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K:Cl Cotransport in Red Cells of Transgenic Mice Expressing High Levels of Human Hemoglobin S

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K:Cl cotransport is involved in generating dense red blood cells (RBCs) in homozygotes for HbS (SS). We report on the properties of this transport system in RBCs from control and transgenic mice expressing high levels of human α^H and β^S chains. Unlike human SS RBCs, mouse RBCs incubated in *isotonic* media exhibited a Cl^--dependent K^+ efflux and therefore have a different set-point for activation. This basal efflux was slightly stimulated by cell swelling to values five times smaller than that in human SS cells; in addition, the delay time for activation was shorter in transgenic than in control mice, but fourfold longer than that of human SS cells. These properties cast doubt on the physiological impact of the mouse K:Cl cotransporter on RBC volume regulation in the mouse and suggest that there are intrinsic differences between the human K:Cl cotransporter and the putative transporter in mice. Am. J. Hematol. 55:112–114, 1997. **© 1997 Wiley-Liss, Inc.**

Key words: sickle-cell anemia; cation transport; volume regulation; erythrocytes

INTRODUCTION

Fabry et al. [1,2] have characterized a strain of transgenic mice with high expression of human β^S-globin and human α-globin (α^H). Transgenic mice homozygous for the β^{major} deletion which are referred to as $\alpha^H\beta^S[\beta^{MDD}]$ have 72.5 ± 2.4% of β chains as β^S. The average mean cellular hemoglobin concentration (MCHC) of transgenic mouse red cells (RBCs) is higher than in control mice (38 ± 1 g/dl vs. control, 36 ± 1 g/dl), and sickling occurs in 95% of cells. In addition, the transgenic mice have moderate reticulocytosis [2] due to enhanced RBC destruction.

The K:Cl cotransport system has been studied in RBCs of SS patients and has been implicated in the generation of very dense (dehydrated) and irreversibly sickled cells [3]. This transport system was found to be only minimally expressed in mature RBCs of normal individuals (Hb AA), but very active in young RBCs from AA and SS subjects [4–6]. In the present study we compared the activity and delay time for activation of K:Cl cotransport of RBCs from control (C57BL/6J) and transgenic mice to those from sickle-cell anemia patients.

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MATERIALS AND METHODS

Blood samples were collected from a tail incision in heparinized (100 U/ml) (Elkins-Sinn, Inc., Cherry Hill, NJ) choline washing solution for mice containing (in

Abbreviations: RBCs, red blood cells; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; NEM, N-ethylmaleimide; Hct, hematocrit; SS2, RBCs with densities or MCHCs between 1.076–1.091 g/ml and 33–37 g/dl, respectively; AA cells, RBCs from normal subjects homozygous for hemoglobin A; SS cells, RBCs from patients homozygous for hemoglobin S.

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mM) 160 choline chloride, 0.15 $MgCl_2$, and 10 sucrose and Tris-MOPS, pH 7.4 at 4°C (330 mOsm). Approximately 20 different mice (10 control, 10 transgenic) were used for these experiments, and no mouse was bled more frequently than once a month.

K^+ efflux from mouse RBCs was determined by incubating cells at 1% hematocrit (Hct) in isotonic (330 mOsm) and hypotonic (250 mOsm) media. The media contained (in mM): A) NaCl, 150 [isotonic Cl^-]; B) $NaNO_3$, 150; C) Na-sulfamate (SFa), 150; D) NaCl, 115 [hypotonic Cl^-]; E) $NaNO_3$, 115; F) NaSFa, 115; G) NaCl, 150, NEM, 1; H) $NaNO_3$, 150, NEM, 1; and I) NaSFa, 150, NEM, 1. All media were supplemented with (in mM): 1 ouabain, 1 $MgCl_2$ or 1 $Mg(NO_3)_2$, 0.01 bumetanide, 10 glucose, 10 sucrose, and 10 Tris-MOPS, pH 7.4 at 37°C. Net K^+ efflux from mouse RBCs was started by adding the cells to the prewarmed efflux media and was sampled in duplicates at 0, 5, 10, 25, 40, and 60 min [7]. K^+ efflux was calculated from the nonlinear regression analysis of K^+ concentration vs. time using the equation describing K^+ efflux for the two-state model proposed by Jennings and Al-Rohil (their Equation 7, [8]) as described in [7].

