Journal of Theoretical Biology 393 (2016) 194–202

Contents lists available at ScienceDirect

Journal of Theoretical Biology

journal homepage: www.elsevier.com/locate/yjtbi

Influence of magnesium sulfate on HCO_3/Cl transmembrane exchange rate in human erythrocytes

Ekaterina S. Chernyshova [a,b], Yulia S. Zaikina [c], Galina A. Tsvetovskaya [d,e], Dmitry I. Strokotov [a,f], Maxim A. Yurkin [a,b], Elena S. Serebrennikova [c], Leonid Volkov [g], Valeri P. Maltsev [a,b,f], Andrei V. Chernyshev [a,b,*]

[a] *Voevodsky Institute of Chemical Kinetics and Combustion, Institutskaya 3, 630090 Novosibirsk, Russia*
[b] *Novosibirsk State University, Pirogova 2, 630090 Novosibirsk, Russia*
[c] *Medical Centre of Siberian Branch of the Russian Academy of Science, Pirogova 25, 630090 Novosibirsk, Russia*
[d] *ANO "Center of New Medical Technologies in Akademgorodok", Pirogova 25/4, 630090 Novosibirsk, Russia*
[e] *Institute of Chemical Biology and Fundamental Medicine, Lavrentiev Avenue 8, 630090 Novosibirsk, Russia*
[f] *Novosibirsk State Medical University, Krasny Prospect 52, 630091 Novosibirsk, Russia*
[g] *Centre de Recherches Cliniques Etienne-Le Bel, 3001 12 Av. Nord Sherbrooke loc. # 4867, Quebec, Canada J1H 5N4*

HIGHLIGHTS

- A novel molecular-kinetic model of Band 3 activation by Mg^{2+} is introduced.
- The model is verified experimentally with the use of a scanning flow cytometer.
- The association constant of Mg^{2+} with Band 3 is evaluated as 0.07 mM.
- The method developed allows quantitative control and optimization of $MgSO_4$ treatment.
- Results of the work are useful particularly for early detection of risk of hypoxia.

ARTICLE INFO

Article history:
Received 7 July 2015
Received in revised form
16 December 2015
Accepted 18 December 2015
Available online 15 January 2016

Keywords:
Magnesium sulfate
Band 3
Red blood cells
Kinetic modeling

ABSTRACT

Magnesium sulfate ($MgSO_4$) is widely used in medicine but molecular mechanisms of its protection through influence on erythrocytes are not fully understood and are considerably controversial. Using scanning flow cytometry, in this work for the first time we observed experimentally (both *in situ* and *in vitro*) a significant increase of HCO_3^-/Cl^- transmembrane exchange rate of human erythrocytes in the presence of $MgSO_4$ in blood. For a quantitative analysis of the obtained experimental data, we introduced and verified a molecular kinetic model, which describes activation of major anion exchanger Band 3 (or AE1) by its complexation with free intracellular Mg^{2+} (taking into account Mg^{2+} membrane transport and intracellular buffering). Fitting the model to our *in vitro* experimental data, we observed a good correspondence between theoretical and experimental kinetic curves that allowed us to evaluate the model parameters and to estimate for the first time the association constant of Mg^{2+} with Band 3 as $K_B \sim 0.07$ mM, which is in agreement with known values of the apparent Mg^{2+} dissociation constant (from 0.01 to 0.1 mM) that reflects experiments on enrichment of Mg^{2+} at the inner erythrocyte membrane (Gunther, 2007). Results of this work partly clarify the molecular mechanisms of $MgSO_4$ action in human erythrocytes. The method developed allows one to estimate quantitatively a perspective of $MgSO_4$ treatment for a patient. It should be particularly helpful in prenatal medicine for early detection of pathologies associated with the risk of fetal hypoxia.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Magnesium sulfate ($MgSO_4$) is a pharmaceutical preparation of magnesium, widely used in medicine (Fawcett et al., 1999; McLean, 1994). It is effective in numerous diseases associated with

---

[*] Corresponding author at: Voevodsky Institute of Chemical Kinetics and Combustion, Institutskaya 3, Novosibirsk 630090, Russia. Tel.: +7 383 3333240; fax: +7 383 3307350.
E-mail address: chern@ns.kinetics.nsc.ru (A.V. Chernyshev).

http://dx.doi.org/10.1016/j.jtbi.2015.12.023
0022-5193/© 2016 Elsevier Ltd. All rights reserved.

