Physiological Concept for a Blood Based CFTR Test

Astrid Stumpf¹, Kerstin Wenners-Epping¹, Mike Wälte¹, Tobias Lange¹, Hans-Georg Koch³, Johannes Häberle², Angelika Dübbers², Sabine Falk², Ludwig Kiesel⁴, Dessy Nikova¹, Reimer Bruns¹, Helga Bertram¹, Hans Oberleithner¹ and Hermann Schillers¹

¹Institute of Physiology II, University of Muenster, ²Department of Pediatrics, University Hospital of Münster, ³Department of Pediatrics, Klinikum Braunschweig, ⁴Department of Obstetrics and Gynecology, University Hospital of Münster

Key Words

Gadolinium • Hemolysis • Cystic fibrosis • Red cells • Atomic force microscopy

Abstract

We tested the hypothesis that the cystic fibrosis transmembrane conductance regulator (CFTR) could be involved in the volume regulation of human red blood cells (RBC). Experiments were based on two gadolinium (Gd³⁺) sensitive mechanisms, i.e. inhibition of ATP release (θ ATP_i) and membrane destabilization. RBC of either cystic fibrosis (CF) patients or healthy donors (non-CF) were exposed to KCl buffer containing Gd³⁺. A significantly larger quantity of non-CF RBC (2.55 %) hemolyzed as compared to CF RBC (0.89 %). It was found that both of the Gd³⁺ mechanisms simultaneously are needed to achieve hemolysis, since either overriding θ ATP_i by exogenous ATP addition prevented Gd³⁺ induced hemolysis, or mimicking θ ATP_i by apyrase in absence of Gd³⁺ could not trigger hemolysis. Additionally, ion driven volume uptake was found to be a prerequisite for Gd³⁺ induced hemolysis as chloride and potassium channel blockers reduced the Gd³⁺ response. The results show that in non-CF RBC Gd³⁺ exerts its dual effect

leading to hemolysis. On the contrary, in CF RBC, lacking CFTR dependent ATP release, the sole Gd³⁺ effect of membrane destabilization is not sufficient to induce hemolysis similar to non-CF. This concept could form the basis of a novel method suitable for testing CFTR function in a blood sample.

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Introduction

Cystic fibrosis is the most common congenital disease among Caucasians, characterized by an impaired salt and fluid balance of epithelial cells [1-4]. This disease is caused by a defective protein, called cystic fibrosis transmembrane conductance regulator (CFTR). In healthy organisms CFTR acts both as a chloride channel and as a membrane conductance regulating protein. The most common mutation of the CFTR gene is Δ F508, resulting in the deletion of phenylalanine in the first nucleotide binding domain at position 508. This deletion is responsible for the misfolding of the CFTR protein, which leads to an impaired trafficking and results in intracellular degradation of CFTR [5]. Therefore, cells with homozygous Δ F508

($\Delta F508$ -/-) are supposed to lack CFTR in plasma membrane. Indeed, it has been shown that cells with this mutation contain only a low amount of CFTR in the plasma membrane [6, 7]. One of the regulating roles of a functional CFTR is a mechanism known as regulatory volume decrease (RVD) [8]. RVD is characterized by CFTR-dependent ATP release, in which ATP activates, in an autocrine way [9], a purinergic receptor [10] and subsequently initiates an intracellular pathway resulting in osmolyte and water efflux [11, 12].

CFTR is expressed nearly in all human cells, including RBC. There, CFTR has been revealed functionally [13, 14] and with western blot analysis [15, 16]. Furthermore, it has been shown that CFTR of RBC plays a role in malaria infection [14] and in regulating vessel tone by mediating ATP release and subsequent activation of endothelial nitric oxide synthesis [17-19].

RBC have long been proven to be very useful as a diagnostic tool since they are simply obtainable and easy to handle. These properties, together with the perspective that RBC could reflect any CFTR dysfunction in the organism, make RBC particularly valuable for CF diagnostic purposes. Here we describe a novel approach showing that altered cell membrane properties due to a defective CFTR can be efficiently detected in RBC of CF patients.

Materials and Methods

Blood samples

Freshly drawn venous blood was used from healthy volunteers and from patients with the homozygous $\Delta F508$ mutation. Newborn blood was taken from isolated umbilical cords after birth. A consensus statement according to the declaration of Helsinki was signed from all blood donors. The number of tested individuals is given by n.

