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Red Blood Cells of a Transgenic Mouse Expressing High Levels of Human Hemoglobin S Exhibit Deoxy-stimulated Cation Flux*

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Abstract. Deoxy-stimulated cation fluxes have been implicated in the generation of the dense and irreversibly sickled red blood cells (RBCs) in patients homozygous for hemoglobin S (SS). We now report on the effect of short term deoxygenation on K^+ and Na^+ transport in RBCs from control mice (C57Bl/6J) and a transgenic ($\alpha^H\beta^S[\beta^{MDD}]$) mouse line that expresses high levels of human α^H and β^S-chains and has a small percent dense cells but does not exhibit anemia. In transgenic mouse RBCs ($n = 5$) under oxygenated conditions, K^+ efflux was 0.22 ± 0.01 mmol/L cell × min and Na^+ influx was 0.17 ± 0.02 mmol/L cell × min. Both fluxes were stimulated by 10 min deoxygenation in transgenic but not in control mice. The deoxy-stimulated K^+ efflux from transgenic mouse RBCs was about 55% inhibited by 5 nM charybdotoxin (CTX), a blocker of the calcium activated K^+-channel. To compare the fluxes between human and mouse RBCs, we measured the area of mouse RBCs and normalized values to area per liter of cells. The deoxy-simulated CTX-sensitive K^+ efflux was larger than the CTX-sensitive K^+ efflux observed in RBCs from SS patients. These results suggest that in transgenic mice, deoxygenation increases cytosolic Ca^{2+} to levels which open Ca^{2+}-activated K^+ channels. The presence of these channels was confirmed in both control and transgenic mice by clamping intracellular Ca^{2+} at 10 μM with the ionophore A23187 and measuring Ca^{2+}-activated K^+ efflux. Both types of mouse had similar maximal rates of CTX-sensitive, Ca^{2+}-activated K^+ efflux that were similar to those in human SS cells. The capacity of the mouse red cell membrane to regulate cytosolic Ca^{2+} levels was examined by measurements of the maximal rate of calmodulin activated Ca^{2+}-ATPase activity. This activity was 3-fold greater than that observed in human RBCs thus indicating that mouse RBC membranes have more capacity to regulate cytosolic Ca^{2+} levels.

In summary, transgenic mouse RBCs exhibit larger values of deoxy-stimulated K^+ efflux and Na^+ influx when compared to human SS cells. They have a similar Ca^{2+}-activated K^+ channel activity to human SS cells while expressing a very high Ca^{2+} pump activity. These properties may contribute to the smaller percent of very dense cells and to the lack of adult anemia in this animal model.

Key words: Sickle cell anemia — Cation transport — Volume regulation — Erythrocytes — Calcium pump

Introduction

Transgenic mouse lines expressing the human α and β^S globin genes have aroused considerable interest in sickle cell anemia research because they allow testing of hypotheses and permit experimental interventions that are not possible in humans. Fabry et al. [13, 15] described a strain of transgenic mice with high red cell expression of human β^S-globin and human α-globin (α^H) and reduced levels of mouse globins. This line of transgenic mice, which has two co-integrated transgenes, the human β^S and α2 globin genes linked to the β-globin locus control region, was bred with mice carrying a deletion of the mouse β^{major} globin gene (Hbb^{d3th} [32]). 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 the β-chains as β^S. The ratio of α^H- to β^S-globin is 0.73 for

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these mice. The average mean cellular hemoglobin concentration (MCHC) of transgenic mouse red blood cells (RBCs) is higher than in control mice (38 ± 1 g/dL *vs.* control 36 ± 1 g/dL) and sickling occurs in 95% of the cells after slow deoxygenation. The transgenic mouse does not exhibit adult anemia but it does have some abnormal hematological features: moderately increased red cell density and reticulocytosis with increased stress reticulocytes [15].

A unique property of RBCs from sickle cell anemia patients [homozygous for hemoglobin S (SS)] is stimulation of downhill passive K^+, Ca^{2+} and Na^+ fluxes by deoxygenation [12, 22, 29, 34, 35]. The deoxy-stimulated K^+ efflux is partially driven by opening of Ca^{2+}-activated K^+ channels [1, 2, 5, 28, 38]. Young and mature human RBCs have Ca^{2+}-activated K^+ channels [1, 2, 4, 5, 28, 31, 33, 38] which open when Ca^{2+} influx into RBCs increases markedly with deoxygenation. This results in red cell dehydration and is particularly important in SS red cells because the rate and to a lesser degree the extent of the polymerization of human hemoglobin S (HbS) under deoxygenated conditions depends on the initial MCHC [11]. Hence, the activity of membrane transporters which decrease RBC volume and increase the MCHC will enhance HbS polymerization. Another transport system which has been implicated in the generation of the very dense and irreversibly sickled cells is the K:Cl cotransport system [3, 6, 20].

The first rationale for examining K^+ transport in the transgenic mouse RBC is to determine whether or not the presence of HbS is sufficient to elicit a deoxy-stimulated K^+ efflux. The second rationale is understanding the mechanisms leading to a mildly elevated MCHC and the absence of very dense cells and anemia observed in transgenic mouse lines [13]. Transgenic mouse RBCs may differ substantially from SS RBCs because mouse RBCs may not express the same ion transport systems that are present in human red cells. Large species differences have been described for the activities of Na^+-K^+ pump, Na^+-K^+-$2Cl^-$ cotransport, Na^+/H^+ exchange, glucose transporter, Ca^{2+} pump and Ca^{2+}-activated K^+ channels in RBCs from human, dog, sheep, cat, pig, and rat.

In the present study we found that only transgenic mice exhibit a deoxy-stimulated Na^+ influx and K^+ efflux that was CTX in inhibitable. Evidence is provided for the presence of Ca^{2+}-activated K^+ channels inhibited by CTX and for very high activity of the membrane Ca^{2+}-ATPase that may protect mouse RBCs expressing HbS from dehydration.