With the exception of solutions containing NEM, hemolysis was found to make a negligible contribution to extracellular K^+. The Cl^--dependent K^+ efflux (K:Cl cotransport activity) was estimated by subtracting the flux in NO_3^- or SFa^- media from that in Cl^- media. Density gradient centrifugation at 30 and 60 min revealed that after a 30-min exposure to NEM most of the unlysed cells had an MCHC of <30g/dl and that after a 60-min exposure to NEM all unlysed cells had an MCHC of <28g/dl.

RESULTS AND DISCUSSION

SS RBCs have a high volume-stimulated K^+ efflux which is Cl^--dependent and which has been identified as K:Cl cotransport (Table I). Previous reports [5,9,10] have shown that swelling-stimulated K:Cl cotransport is highly active in RBCs from SS patients. In addition, K:Cl cotransport activity [4–6,10] and its delay time for activation [7] were shown to be cell age-dependent. Swelling activates this cotransport with a shorter delay time in SS cells than in normal AA cells (1.7 vs. 10.2 min, respectively [7]).

Several protocols for activation of K:Cl cotransport in mouse RBCs were investigated. Initially, measurements of K^+ efflux were performed in isotonic and hypotonic Cl^- media for 15 min to define the volume-stimulated component. This protocol did not show stimulation by cell swelling in normal or high reticulocyte mice; nor was K^+ efflux inhibited when Cl^- was replaced by NO_3^- in either isotonic or hypotonic media, as was the case for human SS cells. Thus, these data indicate that mouse RBCs lack a volume-stimulated K:Cl cotransport. Recently, Armsby et al. [11] reported that mouse RBCs exhibit a volume-stimulated K^+ efflux when the flux was followed for 25 min; furthermore, K^+ efflux into hypotonic media was demonstrated to be Cl^--dependent when Cl^- was replaced by sulfamate. This report led us to further investigate the presence of volume-stimulated K^+ fluxes in normal and transgenic mouse RBCs. In each of these experiments, blood from 4 mice was pooled to follow K^+ efflux for up to 60 min and sulfamate was used to replace Cl^- in isotonic and hypotonic media (Fig. 1).

TABLE I. Cl-Dependent K^+ Efflux From Red Blood Cells of Mouse and SS Patients†

	K^+ efflux, mmol/l cell × hr		
Media	Control mice	Transgenic mice	SS patients [7]
Isotonic Cl^-	9.5 ± 1.6	7.1 ± 1.4	0.3 ± 1.0
Hypotonic Cl^-	11.5 ± 1.2	10.2 ± 1.4	15.5 ± 0.9
Volume-stimulated	2.0 ± 1.8	3.1 ± 1.6	15.8 ± 1.2
Delay time (τ) (min)			
For isotonic conditions	32.7 ± 3.4	28.4 ± 3.1	
For volume stimulation	21.3 ± 3.3	9.0 ± 1.4*	1.7 ± 0.3

†Mean ± SE, n = 3 control, 3 transgenic, and 7 SS patients. For mouse experiments, blood from 2–4 mice was pooled for each case. The number of mouse RBCs per unit volume is approximately twice that of human red cells, but the surface area is only half that of human red cells (D.K. Kaul, personal communication). Therefore, the surface area per liter of cells is approximately equal for both types of cell, and the fluxes are directly comparable.

*$P < 0.03$ when compared to control mice.

Table I summarizes results obtained from measurements of Cl^--dependent K^+ efflux into isotonic and hypotonic media (N = 3). These experiments show that control mouse RBCs, in contrast to human cells, have a Cl^--dependent K^+ efflux into isotonic media with a long delay time that was shortened by about 30% by cell swelling. In RBCs from transgenic mice, cell swelling shortened the delay time for activation of the Cl^--dependent component by about 60%.