magnesium deficiency (Swaminathan, 2003; Ulger et al., 2010). Magnesium sulfate is known as an antiarrhythmic agent to prevent cardiac arrest (Sugiyama et al., 1996), and as a bronchodilator to reduce the symptoms of acute asthma (Blitz et al., 2005). It is also effective as a tocolytic agent in decreasing the risk of eclampsia and preterm birth (Duley and Neilson, 1999; Euser and Cipolla, 2009). Intravenous administration of magnesium sulfate before birth could reduce the risk of cerebral palsy and motor dysfunction in preterm infants (Doyle et al., 2009). However, the effect of the treatment is dose sensitive. A high dosage of magnesium sulfate for attempted tocolysis in preterm labor was shown to cause more harm than do good (Mittendorf et al., 2004). It was found that “aggressive tocolysis” increases a risk of intracranial hemorrhage as well as deaths of fetus and very low body weight neonates (Mittendorf et al., 2002; Murata et al., 2005). In addition, the prolonged exposure to magnesium sulfate was associated with an increased risk of clinical chorioamnionitis (Elimian et al., 2002). By contrast, low doses of magnesium sulfate, when used as prophylaxis in cases of preterm labor, appeared to be neuroprotective for a number of children (Deering et al., 2005). So far, there is no method for determining the optimal dosage of magnesium sulfate for a particular patient before the injection. This is largely due to the lack of understanding of molecular kinetic mechanisms of $MgSO_4$ action. In particular, the role of the influence of magnesium on erythrocytes in blood is known (Abad et al., 2005, 2010; Ariza et al., 2005; Gulczynska et al., 2006), but far from completely understood. In some cases, the anti-hypoxic mechanism of the protection through the activation of erythrocytes physiological properties by magnesium is supposed (Szemraj et al., 2005), but not explained quantitatively.

Magnesium is the second most abundant cation in erythrocytes. The total concentration of Mg^{2+} in human erythrocytes is around 2 mM, but only a small fraction ($\sim$0.2 mM) is free (uncomplexed) (Grubbs, 2002; Gunther, 2007). Many enzymes require the presence of Mg^{2+} for their catalytic action (Beydemir et al., 2000). Magnesium influences ionic traffic through Ca^{2+}, K^+, and Na^+ channels (Antonov and Johnson, 1999; Bara et al., 1993; Michelet-Habchi et al., 2003) and reduces lipids peroxidation of erythrocytes membranes (Abad et al., 2005). It is known that the elevation of intracellular magnesium modifies the main anion exchanger Band 3 (or AE1) in human erythrocytes (Barbul et al., 1999; Gulczynska et al., 2006). In particular, the rate of SO_4^- anions influx (which is going through Band 3) is higher (Teti et al., 2002) in the erythrocytes with increased concentration of intracellular Mg^{2+}.

The main anion exchanger Band 3 is a major membrane glycoprotein in human erythrocytes presented in approximately one million copies per cell and making up to 25% of the cell membrane (Poole, 2000). It mediates a rapid transmembrane exchange of Cl^- for HCO_3^-, thereby enhancing the CO_2/O_2 physiological exchange (since more than 90% of dissolved CO_2 are in the form of HCO_3^- at physiological conditions (Kummerow et al., 2000; Swietach et al., 2010) of pH between 7.0 and 8.0). Also, erythrocytes glycolysis was reported to be modulated by the interaction of Band 3 with glycolytic enzymes (von Ruckmann and Schubert, 2002) and hemoglobin (Weber et al., 2004). While the structure and properties of Band 3 have been extensively studied (Yeagle, 2005; Zhang et al., 2000), much less is known about molecular mechanisms of regulation of anion-exchange function of Band 3. The cytoplasmic domain of Band 3 can be phosphorylated at tyrosine residues (Ferru et al., 2011), and its phosphorylation state is critical for glycolysis, cell shape and anion transport (Merciris et al., 1998; Minetti et al., 2004, 1998; Puceat et al., 1998). The phosphorylation state of Band 3 is determined by the balance between the activities of protein tyrosine kinases p72syk (Harrison et al., 1994) and p53/56lyn (Brunati et al., 1996) (PTKs) and of phosphotyrosine phosphatase PTP1B (Zipser and Kosower, 1996). From *in vitro* studies, employing purified proteins and RBC ghosts, it was shown that Band 3 PTKs are activated by Mg^{2+} ions (Vasseur et al., 1987). The band 3 phosphorylation by PTKs involves the Mg/ATP as a substrate; therefore, a general point of view is that the elevation (Abad et al., 2005) of intracellular Mg^{2+} during magnesium sulfate treatment increases Band 3 phosphorylation state leading to the activation of its anion exchange functionality. However, detailed mechanism of Band 3 activation has not been modeled quantitatively. The situation is complicated by the reports that Mg^{2+} enhances also Band 3 dephosphorylation (Zipser and Kosower, 1996) mediated by PTP1B, which may lead to an opposite (*i.e.* Band 3 deactivation) effect. By contrast, Mg^{2+} may bind directly with Band 3 that affects the charge and functionality of Band 3 (Weber and Voelter, 2004; Weber et al., 2004). Until now, it is not established whether solely the molecular kinetics mechanism of direct complexation of Band 3 with intracellular free Mg^{2+} can quantitatively explain the influence of intracellular free Mg^{2+} on the anion exchange activity of Band 3. Our current research is devoted to the study of this question.