Materials and Methods

Preparation of Red Blood Cells

Sickle Cell Anemia Patients

Blood was drawn from homozygous sickle cell anemia (SS) patients followed in the Heredity Clinic of the Bronx Comprehensive Sickle Cell Center after informed consent. The hemoglobin (Hb) phenotype of all patients was characterized by a combination of cellulose acetate (pH 8.6) and citrate agar (pH 6.4) electrophoresis and a solubility test for HbS. Cells were then density separated into four fractions on a Percoll (colloidal silica coated with polyvinylpyrrolidone: Pharmacia Fine Chemicals, Piscataway, NJ) and Larex (arabinogalactan polysaccharide; Larex International, Roseville, MN) gradient as previously described [14, 16]. We have studied the discocyte fraction (SS2, density (d) = 1.076 to 1.093 g/mL) which contains reticulocytes and young cells with a mean cellular hemoglobin concentration (MCHC) between 31 and 37 g/dL [14]. On the Percoll-Larex gradient, the white blood cells (buffy coat) form a distinct band which is well separated from the red cells and is easily removed. The isolated SS2 fraction was washed twice with cold preservation solution containing (mM) 135 KCl, 15 NaCl, 10 Tris-MOPS, pH 7.4 at 4°C when red cells were to be used on the next day or shipped to Boston. Red blood cells (RBCs) that were used the same day were washed 4 times with cold choline washing solution (CWS) containing (mM) 149 choline chloride, 0.15 $MgCl_2$ and 10 Tris-MOPS, pH 7.4 at 4°C. A cell suspension of approximately 50% hematocrit (Hct) was made in CWS to measure the exact Hct, Hb, cellular K^+ and Na^+ content. A 50 μL sample of the cell suspension in CWS was lysed (1/50 dilution) with Acationox detergent (0.02% in double distilled water) for Hb, K^+ (1/500 dilution) and Na^+ (1/50 dilution) determinations. Cellular K^+ and Na^+ were determined with a Perkin Elmer 3030B atomic absorption spectrophotometer (Norwalk, CT) using appropriate standards. The initial Hb concentration was estimated by measuring the Hct and the optical density of Hb at 540 nm after conversion to the cyanmet form. All experiments on blood from SS patients were performed within 24 hr of venipuncture.

Control and Transgenic Mice

Blood samples were collected from a tail incision in heparinized (100 U/mL) [Elkins-Sinn, Cherry Hill, NJ] choline washing solution for mice containing (mM) 160 choline chloride, 0.15 $MgCl_2$, 10 sucrose and Tris-MOPS, pH 7.4 at 4°C (330 mOsm). For all mouse experiments the isotonic osmolarity was adjusted to 330 mOsm which is the value which we measured in mouse plasma and is reported in the literature [8]. Approximately 0.5 mL of blood was obtained from each mouse and washed twice in the washing solution for mouse. RBCs were then suspended at approximately 30% Hct in washing solution for measurements of cellular K^+, Na^+, Hct and Hb as mentioned in the previous section for SS patients. Ion transport experiments on mouse RBCs were always preformed on the day the blood was collected. Approximately 60 different mice (30 control, 30 transgenic) were used for these experiments and no mouse was bled more frequently than once a month.

Reticulocyte Count in Mice

Reticulocytes were determined by suspending aliquots of cells in centrifuged plasma at 50% Hct. Equal volumes of blood and new methylene blue reticulocyte stain were mixed and the samples were allowed to incubate at room temperature for at least 10 min after which smears were made for counting.

Area Measurements

The maximum sphering without hemolysis was determined for C57Bl and transgenic mice and was found to be 160 and 140 mOsm respectively. Red cell diameter was measured at maximum sphering using an electronic image shearing device with an optico-electronic magnification of 3700× (Model 907, Instruments for Physiology and Medicine,

La Jolla, CA) and the area was calculated from the diameter using the formula for the area of a sphere. Thirty cells from each type of mouse were measured and, since the cells were directly observed, the small number of deformed cells which did not sphere were excluded. We found that control mouse RBCs had a surface area of 69.2 ± 5.7 μm^2 (mean ± SD) and transgenic mouse RBCs had a surface area of 64.1 ± 5.8 μm^2 (Table 1).

Measurements of Deoxy-Stimulated Cation Fluxes

K^+ Efflux from RBCs of SS Patients

K^+ efflux under deoxygenated conditions was determined by incubating washed RBCs from SS patients in Na^+ media containing (mM): 140 $NaNO_3$, 10 Tris-Mops pH 7.4 at 37°C, 0.1 ouabain, 10 glucose, 1 $CaCl_2$, 0.15 $Mg(NO_3)_2$ and 0.01 bumetanide. A sealed flask containing the Na^+ media was deoxygenated for 20 min at 37°C with bubbling nitrogen; at the end of 20 min 100 μL of RBCs, Hct 50%, were injected into the sealed flask and gently mixed by hand. Initial rates of K^+ loss were used to calculate K^+ efflux by sampling the efflux media with a syringe in duplicate at various time points for 15 min in the presence or absence of 5 nM CTX. During the incubation period the flask had a continuous supply of N_2 although it was not bubbling the media. The samples were then pipetted into 1.5 mL ice-cold Eppendorf tubes containing 0.4 mL of dibutylphthalate, centrifuged for 10 sec in a Fisher microcentrifuge. The supernatants were removed for K^+ measurements by atomic absorption spectrophotometry. K^+ efflux was calculated from the slope of the regression line of K^+ concentration versus time taking into account the volume of red cells used. The slopes ± SE of the linear regressions were calculated with the Enzfitter software for the PC. To measure the K^+ efflux under control conditions, RBCs were treated in a similar manner except that the media was oxygenated by air.

K^+ Efflux from Mouse RBCs

K^+ efflux under deoxygenated conditions was determined by incubating mouse RBCs in Na^+ media containing (mM): 150 NaCl, 10 Tris-Mops pH 7.4 at 37°C, 0.5 ouabain [to inhibit the Na^+ pump], 10 glucose, 10 sucrose, 0.15 $MgCl_2$, 0.01 bumetanide [to inhibit Na^+:K^+:$2Cl^-$ cotransport] and 1 $CaCl_2$. Measurements under deoxygenated conditions were performed as described above and shown on Fig. 3. In a single experiment, deoxy-stimulated K^+ efflux from mouse RBCs was also studied in the absence or presence of 100 μM clotrimazole.