Hemolysis measurements

Venous blood from healthy donors and cystic fibrosis (CF) patients was freshly drawn in K_2 -EDTA Monovettes (Sarstedt, Germany) and centrifuged for 10 min at 1000 g. Supernatant and buffy coat were removed and RBC washed two times in the 2-fold volume of 155 mM NaCl, 10 mM Hepes, pH 7.4. 50 μ l of washed RBC were resuspended in 450 μ l of 155 mM KCl, 10 mM Hepes, pH 7.4 (hematocrit 0.1). This suspension was incubated at 37°C in a bacteria shaker at 280 rpm for 60 min. We chose to incubate RBC for 60 min as hemolysis starts after 50 min reaching values sufficient to distinguish between non-CF and CF RBC after 60 min. Then, the suspension was centrifuged at 2000 g for 5 min and the free hemoglobin in the supernatant was quantified by optical density measurements at 546 nm (OD_{546}). Results were correlated to 100 % hemolysis

to avoid errors elicited by the natural individual distribution of different mean corpuscular hemoglobin content (MCHC). For each blood we lysed 50 μ l washed RBC in 450 μ l H_2O (the optical density of this solution represents 100 % hemolysis) and made a calibration curve with appropriate dilutions.

Osmotic fragility measurements

RBC were obtained and treated as described above. Immediately after incubation, RBC were suspended in several dilutions of a KCl solution (155 mM KCl, 10 mM Hepes, pH 7.4) at a hematocrit of 0.01. Suspension was centrifuged for 5 min at 2000 g and free hemoglobin was quantified by OD_{546} measurements compared with hemolysis in water (relative tonicity = 0), which was taken as 100%.

RBC membrane preparation

Glass coverslips were cleaned and 150 μ l of an aqueous solution of poly-L-lysine (0.01 % w/v) was applied to the clean side of each glass coverslip, removed 30 min later and rinsed two times with double distilled water. RBC were washed with isotonic phosphate buffered saline (PBS) immediately after incubation. 150 μ l of a diluted suspension (1:600 in PBS) of the washed cells was placed on the substrate and the RBC were allowed to adhere to the substrate for about 15 min. RBC were then sheared open by subjecting them to a jet stream of 8 ml of buffer (10 mM NaPi, 0.2 mM EGTA, 140 mM NaCl, pH 7.4, in which NaPi represents a mixture of Na_2HPO_4 and NaH_2PO_4 at given pH) at a flow rate of 0.6 ml s^{-1} through a 21 gauge needle from about 2 inches distance and at an angle of about 15° to the substrate. After this shear stress samples were further treated to selectively extract membrane skeletal proteins. To remove the proteins spectrin, actin and band 4.1, the sample was shaken in a low salt buffer (0.3 mM NaPi, 0.2 mM EDTA, pH 7.4) at 37°C for 20 min. Isolated RBC membranes were fixed by 4% paraformaldehyde in isotonic buffer at room temperature for 40 min.

Atomic force microscopy

AFM on isolated membranes was performed in contact mode using a Nanoscope III Multimode-AFM (Digital Instruments, Santa Barbara, California, USA) with a J-type scanner (maximal scan area: 100 x 100 μ m) as described earlier [20]. Images were processed using the SPIP (Image Metrology, Lyngby, Denmark) and Nanoscope III software (Digital Instruments).

All chemicals were obtained from Sigma (Sigma, Deisenhofen, Germany). Grade VII Apyrase (with high ADPase activity) was used in experiments to scavenge ATP. NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) was dissolved in DMSO and stored as a 100 mM stock solution. $GdCl_3$ is a highly hygroscopic salt. Therefore the concentration of the gadolinium stock solution (100 mM) was controlled with an osmometer.

Statistics

All data are presented as mean $\pm$ s.e.m. and significance of differences between mean values was determined with the Students t-test (Significance level is $P = 0.05$).