In this work, we carried out experiments to observe the influence of magnesium sulfate treatment (both *in vitro* and *in situ*) on the anion exchange activity of Band 3 in human erythrocytes. We determined the anion exchange activity in conventional units of the “number of active Band 3” per erythrocyte applying a scanning flow cytometry (Maltsev, 2000; Shvalov et al., 1999) method of measuring single erythrocytes during their hemolysis in isotonic solution of ammonium chloride (Chernyshev et al., 2008). For the quantitative investigation of the influence of $MgSO_4$ on Band 3, we introduced a molecular kinetic model, which consists of three parts: 1) influx of Mg^{2+} into erythrocytes obeying Michaelis–Menten kinetics (Chanson et al., 2005); 2) intracellular magnesium buffering (Bock et al., 1991; Gunther, 2007; Raftos et al., 1999); and 3) Band 3 activation by its complexation (Weber and Voelter, 2004; Weber et al., 2004) with free intracellular Mg^{2+}.

Fitting the developed model to the obtained experimental data, we observed a good correspondence between theoretical and experimental curves that allowed us to estimate the model parameters with good confidence. The values of these parameters are in agreement with known literature data on the apparent Mg^{2+} dissociation constant (from 0.01 to 0.1 mM) that reflects experiments on enrichment of Mg^{2+} at the inner erythrocyte membrane (Gunther, 2007). The estimated value of magnesium influx is close to the known range from 35 to 500 $\mu M\,h^{-1}$ (depending on a donor) in human erythrocytes (Flatman, 1991). This work contributes to the molecular kinetic study of the anti-hypoxic effect of magnesium, since the transmembrane anion exchange of Cl^- for HCO_3^- through Band 3 in erythrocytes is associated with CO_2/O_2 physiological exchange. Results of this work extend the opportunities for control and adjustment of the magnesium sulfate treatment for a particular patient.

2. Materials and methods

2.1. Instrumentation

We used a scanning flow cytometer (SFC) (Maltsev, 2000; Strokotov et al., 2011) in order to obtain the number of active Band 3 channels per erythrocyte, as described in detail elsewhere (Chernyshev et al., 2008). SFC allows the measurement of the light-scattering pattern (LSP) from single cells for their morphological characterization (*i.e.* determination of cell's size, refractive index, shape parameters, *etc.*). Accurate LSP measurements were performed with the rate of up to 300 cells per second.

2.2. Blood donors

All donors were women on 30–36 week of pregnancy from the hospital (Medical center of Siberian Branch of the Russian Academy of Science in Novosibirsk), participated in this study in accordance with the ethical standards established by the Declaration of Helsinki. Among them, there were patients with placental insufficiency (at risks for fetal hypoxia and preterm labor) and healthy donors. Patients were treated in the participating hospital by magnesium sulfate during a few days according to the standard protocol of the hospital (5 g of $MgSO_4$ once a day, at the same time each day, in 25% w/w physiological solution intravenously followed by a maintenance dose of 2 g/h (Idama and Lindow, 1998)).

Whole blood was taken by venopuncture and collected in a vacuum tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (9:1 blood:EDTA). Then the cells were used in experiments at room temperature (22 °C) within 3 h (assuming no significant changes of the erythrocytes in whole blood during this time (Hoffmann et al., 2012)).

For *in situ* measurements, the blood was taken once a day, at the same time each day (at 10 a.m.). The first sample of blood was obtained at admission of the patient. Each next sample of blood was obtained 24 h after each next magnesium sulfate injection (during a few days of the therapy).