Na^+ Influx into Mouse RBCs

Na^+ influx under deoxygenated conditions was determined by incubating mouse RBCs in the same Na^+ media as for K^+ efflux in mice. Deoxygenation of the media was performed as above. Initial rates of Na^+ gain were used to calculate Na^+ influx by sampling with a syringe the influx media in duplicates at 1, 15 and 30 min in the presence or absence of 5 nM CTX. During the incubation period the flask had a continuous supply of nitrogen although it was not bubbling the media. The aliquots were placed in 1.5 mL ice-cold Eppendorf tubes containing 0.2 mL of dibutylphthalate and 0.4 mL choline washing solution for mice and immediately centrifuged for 10 sec in a microcentrifuge to separate the cells from the Na^+ incubation media and avoid subsequent cell washings. The choline washing solution was used to dilute the sodium concentration of the influx media and to reduce the trapping of sodium in the cell pellet. The supernatant was removed by aspiration and the tubes wiped to remove external sodium contamination. The bottom of the tubes were cut with a microcentrifuge tube cutter (Research Products, Mount Prospect, Illinois) into 3 mL plastic tubes containing 1 mL of 0.02% Acationox to lyse the cells. The tubes were vortexed vigorously. The cell Na^+ was estimated from Na^+ measurements of the lysate by atomic absorption and corrected for Hb concentration of cell lysate. Sodium influx was then calculated from the slope of the linear regression of the cellular Na^+ *vs.* time. The slopes ± SE of the linear regressions were calculated with the Enzfitter software. To measure the Na^+ influx under control conditions, RBCs were treated in a similar manner as for deoxygenated conditions except that media was oxygenated with air.

Measurements of Ca^{2+}-Activated K^+ Efflux in RBCs

SS Patients

The maximal activity of Ca^{2+}-activated K^+ efflux was determined under optimized conditions in the presence of the calcium-ionophore A23187 as previously described by Wolff et al. [38] in the presence and absence of 5 nM charybdotoxin (CTX). CTX is a small polypeptide that blocks Ca^{2+}-activated K^+ channels in human RBCs with high affinity. The ionophore A23187 was used to increase and clamp cytosolic ionized Ca^{2+} to 5 μM. Net K^+ efflux was determined by incubating cells in a Na^+ media containing the A23187 (60 μmol/L of red cells). Cytosolic ionized Ca^{2+} was buffered at <0.1 nM Ca^{2+} with 1 mM EGTA or at 5 μM Ca^{2+} with 1 mM citrate to maximally stimulate the Ca^{2+}-activated K^+ efflux.

Control and Transgenic Mice

To study Ca^{2+}-activated K^+ efflux from mouse RBCs, net K^+ efflux was measured in the presence A23187, as above. Red blood cells were incubated in Na^+ media containing (mM): 150 NaCl, 10 Tris-Mops pH 7.4 at 37°C, 0.5 ouabain, 10 glucose, 10 sucrose, 0.15 $MgCl_2$, 0.1 bumetanide, 1 EGTA or 1 Tris-citrate, total $CaCl_2$ either zero of 50 μM, A23187 (60 μmol/L of red cells), at 1% hematocrit. This gives a cytosolic ionized Ca^{2+} of <0.1 nM Ca^{2+} with 1 mM EGTA or 10 μM Ca^{2+} with 1 mM citrate. An aliquot from a 1.9 mM ionophore stock solution in DMSO (protected from light) was added to the media just before the initiation of the efflux measurements. The transport reaction was initiated under dim light by adding the cells to prewarmed media. Initial rates of K^+ loss were used to calculate K^+ efflux in RBCs by sampling the efflux media in duplicates at 1, 2, 3.5, and 5.0 min in the presence or absence of 5 nM CTX. In experiments where the Ca^{2+} was reduced to <0.1 nM, the samples were taken at 1, 3.5, and 5 min. The samples were pipetted into 1.5 mL ice-cold Eppendorf tubes containing 0.4 mL of dibutylphthalate microcentrifuge and treated as above for measurements of deoxy-stimulated cation fluxes. We have chosen 10 μM cytosolic ionized Ca^{2+} to maximally stimulate the Ca^{2+}-activated K^+ efflux to compensate for the higher Ca^{2+}-ATPase seen in mouse RBC membranes when compared to human. We have demonstrated that 10 μM extracellular Ca^{2+} results in maximal stimulation by increasing extracellular Ca^{2+} to 100 and 150 μM in the presence of A23187 and the appropriate buffers. This did not result in any further increase in Ca^{2+}-stimulated K^+ efflux.

The total calcium concentration of the flux media was measured by atomic absorption spectrophotometry using calcium standards (EM Sciences, Cherry Hill, NJ) in sodium media. A custom made computer program was used to calculate the Ca^{2+} concentrations using the dissociation constant and correcting for ionic strength at pH 7.4 and 0.15 mM $MgCl_2$ as previously described [38].

CTX is obtained from the venom of *Leiurus quinquestriatus* (Latoxan Scorpio Farm, Rosans, France) and was stored at −20°C as