Results

Gd³⁺ response in RBC of healthy donors and CF patients

We used the trivalent lanthanide gadolinium (Gd^{3+}) firstly to block CFTR-dependent RVD, and secondly to induce structural transitions in RBC membranes. The Gd^{3+} -induced hemolysis is shown in figure 1. During incubation of RBC in KCl buffer with 100 μM Gd^{3+} we observed hemolysis of non-CF RBC. In contrast, RBC from CF donors with the $\Delta F508$ $-/-$ mutation showed a significantly reduced hemolysis under the same conditions. Quantification by optical density measurements at 546 nm (OD_{546}) revealed a hemolysis degree of $2.55 \pm 0.125\%$ ($n = 51$) for non CF RBC and $0.89 \pm 0.087\%$ ($n = 33$) for CF RBC. The incubation of RBC in KCl buffer without Gd^{3+} (indicated as control in figure 1A) elicited only little hemolysis. The mean values between non-CF and CF RBC did not differ significantly ($0.51 \pm 0.011\%$, $n = 171$ and $0.52 \pm 0.012\%$, $n = 36$, respectively). Differences in the degrees of hemolysis between control and Gd^{3+} exposed RBC can be already qualitatively detected by eye (figure 1B and 1C). The supernatants of the control experiments (figure 1B and C, left vials) are relatively colorless, corresponding to a very low hemolysis of about 0.5 %. In contrast, Gd^{3+} exposure of non-CF RBC (figure 1B, right vial) leads to a bright red-colored supernatant that corresponds to a hemolysis of about 2.6 %. Only a faintly colored supernatant results from Gd^{3+} treatment of CF RBC corresponding to a hemolysis degree of only about 0.9 % (figure 1C, right vial). These results clearly demonstrate that Gd^{3+} -induced hemolysis could be used to distinguish between CF and non-CF RBC.

Dual Gd^{3+} effect on RBC

Gd^{3+} is known to block CFTR dependent cell volume regulation by inhibiting ATP release (θATP_i) and to induce destabilization of the RBC membrane. We investigated the duality of the Gd^{3+} effect, i.e. we tested whether hemolysis results from either blocking ATP release and/or membrane destabilization. We applied Gd^{3+} and, at the same time, overcame θATP_i by exogenous addition of 50 μM ATP. Under these conditions both non-CF RBC and CF RBC display no hemolysis (Figure 2). This is an indication that the exogenously added ATP compensates for θATP_i by triggering the purinergic receptor-mediated signal cascade, which leads to RVD. We also tested whether an ATP-stimulated ATP-release [21] in amounts, which are osmotically effective, could induce a cell shrinkage. Thus, this cell volume decrease would prevent

Fig. 1. Gd^{3+} -induced hemolysis of non-CF RBC and CF RBC. (A) RBC of healthy donors (non-CF) and CF patients were incubated without (control) or with 100 μM Gd^{3+} . Number of tested individuals is given by n. Supernatants of non-CF RBC (B) and CF RBC (C) are shown after incubation without (left vials, respectively) and with 100 μM Gd^{3+} (right vials, respectively). The error bars indicate the SD.

Gd^{3+} induced hemolysis. If so, annulling the outwardly directed ATP gradient across the plasma membrane by adding 5 mM ATP to the incubation medium should also cancel the ATP effect. Since, we do not observe any differences between application of either 50 μM ATP or 5 mM ATP on hemolysis values in control experiments and Gd^{3+} treated RBC (data not shown), we conclude that the exogenously added ATP compensates for θATP_i .

In a second approach we administered the ATP scavenger apyrase. In these series of experiments Gd^{3+} was omitted since the strategy was to mimic the Gd^{3+} effect θATP_i without simultaneously inducing the Gd^{3+} effect of membrane destabilization. As indicated in figure 2, extracellular ATP depletion was not followed by hemolysis. This indicated that θATP_i alone, i.e. inhibition of CFTR dependent RVD, is not sufficient for hemolysis, and that membrane destabilization, as it occurs with Gd^{3+} , is a second prerequisite. Taken together the results demonstrate that both of the Gd^{3+} effects, θATP_i and membrane destabilization must occur simultaneously to induce hemolysis.

Fig. 2. Hemolysis of RBC obtained from healthy donors (filled columns) and CF patients (open columns) were incubated without (control) or with 100 μM Gd^{3+} (Gd^{3+}), with 100 μM Gd^{3+} plus 50 μM ATP (Gd^{3+} + ATP), with 10 U/ml apyrase (apyrase), 100 μM NPPB (NPPB), with 100 μM Gd^{3+} plus 100 μM NPPB (Gd^{3+} + NPPB), with 200 nM ChTx (ChTx), with 100 μM Gd^{3+} plus 200 nM charybdotoxin (Gd^{3+} + ChTx). Incubation of RBC with 100 μM Gd^{3+} in NMDG-Cl (Gd^{3+}) or with K^{+} -gluconate (Gd^{3+}) are shown on the right side. An asterisk indicates a significant difference from the corresponding Gd^{3+} incubation value. The number of experiments is indicated in the columns.