For *in vitro* study, the blood was taken from the donors, which were not under $MgSO_4$ treatment. Time series of the "number of active Band 3" per erythrocyte, hereinafter referred to as kinetics, were measured as follows. Volume of 0.01 ml of 25% w/w physiological solution of $MgSO_4$ was added to 2 ml sample of whole blood (reaching the concentration of 10 mM for $MgSO_4$ in whole blood) to initiate the reaction of Band 3 activation Then, at certain time points (during 90 min) 8 μl of the mixture was taken (each time) in order to determine the number of active Band 3 per erythrocyte, as described below.

2.3. Determination of the "number of active Band 3" per erythrocyte

The "number of active Band 3" per erythrocyte was obtained using dynamic flow cytometry by a method (Chernyshev et al., 2008) of erythrocytes sphering in a lysing solution of ammonium chloride. To start the measurements, a volume containing 5 μl of blood from a sample tube was placed into a testing tube with 1 ml of the lysing solution to achieve the initial concentration in the range from $0.5\cdot10^7$ to $2\cdot10^7$ cells per milliliter. As lysing solution we used an isotonic (0.15 M) concentration of ammonium chloride (NH_4Cl) in degassed (distilled water was boiled for 10 min) water solution with 1.5 mM buffer HEPES (Sigma) and 1 mM sodium bicarbonate ($NaHCO_3$). The solution provided a constant pH = 7.2 in the external media during hemolysis that was verified independently. The solution was prepared prior to experiments and used within an hour. To ensure homogeneity of the cell suspension the sample was stirred up by pipetting. The overall delay for the sample preparation (before measurements) was about 15 s. Then the testing tube was attached to SFC, and the number of "active Band 3" per cell was obtained from real-time measurements of the erythrocytes sphering rate in the course of hemolysis (Chernyshev et al., 2008).

2.4. Theoretical modeling

For a quantitative analysis of *in vitro* experimental data, we consider a molecular kinetic model of Band 3 activation by $MgSO_4$, which consists of the following three parts:

a) Influx of Mg^{2+} obeys Michaelis–Menten kinetics (Chanson et al., 2005; Flatman, 1991)

$$Mg^{2+}_{out}+M_{ch}\overset{K_m}{\longleftrightarrow}Mg^{2+}_{out}M_{ch}\overset{k_m}{\Longrightarrow}Mg^{2+}_{in}+M_{ch} \tag{1}$$

where M_{ch} is a magnesium transport channel; K_m and k_m are Michaelis–Menten constants of the reaction, and the maximum flux rate is $v_{\max}=k_m[M_{ch}]$;

b) Total intracellular Mg^{2+} content is partitioned between free and bound forms (Bock et al., 1991; Grubbs, 2002; Gunther, 2007; Raftos et al., 1999) (magnesium buffering)

$$\begin{cases} Mg^{2+}_{in}+A_1\overset{K_1}{\longleftrightarrow}MgA_1 \\ Mg^{2+}_{in}+A_2\overset{K_2}{\longleftrightarrow}MgA_2 \\ \ldots \\ Mg^{2+}_{in}+A_m\overset{K_m}{\longleftrightarrow}MgA_m \end{cases} \tag{2}$$

where A_i ($i=1,..,m$) are cytoplasmic buffers; K_1, K_2 and K_i are corresponding association constants;

c) Band 3 is activated due to its complexation (Weber and Voelter, 2004; Weber et al., 2004) with free intracellular Mg^{2+}

$$Mg^{2+}_{in}+B\overset{K_B}{\longleftrightarrow}B_a \tag{3}$$

where B and B_a are the non-active and the active Band 3, correspondingly; K_B is the corresponding association constant.

We follow a traditional assumption that constants (*i.e.* kinetic rate constants, dissociation constants, *etc.*) of the model does not depend (but concentrations of metabolites may depend) on normal or hypoxic states of donors.

