glycerol permeation occurs mainly through the lipid bilayer with no contribution of protein channels.

Bovine erythrocytes show higher glycosylated AQP1 expression

Membrane proteins obtained from erythrocytes were analyzed by Western blotting after SDS-PAGE (Figure 3). To identify AQP1 the immunoblots were probed with an anti-AQP1 antibody directed against the C-terminal 19 aminoacids of human AQP1 (residues 243-261: KVWTSGQVEEYDL DADDIN; UniProtKB ID: P29972), a region that is completely conserved in bovine AQP1 (residues 245-263; UniProtKB ID: P47865). Immunoblots of hRBC and bRBC, probed with anti-hAQP1, showed the presence of three bands of ~ 53, 40 and 29 kDa as previously reported for the purified forms of this protein [25]. After PNGase F treatment, in both samples (hRBC and bRBC) the bands of higher molecular mass were completely absent yielding a single band of ~ 29 kDa, which might represent the deglycosylated AQP1 as formerly reported [15]. However, and considering that for both samples 20 µg of total protein were loaded in each lane, the Western blot analysis also indicate that the expression level of AQP1 in bRBC is higher than that found in hRBC.

AQP3 is not detected in bovine membrane erythrocytes

To identify AQP3, the immunoblots were probed with anti-AQP3 directed against the C-terminal 18 aminoacids of rat AQP3 (residues 275-292: STEAENVKLAHMKHKEQI; UniProtKB ID: P47862), a region that is highly conserved in human (UniProtKB ID: Q92428) and bovine AQP3 (UniProtKB ID: Q08DE6). Immunoblotting with anti-rAQP3 revealed that AQP3 is abundantly

expressed in hRBC (Figure 3B). In this sample the AQP3 antibody recognized a broad band at ~ 40-50 kDa, which was reduced to 29 kDa after PNGase F treatment, indicating the expression of glycosylated AQP3 in hRBC as previously described [15,26]. In bRBC two faint bands at ~ 40-50 kDa were detected. However, after PNGase F treatment no change in the Western blot profile was observed. These results suggest that AQP3 is absent in bRBC.

To further examine AQP3 membrane localization in hRBC and bRBC, an immunofluorescence analysis was carried out. Figure 4A shows high levels of AQP3 expression in hRBC, consistent with data in the literature [27]. In contrast, AQP3 antibody labelling was not observed in bRBC (Figure 4B), confirming the results achieved by immunoblotting. Negative controls obtained by incubating bRBC directly with the secondary antibody showed no background, validating the specificity of the secondary antibody to anti-AQP3 observed with hRBC. These results confirm the absence of AQP3 in bovine erythrocytes.

DISCUSSION

In general, erythrocytes are highly permeable to water, urea and glycerol. However, expression of aquaporin isoforms in erythrocytes appears to be species characteristic; the chicken and echidna RBCs have no functional AQP1 compared with humans. Our results showed relatively similar P_f values in human and bovine erythrocytes, although the latter being nearly 1.5-fold higher. This same relation has been recently reported in a study that compares the osmotic water permeability from 11 different mammals [28]. The E_a for water transport was low and similar for both hRBC and bRBC confirming the presence of water channels.

To investigate the relevance of AQP3-mediated glycerol transport in both hRBC and bRBC, we assessed P_{gly} using two distinct methods: the rates of volume changes due to glycerol influx in hRBC were estimated using stopped-flow light scattering, while the osmotic lysis method was chosen for bRBC due to the larger time scale needed to observe the much slower glycerol permeation. The P_{gly} obtained for hRBC using the stopped-flow were of the same order of magnitude as those reported for dihydroxy alcohols using this same methodology [29], but were one order larger than the described using rapid filtration with radioactive labeled glycerol [13].

As for bovine erythrocytes and confirming previously reported values [17], the P_{gly} obtained was roughly three orders of magnitude lower than human, pointing to a totally diverse pathway of permeation. This notion is reinforced by the high E_a value found, indicating a lipid- rather than protein-mediated glycerol transport.

Given that the AQP3 protein has been recognized as a main glycerol channel in human and rat erythrocytes [13] and that *AQP3* gene was recently assigned to bovine

chromosome 8 [16], our first approach was to examine AQP3 expression in bRBC and correlate it with the channel function.