Block of ion/volume flux

It has been shown that ATP release in RBC is stimulated by deformation [22], cell swelling [11], hypoxia and reduction of pH [23, 24]. In high potassium chloride medium RBC swell due to an influx of K^{+} and Cl^{-} followed by water. This causes ATP release, which activates RVD. As a result an efflux of osmolytes and water occurs. In order to test whether in addition to the both effects of Gd^{3+} , ion driven water influx was necessary for hemolysis, we added to the Gd^{3+} exposed RBC either a chloride or potassium channel blocker. We used 100 μM of the weakly selective chloride channel blocker NPPB [25] and 200 nM of the highly selective potassium channel blocker charybdotoxin (ChTx), a selective inhibitor of the RBC Gardos channel (also named IK1 or hSK4 or KCNN4) [26].

In detail, RBC from healthy donors or CF patients incubated with 100 μM Gd^{3+} and 100 μM NPPB revealed hemolysis of $0.83 \pm 0.049\%$ ($n=16$) and of $0.59 \pm 0.080\%$ ($n=12$), respectively; with 100 μM Gd^{3+} and 200 nM

Fig. 3. Osmotic fragility of RBC. Relative tonicity (water = 0%) is plotted versus hemolysis (100% hemolysis in water). After incubation without Gd^{3+} (filled squares), with Gd^{3+} (filled circles) or with Gd^{3+} plus NPPB (open triangles) RBC were diluted at a hematocrit of 0.01 in KCl solutions with different tonicities. The right shift indicates an increased RBC fragility induced by Gd^{3+} .

charybdotoxin the hemolysis of non-CF RBC was $1.19 \pm 0.208\%$ ($n=12$), and of CF RBC: $0.81 \pm 0.175\%$ ($n=11$); and with 200 nM ChTx alone the values were $0.46 \pm 0.012\%$ ($n=12$), and $0.49 \pm 0.017\%$ ($n=11$), respectively.

Along the same line of arguments, instead of KCl we applied impermeable cations (NMDG-Cl) or anions (potassium gluconate) [27] at isomolar concentrations. As displayed in figure 2, all experimental procedures mentioned above significantly suppressed the Gd^{3+} induced hemolysis. Incubation in NMDG-Cl (hemolysis of non-CF RBC: $0.48 \pm 0.011\%$ ($n=19$), hemolysis of CF RBC: $0.50 \pm 0.013\%$ ($n=8$)) and K^{+} -gluconate (hemolysis of non-CF RBC: $0.49 \pm 0.035\%$ ($n=18$), hemolysis of CF RBC: $0.44 \pm 0.027\%$ ($n=8$)) showed a low hemolysis in the range of the control value.

Gd³⁺ induced osmotic fragility

Gd^{3+} is known to induce structural transitions in erythrocyte membranes [28], which should be accompanied by increased fragility of these membranes. To evaluate this effect, we tested osmotic resistance of RBC after incubation with or without Gd^{3+} .

Exposure to Gd^{3+} resulted in a clear right shift of the curve indicating increased fragility of the membrane (Figure 3). Furthermore, we examined changes in osmotic

Fig. 4. AFM images (20x 20µm) of isolated RBC membranes. The scale bar represents the gray-scale coded height. Isolation was performed immediately after incubation of RBC without Gd³⁺ (A), with Gd³⁺ (B) and with Gd³⁺ plus NPPB (C). Please, note the porous structure in B and C.

resistance in presence of Gd³⁺ together with NPPB (Figure 3). Clearly, Gd³⁺ increased osmotic fragility independent of NPPB. It should be noted here that Gd³⁺ induces hemolysis, whereas NPPB prevents Gd³⁺ induced hemolysis (see Figure 2). It became clear that the change of osmotic resistance is not correlated with hemolysis. This result is consistent with the view that Gd³⁺ destabilizes the membrane irrespectively of changes in membrane chloride conductance.

