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Role of RhAG and AQP1 in NH_3 and CO_2 gas transport in red cell ghosts: a stopped-flow analysis

Rôle des protéines RhAG et AQP1 dans le transport de NH_3 et de CO_2 des stromas érythrocytaires recellés : analyse par fluorimétrie au flux interrompu

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Abstract

To clarify the potential role Rh/RhAG and AQP1 proteins in erythrocyte gas transport, NH_3 and CO_2 transport was measured in erythrocyte ghost membrane vesicles from rare human variants (Rh_{null}, CO_{null},) and knockout mice (homozygous $AQP1^{-/-}$, $Rh^{-/-}$ and $Rhag^{-/-}$) exhibiting well-characterized protein defects. Transport was measured from intracellular pH (pH_i) changes in a stopped-flow fluorimeter. NH_3 transport was measured in chloride-free conditions with ghosts exposed to 20 mM inwardly directed gradients of gluconate salts of ammonium, hydrazine and methylammonium at 15 °C. Alkalinization rates of control samples were 6.5 ± 0.3, 4.03 ± 0.17, 0.95 ± 0.08 s^{-1} for each solute, respectively, but were significantly reduced for Rh_{null} and CO_{null} samples that are deficient in RhAG and AQP1 proteins, respectively. Alkalinization rates of Rh_{null} ghosts were about 60%, 83% and 94% lower than that in control ghosts, respectively, for each solute. In CO_{null} ghosts, the lack of AQP1 resulted in about 30% reduction of the alkalinization rates as compared to controls, but the transport selectivity of RhAG for the three solutes was preserved. Similar observations were made with ghosts from KO mice $Rhag^{-/-}$ and $AQP1^{-/-}$. These results confirm the major contribution of RhAG/Rhag in the NH_3 conductance of erythrocytes and suggest that the reduction of transport rates in the absence of AQP1 would be better explained by a direct or indirect effect on RhAG/Rhag-mediated transport. When ghosts were preloaded with carbonic anhydrase and exposed to a 25 mM CO_2/HCO_3^- gradient at 6 °C, an extremely rapid kinetics of acidification corresponding to CO_2 influx was observed. The rate constants were not significantly different between controls and human variants (125 ± 6 s^{-1}), or between wild-type and KO mice, suggesting no major role of RhAG or AQP1 in CO_2 transport, at least in our experimental conditions.

Keywords: Gas permeability; NH_3 transport; CO_2 transport; Stopped-flow; Red cells; Human and mouse variants

Mots clés : Perméabilité aux gaz ; Transport de NH_3 ; Transport de CO_2 ; Fluorimétrie de flux interrompu ; Érytrocytes ; Variants humains et murins

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1. Introduction

It has been assumed for a long time that permeability of cell membranes to gases is mediated by free diffusion through the lipid bilayer. However, this view has been challenged in recent years as some membranes have an extremely low permeability to NH_3 or CO_2 and specific proteins have been suspected to facilitate gas transport [1–4, and references therein], although this is still a matter of debate.

The RH (Rhesus) blood group system [5–7] is an interesting example, as current studies suggest a role of RhAG (Rh-associated glycoprotein) in red cells, and of RhBG and RhCG homologs in non-erythroid tissues, either in NH_3 or CO_2 transport function (see Transf Clin Biol. 2006, this issue). The physiological significance of NH_3 movement across RBC membranes is unclear but it might be involved in organ detoxification or it might participate in the fine tuning of intracellular pH regulation. It was shown many years ago that the lipid permeability of RBCs to NH_3 is very high [8,9] and we recently demonstrated that at 15 °C about 50% of the NH_3 conductance of RBCs was protein-mediated, by showing that RhAG facilitates NH_3 influx [10]. Other studies suggested that the water channel AQP1 [11,12], which is abundant on RBCs, might also transport NH_3 [3], but this was controversial [13–15].

To get further insights into NH_3 and CO_2 gas transport mediated by red cell membrane proteins, we analyzed the kinetics of intracellular pH (pH_i) changes of resealed ghosts prepared from several human and mouse variants. These variants were selected because they exhibit specific membrane protein defects, thus providing the opportunity to document the relative contribution of each of these proteins to gas transport function. Rh_{null} and CO_{null} RBCs, and their knockout mice counterparts ($Rhag^{-/-}$ and $AQP1^{-/-}$), lack Rh/RhAG [16,17] and AQP1 [18,19], respectively. We also studied other variants such as Jk_{null} or GIL_{null} lack the urea transporter UTB1 [20] or aquaglyceroporin AQP3 [21], whereas Fy_{null} lack the Duffy/DARC membrane protein involved in *Plasmodium vivax* invasion and chemokine receptor [22] and $Kell_{null}$ cells lack the Kell protein, a Zn-dependent endopeptidase of the neprilysin family [23].