Human erythrocyte AQP1 and AQP3 showed the typical profile [15,30], namely the presence of several forms with molecular masses around 50 and 40 kDa that were reduced to ~ 29 kDa after PNGase F treatment, corresponding to the molecular mass of AQP1 (28 kDa) and AQP3 (32 kDa). Since a denaturing gel was used, the glycosylation rather than the oligomeric state could be inferred. When compared to human, bovine AQP1 presented a similar expression pattern of glycosylated forms although in slight higher levels. This subtle difference may explain the 1.5-fold larger P_f found for bRBC relative to hRBC.

On immunoblotting, no AQP3 expression could be detected for bRBC. In human and rat erythrocytes, AQP3 was shown to be highly glycosylated thus masking immunodetection [15]. Upon treatment with glycanase PNGase F, AQP3 was dissociated into its deglycosylated forms visible in hRBC membranes, but it persisted not detectable in samples from bRBC. Consistently, immunofluorescence analysis of AQP3 in bRBC membranes, confirmed the absence of AQP3 expression.

The physiological role of aquaglyceroporins in erythrocytes is still not clear. Absence of AQP3 has also been described in mouse erythrocytes [15], where another aquaglyceroporin isoform (AQP9) plays the role of glycerol channel maintaining the red cells highly permeable to glycerol [26]. The reason why bovine red cells do not contain glycerol channels is not obvious.

Glycerol is a small metabolite, which is important for gluconeogenesis in the liver. During fasting and exercise, up-regulated AQP7 expression in adipocytes facilitates the release of glycerol resultant from triglyceride hydrolysis, which is

transported through the blood and is taken up by the liver via another aquaglyceroporin isoform, AQP9, also up-regulated by fasting [31]. In the hepatocyte, phosphorylation by glycerol kinase is the first step to convert glycerol to glucose. In ruminants, rumen fermentation of dietary carbohydrate results in only small amounts of glucose being absorbed from the gastrointestinal tract and therefore, gluconeogenesis is a major source of glucose [32].

In contrast to liver or kidney, glycerol seems not to be metabolized in erythrocytes. The presence of an aquaglyceroporin in these red cells imply that this channel may additionally be involved in other mechanisms, possibly contributing to volume regulation after exposure to local osmotic stresses. After the initial osmotic shrinkage due to glycerol gradients stimulated by fasting, AQP3 expressing erythrocytes would be enabled to rapidly recover their volume, a similar phenomenon hypothesised for urea when red blood cells cross the kidney medulla [33]. AQP3 can also moderately permeate urea although to a lesser extent [1] and this function might also play a role in volume regulation in hRBC. As for bRBC, besides a high expression level of the facilitated urea transporter [34] coexistent with urea permeability similar to the human specie [28], which itself may improve erythrocyte osmotic fragility, the physiological reasoning for the absence of a glycerol transporter remains ambiguous.

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FIGURE LEGENDS

Figure 1 – Representative permeability assays in human and bovine erythrocytes at 23°C. **(A)** Water permeability assayed by stopped flow: cell shrinkage due to water outflow after an hyperosmotic shock with 100 mM sucrose gradient, **(B)** Glycerol permeability assayed by stopped flow: fast shrinkage followed by cell reswelling due to glycerol uptake after an inwardly directed 100 mM glycerol gradient, **(C)** Glycerol permeability of bovine erythrocytes assayed by osmotic lysis: time course of haemolysis in glycerol lysis solution.

Figure 2 - Activation energy (E_a) of water and glycerol transport for human (gray bars) and bovine (white bars) erythrocyte membranes. Values are mean \pm SD of three independent experiments (n = 3).

Figure 3 – Expression of **(A)** AQP1 and **(B)** AQP3 in erythrocyte membranes. Membrane proteins (20 μ g per lane) from bovine (bRBC) and human erythrocytes (hRBC), with (+) and without (-) PNGase F digestion, were analysed by Western Blotting using **(A)** anti-AQP1 and **(B)** anti-AQP3 antibodies. The molecular masses (kDa) of the molecular weight markers are indicated. Twenty μ g of membrane proteins of hRBC and bRBC were loaded in individual lanes. Observed faint protein bands are indicated (* and **).

Figure 4 - Immunodetection of AQP3 in (A) human and (B) bovine erythrocytes. Erythrocytes were probed with anti-AQP3 antibody followed by Alexa-Fluor-568-conjugated anti-rabbit IgG (red fluorescence) and visualized by fluorescence microscopy. Scale bar, 5 μ m.