As an alternative way to provide additional support for the hypothesis that Gd³⁺ induced membrane fragility occurs, we examined RBC plasma membrane with atomic force microscopy (AFM). This technique is able to provide topographical information of membrane structures at nanometer resolution [29]. RBC were treated as described above and by use of a jet stream procedure (see methods) we obtained inside-out isolated membranes spread on glass. AFM images of such RBC membranes exhibit intact membrane surfaces (Figure 4A). Membranes of Gd³⁺ exposed RBC show large porous structures with diameters up to 600 nm, independent of the presence of NPPB (Figure 4B and 4C). Obviously, these large pores are formed by the jet stream procedure but only in RBC exposed to Gd³⁺. Membranes treated with Gd³⁺ show an increase of fragility and therefore they would break more easily and form the observed porous structures. These observations strongly support the osmotic resistance measurements indicating that Gd³⁺ destabilizes the membrane.

Effects of 100 µM Gd³⁺ on RBC of adults and newborns

The Gd³⁺-induced hemolysis of non-CF RBC is clearly detectable but only about 2 % of all RBC are lysed. In blood drawn from adult donors, the reticulocytes represent 1 – 2 % of the RBC. Therefore we used newborn blood (NB) from umbilical cords to test the hypothesis that reticulocytes and very young RBC are responsible for the Gd³⁺-induced hemolysis. Umbilical cord blood contains a higher percentage of young RBC as

compared to adult blood and thus should be more sensitive to Gd³⁺. Incubation of newborn RBC without Gd³⁺ shows a hemolysis of 0.71 ± 0.041 % (n = 38) which is comparable to that of adult RBC. Incubation with 100 µM Gd³⁺ reveals a significant higher hemolysis of newborn RBC (3.80 ± 0.427 %, n = 26) as compared to adult non-CF RBC 2.55 ± 0.125 % (n = 51) (Figure 5).

Discussion

CFTR and the dual effect of Gd³⁺

CFTR-dependent ATP release was shown for several cell types [11] and also for human red blood cells [13]. Gd³⁺ is known to block CFTR-dependent RVD [11] and to induce structural transitions in RBC membranes [30-33], which are accompanied by increased fragility of these membranes. We show that the Gd³⁺-induced hemolysis of non-CF RBC is nearly three times higher than that of CF RBC. The difference between these two groups is most likely caused by the lack of CFTR in the plasma membrane of CF RBC. RBC exposure to isoosmotic potassium chloride leads to both KCl influx and cell volume increase. This triggers CFTR dependent ATP release initiating a signaling pathway with the result of RVD [34]. RBC remain intact [35] and no hemolysis occurs. However when administrated, Gd³⁺ interferes with RVD and causes hemolysis. From our experiments it becomes clear that Gd³⁺ must act through at least two ways on the plasma membrane to induce hemolysis in RBC of healthy donors. A scheme of the dual effect of Gd³⁺ is shown in figure 6. The first effect is the inhibition of ATP release with a subsequent inhibition of RVD. Thus, the RBC would remain swollen (but would not hemolyze) in KCl buffer. The second effect is the membrane destabilization that includes aggregation of membrane proteins and phase transition of the lipids. This would result in decreased plasma membrane stability leading, only in combination with θATP_i , to RBC hemolysis.

The results of two series of experiments presented

Fig. 5. Gd³⁺-induced hemolysis of adults and newborn RBC. Washed RBC were incubated without (control) or with 100 μ M Gd³⁺. In control experiments adult non-CF RBC and newborn RBC show a comparable hemolysis. A significantly higher Gd³⁺-induced hemolysis is shown for newborns RBC compared to adults RBC.

here, i.e. resistance and AFM imaging (Figure 3 and 4), support this hypothesis. Firstly, in presence of Gd³⁺, a decrease of the osmotic resistance was observed. Secondly, in vitro shear stress induced artifacts (pores) in the RBC lipid bilayer when cells were pre-exposed to Gd³⁺, as seen in the AFM images. This result is supported also by other structural studies with AFM showing that Gd³⁺ induces pores in RBC membranes [32].

Although Gd³⁺ makes membranes vulnerable, the pore structures occur only in vitro, induced by artificial shear stress (jet stream; see methods). If 600 nm pores would have formed already during Gd³⁺ incubation, hemolysis should have been observed also in the Gd³⁺ together with NPPB experiment. This, however, was not detected.

The dual effect hypothesis of Gd³⁺ is based on several observations: We destabilized the membrane while maintaining RVD by exogenously added ATP, where no hemolysis occurred. We also blocked RVD by apyrase induced extracellular ATP depletion without destabilizing the membrane. Again, no hemolysis occurred. Taken together, neither the sole RVD block nor the sole membrane destabilization lead to hemolysis, however both effects must take place simultaneously to hemolyze RBC of healthy donors.