It was established by Chanson et al. (2005) that both the influx and the efflux of Mg^{2+} in erythrocytes obey Michaelis–Menten kinetics with approximately the same values of the constants K_{m} and $v_{\max}$ (*i.e.* $K_m^{efflux}\approx K_m^{influx}$ and $v_{\max}^{efflux}\approx v_{\max}^{influx}$). Therefore, one can use reported values of the constants $K_m\approx1$ mM and $v_{\max}\approx35$–$500\ \mu\mathrm{M\,h^{-1}}$ (depending on a donor) (Flatman, 1991) for Mg^{2+} efflux in human erythrocytes as approximate values of these constants for Mg^{2+} influx, and vice versa. Thus, since the extracellular magnesium concentrations $[Mg^{2+}_{out}]\gg1\ \mathrm{mM}\approx K_m$ in our *in vitro* experiments (the concentration of $MgSO_4$ is 10 mM), the influx reached its maximum rate of $v_{\max}$. On the other hand, the efflux rate is much less than $v_{\max}$ due to low intracellular magnesium concentration: $[Mg^{2+}_{in}]\approx0.2\ \mathrm{mM}\ll K_m$. Therefore, under our experimental conditions *in vitro*, the efflux can be neglected and the influx can be considered at maximum rate $v_{\max}$, which is independent on the extracellular magnesium concentration $[Mg^{2+}_{out}]$. However, that is not a principal limitation and the model can be easily extended to include both fluxes and any values of $[Mg^{2+}_{in}]$ and $[Mg^{2+}_{out}]$.

The number of active Band 3 per erythrocyte, B_a, can be derived according to reaction scheme (3):

$$B_a=\frac{B_0}{1+\frac{K_B}{[Mg^{2+}_{in}]}} \tag{4}$$

where B_0 is the total number of Band 3 in the cell. The influx of magnesium elevates the amount of intracellular Mg^{2+} that can be expressed according to reactions schemes (1) and (2) and assuming the above mentioned maximum rate $v_{\max}$ for Mg^{2+} influx:

$$\left([Mg^{2+}_{in}]+\sum_{i=1}^{m}\frac{[A_i]_0[Mg^{2+}_{in}]}{[Mg^{2+}_{in}]+K_i}\right)V=N+v_{\max}t \tag{5}$$

where V is the cell volume; N is the total amount of intracellular Mg^{2+} at the beginning (*i.e.* at $t=0$); square brackets denotes concentration; the subscript "0" means the total content of the corresponding reagent (*i.e.* the sum of its free and bound forms). Taking into account typical concentrations (Gunther, 2007; Raftos et al., 1999) of free Mg^{2+} ($\left[Mg_{in}^{2+}\right]\sim 0.2$ mM) and buffers (adenosine triphosphate [ATP]$\sim$1.65 mM; adenosine diphosphate [ADP]$\sim$0.2 mM; 2, 3-diphosphoglycerate [DPG]$\sim$4.5 mM; hemoglobin [Hb]$\sim$5 mM, *etc.*) in human erythrocytes, as well as corresponding association constants (Bock et al., 1991; Gunther, 2007) ($K_{ATP}=0.05$ mM, $K_{ADP}=0.4$ mM, $K_{DPG}=1.2$ mM, $K_{Hb}\sim$ 65–400 mM, *etc.*), the left side of Eq. (5) can be approximated with rather good accuracy (the relative error is less than 10%) by a more simple

Table 1
Values of cell variables and constants required for inclusion in the model equations.

Variables and constants	Values (mM)	References
$[Mg_{in}^{2+}]$	~0.2	(Gunther, 2007)
[ATP]	~1.65	(Gunther, 2007)
[ADP]	~0.2	(Raftos et al., 1999)
[DPG]	~4.5	(Gunther, 2007)
[Hb]	~5	(Raftos et al., 1999)
K_m	1	(Chanson et al., 2005)
K_{ATP}	0.05	(Bock et al., 1991)
K_{ADP}	0.4	(Gunther, 2007)
K_{DPG}	1.2	(Bock et al., 1991)
K_{Hb}	~65–400	(Gunther, 2007)

Table 2
Initial values and day-to-day linear trend of B_a and C_a of patients under magnesium sulfate treatment, as shown in Figs. 1(A–E) and 2(A–E). For each donor, standard errors of B_a and C_a were evaluated using 30 time points of experimental kinetics of 10,000 erythrocytes measured by SFC during their sphering in the lysing solution.