Several differences were observed between mouse and human SS RBCs: first, K^+ efflux into isotonic media was nonlinear in mouse RBCs and showed a significant Cl^--dependent component. This pathway is silent in RBCs from SS patients; therefore, it shows that the set-point for activation of the cotransporter is different in mouse RBCs. Second, stimulation of K^+ efflux by cell swelling is 5-fold higher in SS patients. Third, the anion sensitivity to NO_3^- differs from that found for human, rabbit, sheep, and trout [3]. Fourth, the delay time (τ) for activation of K^+ efflux by cell swelling is 4-fold longer in the transgenic mouse than in human SS cells. Fifth, studies on the effect of 1 mM NEM on K^+ efflux showed marked differences with human SS cells. NEM markedly stimulated a K^+ efflux but also induced a significant lysis and

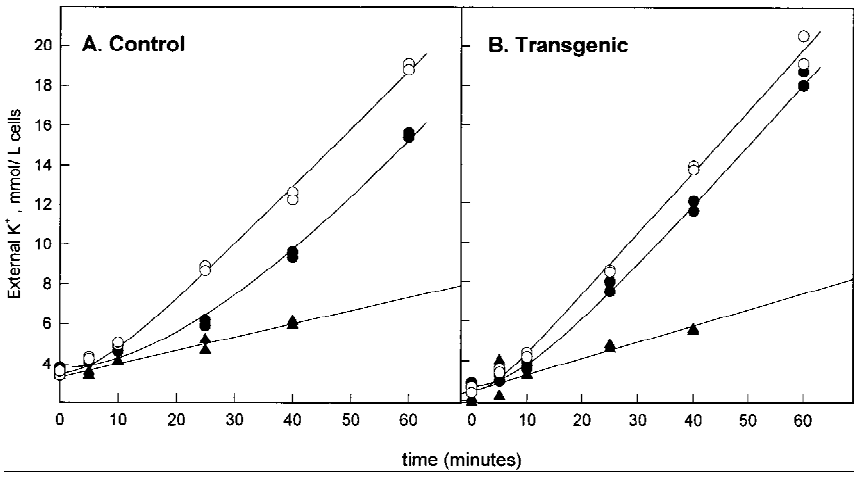

Fig. 1. K^+ efflux from RBCs of control and transgenic mice under isotonic (-●-●-●-) and hypotonic (-○-○-○-) conditions in Cl^- media and in hypotonic sulfamate (-▲-▲-▲-) media. Net K^+ efflux was measured in media containing 1 mM ouabain and 0.01 mM bumetanide, pH 7.4. Solid lines were drawn from values calculated by fitting the experimental points to the minimal two-state model proposed by Jennings and Al-Rohil [8]. Note that in all cases there are at least two and usually three data points from which the rate is calculated. This implies that the observation time of 60 min was long enough to allow calculation of reliable rates and that K^+ efflux and cellular integrity were maintained over the entire observation period.

progressive swelling. Finally, Cl^--dependent K^+ efflux into isotonic and hypotonic media was significantly reduced by preincubation with 300 nM okadaic acid but not by 50 μM DIOA ([(dihydroindenyl)oxy]alkanoic acid) which inhibits human K:Cl (data not shown).

In conclusion, a more detailed analysis of the time dependence of the Cl-dependent K^+ efflux from mouse RBCs showed that the cotransporter is not silent under isotonic conditions and that it has a long delay time for activation. Hypotonic media stimulated this Cl^--dependent K^+ efflux very slightly and slowly, with a shorter delay time in the transgenic (9 min) than in the control (21 min) mice. In all cases, the volume-stimulated component was significantly smaller than in human SS RBCs, and the delay time for activation was significantly longer. These properties of Cl^--dependent K^+ efflux indicate that there are substantial differences between mouse and human SS RBCs, and they cast doubt on its physiological impact on cell volume regulation in the mouse. In human SS RBCs, the high activity and short delay time for activation by cell swelling of this cotransporter implicate it in the generation of dense and irreversibly sickled cells (ISCs) [3].

We speculate that the low activity of this cotransporter and its long delay time for activation may partially protect $\alpha^H\beta^S[\beta^{MDD}]$ mouse RBCs from extreme dehydration. This may account for the small proportion of very dense RBCs found in all transgenic mouse models [2,12] and may also contribute to the absence of adult anemia.

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