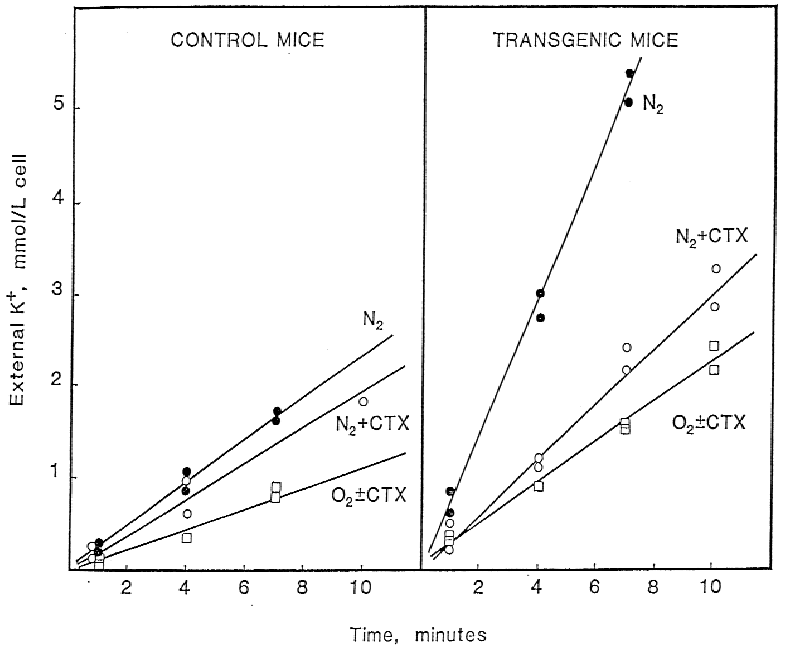

Fig. 1. Effect of deoxygenation on K^+ efflux from a control and a transgenic mouse in the presence and absence of charybdotoxin (CTX). Deoxygenation was induced by bubbling the NaCl media with N_2 as indicated in Materials and Methods. K^+ efflux is low under O_2 (air) conditions (—□—□—□—) and is not different when CTX is present. When RBCs are incubated in deoxygenated media (—●—●—●—) K^+ efflux is stimulated significantly more in transgenic than in control mice in this experiment. In the presence of 5 nM CTX, this K^+ efflux is inhibited (—○—○—○—). These are single experiments which are representative of at least 5 other experiments in 5 different control and 6 different transgenic mice; average values are presented in Fig. 2.

previously described [38]. CTX is an extremely stable molecule which maintained its activity stored frozen in isotonic NaCl solution at 3 μM.

Ca^{2+}-ATPase Activity of RBC Membranes

Preparation of RBC Membranes

Packed RBCs were washed twice in (mM); 120 KCl and 20 Tris-Cl^-, pH 6.8 at 25°C. Calmodulin-free membranes were isolated from RBCs by hypotonic lysis in a solution containing (mM); 10 K^+-EDTA and 20 Tris-Cl^-, pH 6.8 at 25°C and centrifuged in a Sorvall RC2-B centrifuge at 10,000 RPMs for 20 min. The supernatant was discarded and the membranes collected and washed in the hypotonic lysis solution. The membranes were then washed twice with 10 mM Tris-HEPES, pH 7.2 at 25°C and twice with a solution containing (mM); 120 KCl, 0.05 $CaCl_2$, 1 $MgCl_2$ and 30 Tris-HEPES pH 7.2 at 25°C. The hemoglobin-free membrane suspensions were stored in Eppendorf tubes at −70°C until ready to assay.

Ca^{2+}-ATPase Activity Assay

The ATPase activity was determined in an assay system that is coupled to the consumption of NADH (1:1 coupling with ATP) and recorded as a reduction in absorbance at 340 nm in a spectrophotometer at 37°C as described by Vezzoli et al. [37]. The assay was performed by incubating in triplicate each membrane preparation (20 μL) in a media that contained (mM); 120 KCl, 1 $MgCl_2$, 60 Tris-HEPES, pH 7.2 at 25°C, 0.5 K-EDTA, 0.5 K-EGTA and 0.04% Saponin for 20 min at 37°C. Subsequently, the following substrates were added: 0.5 mM phosphoenolpyruvate, 1 U/mL lactate dehydrogenase, 2 U/mL pyruvate kinase, 0.4 mM NADH and 1 mM ATP to the assay media and incubated for 10 min. $CaCl_2$ at final concentration of 0.75 mM was then added to the assay media incubated for 5 min and calmodulin (2 μg/mL) added. The concentration of the substrates were adjusted for maximal activities under our conditions. A continuous recording of the absorbance at 340 nm was taken for 30 min. A linear calcium-ATPase activity was reached within approximately 20 min. Membrane proteins were determined by the Lowry method using BSA as standard. The calmodulin-stimulated ATPase activity was expressed as nmol P_i liberated/mg protein × min.

Abbreviations

RBCs	Red Blood Cells
CTX	Charybdotoxin
Hb	Hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
NEM	N-ethylmaleimide
Hct	Hematocrit
SS2	RBCs with densities and MCHCs between 1.076 to 1.093 g/mL and 31 to 37 g/dL respectively
AA cells	cells from normal subjects homozygous for hemoglobin A
SS cells	cells from patients homozygous for hemoglobin S

Results

Effect of deoxygenation on K^+ Efflux from RBCs of Control and Transgenic Mice

We studied the initial rates of K^+ loss over a 10-min period and used these results to calculate K^+ efflux from mouse RBCs deoxygenated with N_2 in the presence and absence of CTX. Figure 1 shows the effect of deoxygenation on K^+ efflux from RBCs of control and transgenic mice. Basal K^+ efflux from a control mouse RBCs (Fig. 1, left panel) maintained in oxygenated media was 0.16 ± 0.02 mmol/L cell × min. This value was not significantly different when K^+ efflux was measured in the presence of 5 nM CTX. When control mouse RBCs

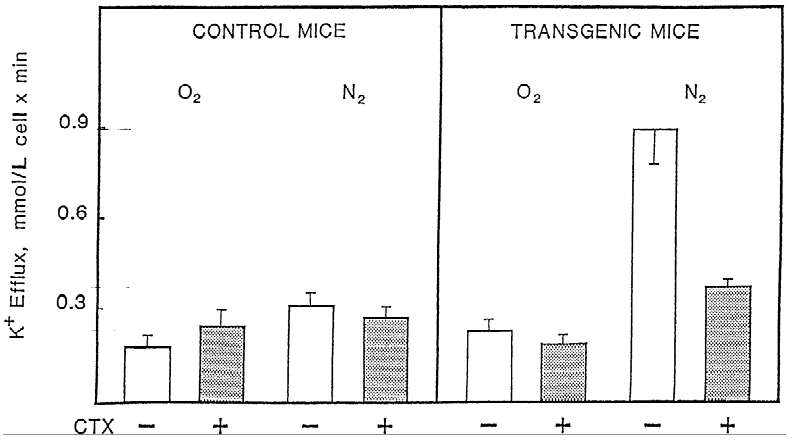

Fig. 2. Summary of the effect of deoxygenation on K^+ efflux in RBCs from control and transgenic mice. Deoxygenation significantly increased K^+ efflux in transgenic but not control mouse RBCs ($P < 0.01$, ANOVA). In transgenic mouse RBCs the deoxy-stimulated K^+ efflux was significantly reduced by 5 nM CTX ($P < 0.01$, ANOVA). Data is expressed as mean ± SE of $n = 5$ and 6 different control and transgenic mice, respectively, in which all parameters were measured from the same RBC preparation.

were incubated under deoxygenated conditions, K^+ efflux increased to 0.24 ± 0.04 mmol/L cell × min. CTX did not significantly decrease K^+ efflux from RBC of control mice under deoxygenated conditions (0.20 ± 0.06 mmol/L cell × min).