Patient	Initial B_a, 10^6	Trend of B_a, 10^6 day^{-1}	Initial C_a, μM	Trend of C_a, μM day^{-1}
1	0.63 ± 0.06	0.21 ± 0.12	0.36 ± 0.19	1.2 ± 0.8
2	0.83 ± 0.08	0.07 ± 0.06	5.2 ± 0.3	0.7 ± 0.3
3	0.42 ± 0.04	0.14 ± 0.03	2.28 ± 0.12	0.8 ± 0.2
4	0.21 ± 0.02	0.09 ± 0.08	1.36 ± 0.06	0.6 ± 0.4
5	0.35 ± 0.04	0.15 ± 0.02	2.48 ± 0.12	0.80 ± 0.12

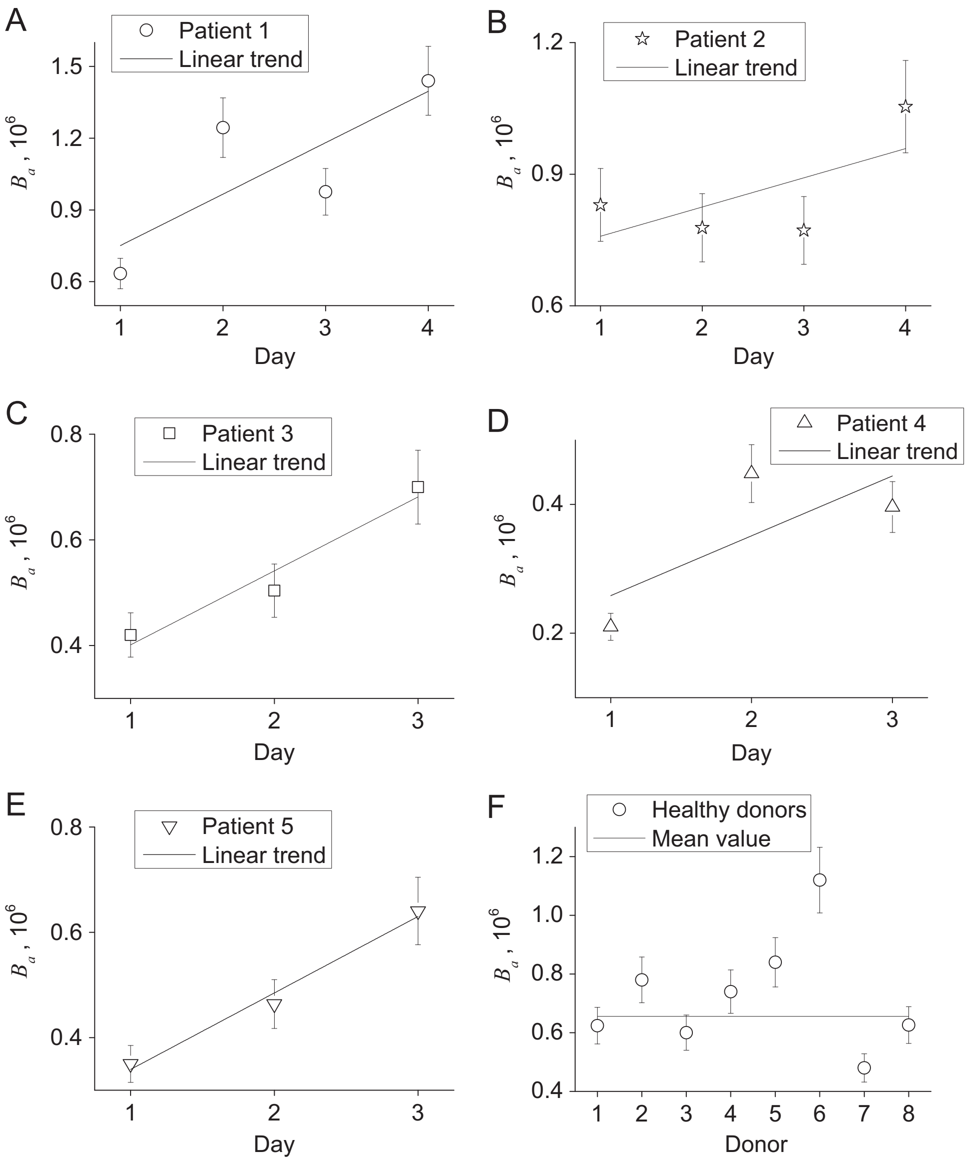

Fig. 1. *In situ* trend of the number of active Band 3 per erythrocyte (dots – experimental data; solid lines – linear fit): A–E – patients under day-to-day $MgSO_4$ treatment; F – healthy donors. For each donor, standard errors of B_a were evaluated using 30 time points of experimental kinetics of 10,000 erythrocytes measured by SFC during their sphering in the lysing solution.

expression:

$$[\mathrm{Mg}^{2+}_{in}]\left(1+\frac{[DPG]_0}{K_{DPG}}\right)V+[ATP]_0\;V=N+v_{\max}\,t \qquad (6)$$