Although Gd³⁺-induced hemolysis of non-CF RBC is clearly detectable, only about 2.5 % of all RBC are

Fig. 6. Scheme of the dual effect of Gd³⁺. First: Gd³⁺ blocks CFTR dependent ATP release and therefore the RVD. Second: Gd³⁺ destabilizes the membrane. Membrane destabilization is independent of θ ATP_i.

lysed. From this observation we derive that most likely a minor population of RBC responds to Gd³⁺. We presume that reticulocytes and very young RBC are potential candidates. Indeed, during erythropoiesis plasma membrane transporters rarify over time [36]. Possibly, also CFTR diminishes during maturation. This view is supported by the finding that incubation of newborn RBC, taken from umbilical cords, with 100 μ M Gd³⁺ reveals a significantly higher hemolysis as compared to adult non-CF RBC. Umbilical cord blood contains more reticulocytes and young RBC as compared to adult blood. Furthermore, matured RBC are supposed to be unable to regulate their volume [37]. It indicates that young RBC and reticulocytes are most likely the RBC population which responds to Gd³⁺. From these results we conclude (i) that reticulocytes and young RBC are the target cells who contain sufficient CFTR to perform volume regulation and, therefore, they are Gd³⁺ sensitive and (ii) that this Gd³⁺ based blood test for CFTR function could be particularly suitable for newborn screening.

Viewing CFTR as a cell volume regulatory protein it is at first sight surprising that RBC of CF patients hemolyze significantly less when exposed to Gd³⁺ as compared to RBC of healthy donors. Since CF RBC do not exhibit a CFTR-dependent ATP release mechanism, RVD must work in a CFTR independent way. Indeed, there is evidence for other volume regulatory pathways that compensate the lack of CFTR. Up-regulation of MDR1 (multi drug resistance transporter) mRNA in response to the absence of CFTR gene expression has

been shown in intestinal epithelium of CFTR knockout mice [38]. Furthermore, the multidrug resistance gene product, P-glycoprotein (Pgp) is known to facilitate ATP release and to participate in RVD [39, 40]. This membrane transporter could serve as a functional substitute of CFTR [41]. Thus the up-regulation of Pgp in CF cells could mediate ATP release [42]. Then, CF RBC do not hemolyze when challenged with Gd^{3+} .

CFTR and volume uptake

We assume that in KCl buffer RBC increase in volume by KCl uptake mainly via potassium and chloride channels. Therefore, application of channel blockers should reduce volume uptake and prevent cell swelling. Consistent with this view we found diminished hemolysis when either NPPB or ChTx was added to the KCl buffer. Therefore we hypothesize that cell swelling is a prerequisite for hemolysis.

Gd^{3+} induced hemolysis of non-CF RBC was also significantly reduced by ChTx, which is a highly specific blocker for the Gardos channel [43]. In isoosmotic KCl solution this K^+ channel functions as an effective inward rectifier facilitating K^+ influx. Activation of the Gardos channel requires an increase of intracellular calcium concentration, which has been shown to occur after Gd^{3+} stimulation in young RBC [44]. Although we did not add calcium to the medium there should be enough calcium available from intracellular sources to allow Gardos channel function. Finally, ion substitution experiments clearly indicate that the influx of potassium and chloride is responsible for cell swelling and a prerequisite for the Gd^{3+} induced hemolysis.

Scatter of RBC hemolysis

Although the Gd^{3+} response is significantly different in RBC of healthy donors and CF patients, there is still a low level of Gd^{3+} -induced hemolysis detectable in CF RBC. This background level is not unexpected. It has been shown that cells with the $\Delta F508$ -/- mutation contain a small amount of CFTR in the plasma membrane [7]. This means that there is also some rudimentary ATP release left that can drive RVD. In addition, residual CFTR activity in CF RBC allows some minor Cl^- influx and, by functional crosstalk with the Gardos channel, equivalent K^+ influx. Thus, the scatter in hemolysis is most likely to be not due to technical reasons but rather reflects the actual CFTR profile of RBC. Therefore, the degree of hemolysis directly correlates with CFTR function. It becomes clear that detection of the CFTR in human RBC will open new perspectives for blood-based diagnosis of cystic fibrosis.

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