In RBCs of a transgenic mouse (Fig. 1, right panel), K^+ efflux under oxygenated conditions was 0.22 ± 0.01 mmol/L cell × min in the absence of A23187 and was not significantly affected by the presence of 5 nM CTX. However, deoxygenation significantly enhanced K^+ efflux from 0.22 ± 0.01 to 0.74 ± 0.04 mmol/L cell × min. The presence of CTX markedly blocked deoxy-stimulated K^+ efflux to 0.31 ± 0.02 mmol/L cell × min. Figure 2 summarizes and compares the results of the effect of low oxygen tension on K^+ efflux obtained in 5 control and 6 transgenic mice. It can be seen that the effect of deoxygenation on K^+ efflux is greater in RBCs from transgenic than from control mice. Under deoxygenated conditions there was a significant stimulation of K^+ efflux in transgenic ($P < 0.01$, ANOVA) but not in control mouse RBCs (ANOVA) when compared to oxygenated conditions. In oxygenated media, K^+ efflux was not inhibited by CTX in control or transgenic mice. However, under deoxygenated conditions K^+ efflux was inhibited significantly ($P < 0.01$, ANOVA) by CTX in the transgenic mice.

In a single experiment, clotrimazole was added at a concentration of 100 μM to transgenic mouse RBC which were then deoxygenated; in the absence of clotrimazole an efflux of 0.67 ± 0.04 mmol/L cell × min was observed while the presence of clotrimazole markedly blocked deoxy-stimulated K^+ efflux to 0.35 ± 0.02 mmol/L cell × min.

Since transgenic mouse RBCs are smaller than human SS cells (Table 1), we have normalized our values to area per liter of cells to compare the fluxes between these two cells types. Figure 3 shows this comparison. In deoxy-conditions, RBCs from SS patients showed a significant increase in K^+ efflux when compared to oxygenated conditions ($P < 0.01$, ANOVA). The total K^+ efflux from transgenic mouse RBC under deoxygenated conditions was higher than that in SS patients. In both cell types, deoxy-stimulated K^+ efflux was significantly inhibited by CTX ($P < 0.01$, ANOVA) and the transgenic mice had higher CTX-insensitive flux values. Therefore, the percent of CTX-sensitive, deoxy-stimulated K^+ efflux was similar in transgenic mice and in SS patients. Note also that the deoxy-stimulated K^+ efflux was more than 10 times smaller than the maximal Ca^{2+}-activated K^+ efflux in both SS patients and transgenic mice.

Table 1. Hematological properties of SS patients and mice

	Retics[a] %	Red cells/ mL × 10^{10}	Surface area[a] μm^2
Human SS	15.0 ± 6.0	1.1	135 ± 10
Control mice	3.4 ± 1.0[b]	2.29	69.2 ± 5.7
Transgenic mice	6.7 ± 1.7[b]	2.29	64.1 ± 5.8

[a] Mean ± SD; $n = 30$. [b] Fabry ME, Sengupta A, Suzuka SM, Constatini F, Rubin EM, Hofrichter J, Christoph GW, Manci EA, Culberson D, Factor SM, Nagel RL: A second generation transgenic mouse model expressing both hemoglobin S and HbS-Antilles results in increased phenotypic severity. *Blood* **86:**2419–2428, 1995.

Effect of Deoxygenation on Na^+ Influx into RBCs from Control and Transgenic Mice

Because deoxygenation has been shown to increase net Na^+ influx into RBCs from SS patients [22, 34], we tested whether this pathway was present in RBCs from control and transgenic mice. Figure 4 shows a summary of these findings. In control mice there was a small but significant ($P < 0.05$, ANOVA) increase in Na^+ influx under deoxygenated conditions when compared to oxygenated conditions. In transgenic mice, Na^+ influx was greatly enhanced (0.8 mmol/L cells × min, $P < 0.03$, ANOVA) and was larger than control mice (0.3 mmol/L cells × min, $P < 0.05$, nonparametric analysis). These

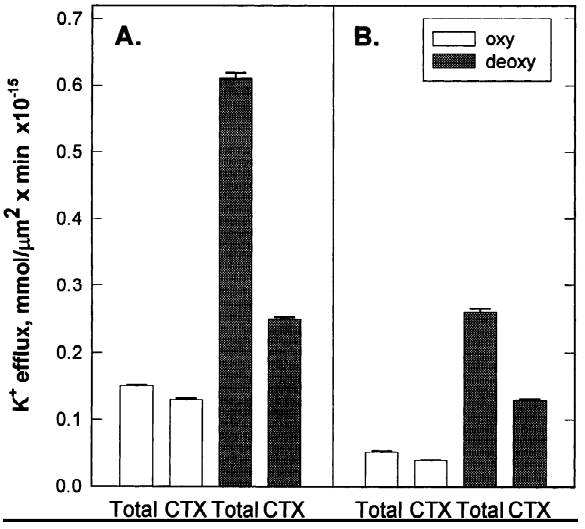

Fig. 3. Comparison of deoxy-stimulated K^+ efflux between RBCs from transgenic mice (*A*) and SS patients (*B*). In both cell types, deoxygenation significantly increased K^+ efflux and was significantly inhibited by 5 nM CTX ($P < 0.01$, ANOVA). There was no significant difference between the fluxes in transgenic mice and SS patients. Results are normalized to area per liter of cells and are expressed as mean ± SE of $n = 6$ and 4 different transgenic mice and SS patients, respectively, in which all parameters were measured from the same RBC preparation. Media was deoxygenated as mentioned in Materials and Methods.

Na^+ fluxes were insensitive to CTX because this peptide did not significantly affect Na^+ influx under oxygenated or deoxygenated conditions in control or transgenic mouse RBCs.