It should be noted that the value of association constant K_{DPG} (that is important for derivation of Eq. (6)) varies significantly in different publications. In this work, we took the value of 1.2 mM for K_{DPG} obtained by Bock et al. (1991) with ^{25}Mg NMR spectroscopy (and it agrees with 1.5 mM measured by Gupta et al. (1978) with ^{31}P NMR spectroscopy), whereas a questionable value of 0.1 mM for K_{DPG} used in 1999 by Raftos et al. (1999) was taken from much yearlier publication of Achilles et al. (1972) and was not supported by later measurements. The development of new techniques during the 1980s has provided more reliable data related to the regulation of cellular magnesium (Grubbs, 2002).

Table 3
Values of B_a and C_a for healthy donors, as shown in Figs. 1F and 2F. For each donor, standard errors of B_a and C_a were evaluated using 30 time points of experimental kinetics of 10,000 erythrocytes measured by SFC during their sphering in the lysing solution.

Donor	B_a, 10^6	C_a, μM
1	0.62 ± 0.06	4.1 ± 0.4
2	0.78 ± 0.08	5.6 ± 0.6
3	0.60 ± 0.06	3.8 ± 0.4
4	0.74 ± 0.07	5.1 ± 0.5
5	0.84 ± 0.08	6.0 ± 0.6
6	1.12 ± 0.11	6.9 ± 0.7
7	0.48 ± 0.05	3.3 ± 0.3
8	0.63 ± 0.06	4.7 ± 0.5

One can see from Eq. (6) that the only significant buffers of magnesium in erythrocytes (in our model) are ATP and DPG. Combining Eqs. (4) and (6) the following expression for the number of active Band 3 per cell as a function of time is derived:

$$B_a(t)=\frac{B_0}{1+\frac{1/\gamma-1}{t/\tau+1}} \qquad (7)$$

where γ is the fraction of initially active Band 3 (*i.e.* $B_a(0)=\gamma B_0$):

$$\gamma=\left[1+\frac{K_B V}{N-[ATP]_0\;V}\left(1+\frac{[DPG]_0}{K_{DPG}}\right)\right]^{-1} \qquad (8)$$