2. Flux measurements in red cell ghosts by stopped-flow analysis

Flux rates into RBC ghosts resealed on a buffer medium containing a fluorescent pH-indicator (pyranine, $pK_a = 7.3$) were calculated from pH_i-dependent fluorescence changes measured in a stopped-flow fluorimeter (SFM3, Bio-Logic, Grenoble, France), essentially as described [10]. Data from five to ten time courses were averaged and were fitted to a single exponential function using the Simplex procedure of the Biokine software (Bio-logic) to calculate kinetic rate constants (k, s^{-1}). Kinetic rate constants measured in transport studies strictly reflected differences in permeabilities as human and mouse ghost preparations have the same buffer capacity as they were resealed in the same buffer medium (distinct for NH_3 and CO_2 transport), and the surface/volume ratio are also identical (but different between human and mouse) [10].

There are significant advantages of using resealed RBC ghost membrane vesicles compared to intact RBCs or transfected cells. Ghost vesicles allow specification of composition of the solutions on either side of the membrane, and do not suffer from potential artifacts of overexpression. We made use of ghost vesicles prepared from human RBC variants and RBCs from transgenic mice that lack the major membrane proteins, some with a well-known transport function. The use of null cells rather than transport inhibitors eliminates non-specific inhibitor actions, or uncertainties about whether protein-facilitated gas transport can be inhibited by the same compounds that inhibit non-gas transport.

3. Red cell transport of NH_3 and related solutes

We showed previously that NH_3 movement across the RBC membrane induces a rapid intracellular alkalinization which was mediated approximately equally by lipid diffusion and by the RhAG subunit of the Rh complex [10]. As illustrated in Fig. 1A for CH_3NH_2 influx into control ghosts (ctrlRh+), there was first a rapid alkalinization phase followed by a slower acidification phase ($t_{1/2}$ about 40 s) to return to external pH (pH_0) of the incubation medium. Alkalinization constants were calculated from the initial phase (Fig. 1B). These studies were performed in a medium containing chloride, thus potentially reducing alkalinization rates as Band 3 largely contributed to acidification through its anion exchanger (Cl^-/HCO_3^-) activity. Here, chloride free conditions (NaCl replaced by potassium gluconate) were used to severely restrict Band 3 activity. Moreover, to better estimate solute fractions crossing the RBC membrane via the protein and lipid diffusion pathways, respectively, we used gluconate salts of ammonium, MeA and of hydrazine (pK_a close to 8; diameter about 4.6 Å [24]). We confirmed our previous observations showing a rapid intracellular alkalinization of erythrocyte ghosts. However, the acidification phase was much slower in the absence of chloride ($t_{1/2} > 1000$ s), when Band 3 function is blocked (Fig. 1C as compared to 1A), and this was unlikely to interfere with alkalinization rate constant determination.

The rate constants of alkalinization (k) of ghosts from control Rh-positive cells was 6.5 ± 0.3 s^{-1} for NH_3, 4.03 ± 0.17 for NH_2–NH_2 and 0.95 ± 0.08 s^{-1} for CH_3NH_2 (Table 1), indicating that movement of NH_3 and other solutes across the RBC membrane occurs efficiently when Band 3 function is blocked, as expected. Moreover, addition of DIDS (10 and 100 μM) had no effect on rate constants (data not shown). The transport efficiency estimated by alkaline constant ratios k_{MeA}/k_{NH3} and k_{NH2NH2}/k_{NH3} were 0.15 and 0.66, respectively, indicating that hydrazine was efficiently translocated. Next, we determined the alkalinization kinetics and rate constants of ghosts from human variants with rare phenotypes (Rh_{null}, CO_{null}, Jk_{null}, GIL_{null}, Fy_{null}, $Kell_{null}$,) and KO mice ($Rh^{-/-}$, $Rhag^{-/-}$, $AQP1^{-/-}$). In all cases, a rapid alkalinization was observed similar to that of con-