Ca^{2+}-activated K^+ Efflux from RBCs of Control and Transgenic Mice

Human RBCs possess a Ca^{2+}-activated, charybdotoxin (CTX) sensitive K^+ efflux [4, 38]. To determine the presence of this channel in mouse RBCs, the maximal activity (V_{max}) of Ca^{2+}-activated K^+ efflux was measured by clamping cytosolic Ca^{2+} at 10 μM using A23187 and sampling the efflux media for K^+ concentration during 5 min (Fig. 5). The K^+ appearing in the media was normalized per volume of cells added to the media. In the experiment of Fig. 1, the presence of 10 μM cytosolic Ca^{2+} and A23187 induced a K^+ efflux of 8.69 ± 0.57 mmol/L cell × min; we call this the total flux (increasing extracellular Ca^{2+} to 100 and 150 μM in the presence of A23187 and the appropriate buffers did not result in a further increase). This efflux was reduced to 3.78 ± 0.19 when mouse RBCs were incubated with 5 nM CTX in the presence of 10 μM cytosolic Ca^{2+} and A23187. We tested that 5 nM CTX induced maximal inhibition. When cytosolic Ca^{2+} was buffered at <0.1 nM with EGTA, in the presence of A23187, the K^+ efflux was significantly reduced to 0.35 ± 0.14 mmol/L cell × min as shown in Fig. 5. The difference between the total flux and the flux in the presence of EGTA is 8.34 mmol/L cell × min for this case and is called the Ca^{2+}-activated K^+ efflux. The difference between Ca^{2+}-activated K^+ efflux in the presence and absence of CTX yields the Δ-CTX sensitive fraction which in this experiment was 4.56 mmol/L cell × min. These experiments measure the maximal rate of Ca^{2+}-activated K^+ efflux because the flux did not increase when the cytosolic calcium was clamped at 100 and 150 μM.

Table 2 summarizes the results of the Ca^{2+}-activated K^+ efflux measurements in RBCs from 3 control and 3 transgenic mice in the absence and presence of CTX. In both cell types, the Ca^{2+}-activated K^+ efflux had similar values. It should be remarked, that higher concentrations of ionized Ca^{2+} were used in the media to estimate the maximal stimulation of K^+ efflux in mouse than in human cells because, as we will show later, the capacity to extrude Ca^{2+} as estimated by the Ca^{2+}-ATPase is significantly higher in mouse than in human red cells.

Figure 6 compares the means of Ca^{2+}-activated K^+ efflux, in RBCs from transgenic mice *vs.* SS patients. As in the case of the deoxy K^+ efflux, the fluxes were normalized to area per liter of cells. The total Ca^{2+}-activated K^+ efflux was 6.4 μmol/μm² × min in SS cells *vs.* 6.0 μmol/μm² × min in mouse RBCs. In the presence of CTX, the Ca^{2+}-activated K^+ efflux was 2.3 μmol/μm² × min in SS cells *vs.* 3.5 μmol/μm² × min in mouse RBCs. Therefore, after normalizing to area per liter of cells, the ΔCTX-sensitive K^+ efflux was 1.8-times higher in SS patients than in either control or transgenic mouse RBCs.

Ca^{2+}-ATPase Activity of Mouse and Human Red Cells

RBC cytosolic Ca^{2+} is maintained at low levels by the plasma membrane Ca^{2+} pump and its associated Ca^{2+}-ATPase activity despite shear stress enhanced Ca^{2+} entry [23]. Deoxygenation has been shown to increase Ca^{2+} influx and ionized cytosolic Ca^{2+} in human SS cells [12]. Because the activity of the Ca^{2+} pump is the main regulator of cytosolic Ca^{2+} levels in RBCs, we have studied the maximal activity of the calmodulin-stimulated Ca^{2+}-ATPase. Table 3 summarizes the Ca^{2+}-ATPase activity of RBC membranes from mice and humans assayed with the same procedure. A potential difficulty with these measurements is the presence of hemoglobin in the membranes from red cells which express HbS and the difficulty of interspecies comparisons. However, the maximum percent of the membrane weight attributed to Hb is between 4 and 10% and the differences presented below far exceed this potential correction. The calmod-

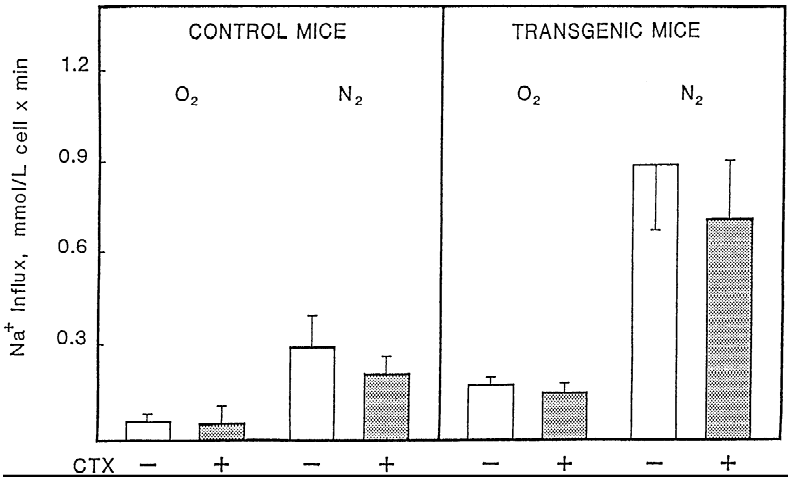

Fig. 4. Effect of deoxygenation on Na^+ influx in control and transgenic mouse RBCs. Data are expressed as mean ± SE of $n = 4$ different control and transgenic mice in which all parameters were measured from the same RBC preparation. Media was deoxygenated as mentioned in Materials and Methods and was of similar composition as in deoxy-stimulated K^+ efflux experiments.

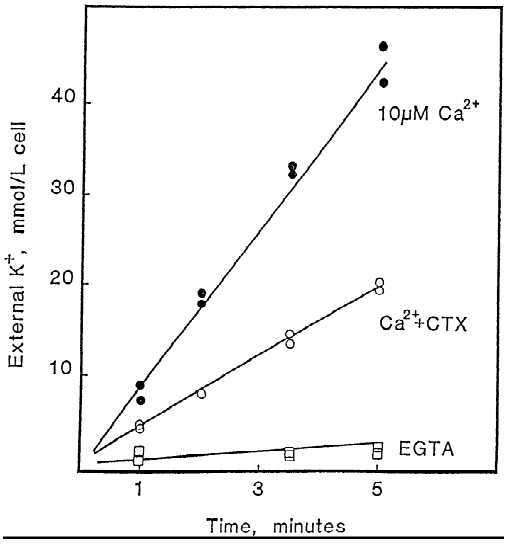

Fig. 5. Activation of K^+ efflux by elevation of cytosolic Ca^{2+} and inhibition by charybdotoxin in mouse red blood cells. K^+ efflux is very low if Ca^{2+} is clamped in the presence of A23187 at <0.1 nM with EGTA (—□—□—□—). When ionized Ca^{2+} is elevated to 10 μM, K^+ efflux is stimulated significantly (—●—●—●—). In the presence of 5 nM CTX this Ca^{2+}-activated K^+ efflux is inhibited (—○—○—○—). This is a single experiment which is representative of six other experiments in six different mice; average values are presented in Fig. 6. Media composition is described in Materials and Methods.

Table 2. Charybdotoxin-sensitive and insensitive Ca^{2+}-activated K^+ efflux from mouse red blood cells

Media	K^+ Efflux, mmol/L cell × min ± SE	
	Control mice	Transgenic mice
Total	7.1 ± 1.0	8.8 ± 0.2
+ CTX	4.0 ± 0.3	5.2 ± 0.8
Δ CTX-sensitive	3.0 ± 1.0	3.6 ± 0.6

K^+ efflux was measured in Na^+ media as described in Materials and Methods. Data is expressed as mean ± SE, $n = 3$ control and 3 transgenic mice. Total rate is the difference between the maximal Ca^{2+}-activated K^+ efflux from RBCs with 10μM cytosolic ionized Ca^{2+} minus the efflux in the presence of EGTA without charybdotoxin (CTX). Δ CTX-sensitive was determined from the difference between total K^+ efflux in the presence and absence of CTX in cells with increased ionized cytosolic Ca^{2+} in each experiment in each mouse. There was no significant difference in the efflux between control and transgenic mice in all conditions (nonparametric analysis).

ulin activated Ca^{2+}-ATPase activity had similar values in control and transgenic mouse RBCs membranes, but the enzyme activity of both strains was greater (121.9 nmol/mg × min transgenic; 105.9 nmol/mg × min control) than in membranes of normal human RBCs (31.6 nmol/mg × min). We also examined the Ca^{2+}-ATPase activity of membranes of SS cells with normal density (SS2) of blood from SS patients. As observed by others [10, 18, 19, 24, 25, 27], SS cells exhibited lower activity (3.1 nmol/mg × min) than normal AA cells.

Discussion

The present paper established that the RBCs of this transgenic mouse expressing HbS has one of the hallmarks of the human disease, the deoxy-stimulated K^+ efflux. This implies that these mice can serve as a model to identify factors involved in its activation. Our findings also show that, when initial rates are measured, this deoxy-stimulated K^+ efflux is balanced by a net Na^+ gain of similar magnitude, in the presence of physiological calcium concentrations. However, the 1:1 exchange of Na^+ for K^+ has been shown to be altered by changing the experimental conditions: for example, prolonged deoxygenation (1–2 hr) and altered external Ca^{2+} concentration [20–22] or oxy-deoxy cycling all affect the ratio [28].

Our results also show that even though control and transgenic mouse RBCs expressed similar maximal activity of Ca^{2+}-activated, CTX-sensitive K^+ efflux, trans-

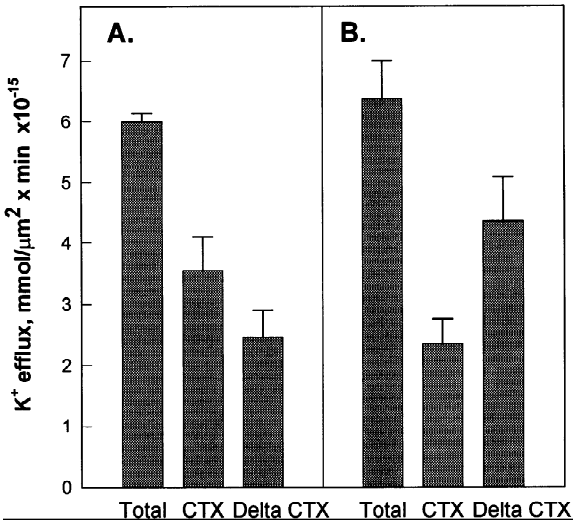

Fig. 6. Comparison of the maximal rates of Ca^{2+}-activated K^+ efflux between transgenic mice (*A*) and human SS (*B*) red blood cells in the presence and absence of charybdotoxin (CTX). Data are expressed as mean ± SE, $n = 3$ transgenic mice and 8 SS patients. K^+ efflux was measured in Na^+ media as described in Materials and Methods. The maximal Ca^{2+}-activated K^+ efflux from mouse RBCs with 10 μM cytosolic ionized Ca^{2+} or human SS RBCs with 5 μM cytosolic ionized Ca^{2+} in the absence of CTX was calculated from the difference between the total efflux and the efflux in the presence of EGTA. CTX-sensitive efflux was determined from the difference between Ca^{2+}-activated K^+ efflux in the presence and absence of CTX in cells with increased ionized cytosolic Ca^{2+} in each experiment in each mouse/subject. The rates were normalized to area per liter RBC and the maximal rate of Ca^{2+}-activated K^+ efflux was similar in SS patients and in transgenic mice. In both cell types, 5 nM CTX significantly inhibited the maximal rate of Ca^{2+}-activated K^+ efflux to similar values.

Table 3. Calmodulin activated Ca^{2+}-ATPase activity in mouse and human red blood cell membranes

	Ca^{2+}-ATPase, nmol/mg × min
Mouse red blood cells	
Control ($n = 4$)	105.9 ± 5.9
Transgenic ($n = 5$)	121.9 ± 7.1
Human red blood cells	
HbA($n = 10$)	31.6 ± 1.8*
HbS($n = 4$)	3.1 ± 0.9

Mean ± SE n = number of subjects studied. HbA are normal subjects homozygous for Hb A. (*) Significantly different from control and transgenic mice and SS patients ($P < 0.01$, nonparametric analysis). ATPase activity was not significantly different between control and transgenic mice.

genic RBCs, but not control mouse RBCs showed enhanced K^+ efflux upon deoxygenation. The deoxy-stimulated K^+ efflux first described by Tosteson in human cells [35] therefore is also unique to mouse RBCs which express HbS. CTX partially inhibited the deoxy-stimulated K^+ efflux in mouse RBC and human SS cells. The deoxy-stimulated, CTX-sensitive K^+ efflux from RBCs of mice expressing HbS was almost twice as large as that observed in SS patients.

Previous studies of human RBCs defined the dependence of K^+ efflux on the concentration of ionized cytosolic Ca^{2+} [38]; those studies determined that the threshold for cytosolic Ca^{2+} to open K^+ channels is over 100 nM as determined by equilibrium measurements with a calcium electrode. Therefore, during the 10-min period of deoxygenation, we expect the Ca^{2+} influx to increase and raise cytosolic Ca^{2+} levels over this threshold. Evidence that opening this K^+ channel can induce red cell dehydration comes from several sources [5, 28, 31]. Tiffert et al. [33] have also estimated in ATP-depleted red cells from normal subjects that the dehydration response to an increased Ca^{2+} influx is extremely steep and seems to occur within a range of cytosolic Ca^{2+} of 40–150 nM.

In addition to the cellular loss of K^+ stimulated by deoxygenation, human SS cells have been shown to have a net gain of Na^+ [22, 34]. Likewise, transgenic mouse RBCs showed a deoxy-stimulated Na^+ influx which was not blocked by CTX and reflects downhill movement of Na^+. The net Na^+ influx was significantly higher in transgenic mouse RBCs than in RBCs from control mice. Moreover, in the presence of external Ca^{2+}, the extent of deoxy-stimulated Na^+ influx of transgenic mouse RBCs was not significantly different from that of deoxy-stimulated K^+ efflux; therefore, the total cation content does not change significantly during short periods of deoxygenation in transgenic mouse RBCs.

Control and transgenic mice have maximal activity of Ca^{2+}-activated K^+ efflux of similar magnitude, and are slightly less sensitive to CTX than are human RBCs. However, on the basis that the CTX-sensitive component of K^+ efflux reflects the number of Ca^{2+}-activated K^+ channels, mouse RBCs express a smaller number of channels per unit area than previously reported for human SS RBCs [28, 38]. The number of Ca^{2+}-activated K^+ channels per cell have been described to be highly variable in SS patients but on the average the level of expression is similar to that of AA cells [38]. It seems likely that these channels open in RBCs expressing HbS when deoxygenation leads to an increased Ca^{2+} influx and increases cytosolic Ca^{2+} because, in both human SS and transgenic mouse RBCs, deoxy-stimulated K^+ efflux is markedly inhibited by CTX which is a characteristic of the Ca^{2+}-activated K^+ channel. Furthermore, the formation of dense cells observed during long term oxy-deoxy cycling has been shown to be inhibited by CTX [5, 28, 31]. The activation of the Ca^{2+}-dependent K^+ channels by deoxygenation may be dependent on the number of channels and/or on the activity of the membrane Ca^{2+} pump which will determine how fast calcium can be extruded from the cell.

The activity of the calmodulin-activated Ca^{2+}-ATPase of the membrane Ca^{2+} pump was studied in mouse and human RBC membranes because this is a mechanism which can rapidly regulate cytosolic Ca^{2+} levels during deoxygenation [7]. Most of these studies have shown that the Ca^{2+} ATPase activity of RBC membranes from SS patients is decreased in comparison to membranes from RBCs of normal subjects [10, 18, 19, 24, 25, 27]. These findings are unexpected because most transport systems have enhanced activity in the very young RBC population of SS blood. We found that in both control and transgenic mice, the RBC membranes had a higher Ca^{2+}-ATPase activity than normal human RBC membranes.

The low activity of the Ca^{2+}-dependent ATPase observed in human sickle cells with normal density (SS2) is in agreement with previous observations made by Dixon et al. [10]. Recent studies of Ca^{2+} efflux under deoxygenated conditions in human AA and SS cells [12] found the reduction to be the same in both cases, but both the conditions of the preparation of the membranes and the assays used were different from those reported earlier and those used in this paper.

Comparison of the deoxy CTX inhibitable K^+ efflux which is attributable to Ca^{2+}-dependent K^+ channels for mouse and human RBCs reveals an apparent contradiction; although the maximal CTX-inhibitable flux per unit area is larger in man than in mouse (implying a larger number of channels per unit area), the deoxy CTX inhibitable K^+ efflux is larger in mouse than in man. The activity of the membrane Ca^{2+} pump which determines how fast calcium can be extruded from the cell is also estimated to be higher in mouse than in man which would again suggest that the deoxy flux in man should be higher. However, calcium enters the deoxygenated HbS red cell due to general permeabilization to cations which is attributed to membrane distortion by the polymer [26] and the rate at which Ca^{2+} enters the deoxygenated mouse red cell has not estimated and may be higher since the membrane distortion observed in transgenic mouse red cells is more extreme than that observed in human cells [13, 15]. Mouse red cells may also have more permeable membranes under all conditions; this supposition is supported by the larger efflux observed under oxygenated conditions in mouse. In addition, both the potassium and sodium content per Kg of Hb are higher in the mouser red cell to balance the higher plasma osmolarity found in the mouse and this should also increase the deoxy potassium efflux. Further study is needed to validate these or other explanations for the high potassium efflux in the sickle mouse.

In conclusion, transgenic mice expressing HbS uniquely have one of the hallmarks of the human disease, deoxy-stimulated K^+ efflux. As in SS patients, a large percent, but not all of the deoxy-stimulated efflux is inhibitable by charybdotoxin which suggests that more than one mechanism may contribute to this efflux. We speculate that the lack of dense cell formation may contribute to the absence of anemia in the sickle cell mouse.

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Dr. Canessa passed away on February 1, 1997. Her talent and imagination will be sorely missed.

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