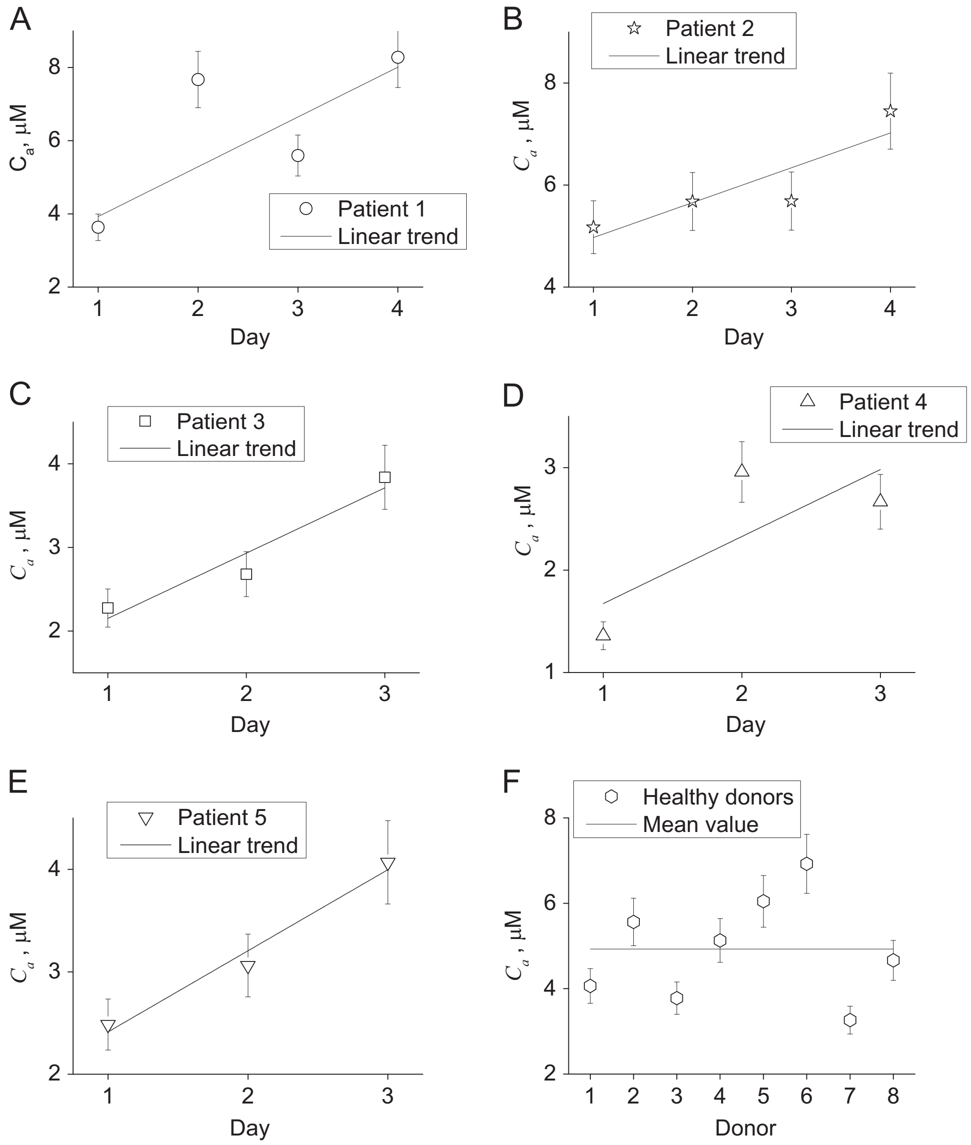

Fig. 2. *In situ* trend of the concentration of active Band 3 in blood (dots – experimental data; solid lines – linear fit): A–E – patients under day-to-day $MgSO_4$ treatment; F – healthy donors. For each donor, standard errors of C_a were evaluated using 30 time points of experimental kinetics of 10,000 erythrocytes measured by SFC during their sphering in the lysing solution.

and τ is the characteristic time of Band 3 activation in the presence of extracellular $MgSO_4$.

$$\tau = \frac{N - [ATP]_0 \;\; V}{v_{\max}} \tag{9}$$

Eq. (7) was used in this work to fit time-resolved experimental data obtained *in vitro*. Values of the model constants and assumed ranges of metabolites concentrations are summarized in Table 1.

3. Results

3.1. *In situ effect of magnesium sulfate treatment on Band 3 of patients*

In situ measurements showed a positive day-to-day trend in the number of active Band 3 per erythrocyte for all five patients (with placental insufficiency), which were under magnesium sulfate treatment, as presented in Fig. 1(A–E) and Table 2. The first day in Fig. 1(A–E) corresponds to the admission of the patient, and the first sample of blood was obtained prior to the first injection of magnesium sulfate. Each next day corresponds to the sample of blood obtained 24 h after each next magnesium sulfate injection. For comparison, the number of active Band 3 per erythrocyte of healthy donors is presented in Fig. 1(F) and Table 3. Interestingly, a positive day-to-day trend had been observed also for the concentration of erythrocytes in blood of some patients under the treatment. Thus, the concentration of active Band 3 in whole blood (*i.e.* the product of the erythrocytes concentration on the number of active Band 3 per cell), which we denoted as C_a, was increasing for these patients even faster than the number of active Band 3 per cell, as presented in Figs 2(A–E), 3(A and B) and Table 2. For a comparison, the concentration of active Band 3 measured in blood of healthy donors is presented in Fig. 2(F) and Table 3. As one can see from Figs. 1–3 and Table 4, active Band 3 values of patients are low initially but increasing during few days of $MgSO_4$ treatment approaching (within the standard deviation) the range of healthy donors.

3.2. *In vitro kinetics of Band 3 activation by extracellular magnesium sulfate*

In vitro experimental kinetic curves on Band 3 activation obtained from 4 heathy donors are presented in Fig. 4 (dots). These data were fitted with Eq. (7) using a standard Levenberg–Marquardt least squares algorithm (Gill and Murray, 1978) as it is realized in Origin software (Wheeler, 1993) produced by OriginLab Corporation. The best fitted theoretical curves are shown in Fig. 4 (solid lines), and corresponding obtained values of the model parameters γ, τ and B_0 are presented in Table 5.

As one can see from Table 5, the relative variation of donors on the parameter τ (corresponding to the magnesium influx rate) is greater than the variation on the parameter γ (corresponding to the concentrations of intracellular Mg^{2+}, ATP and DPG). That is in agreement with known reports in the literature that the relative variation of the magnesium influx rate (Flatman, 1991) among donors is much larger than the relative variation of the intracellular concentrations of Mg^{2+}, ATP and DPG (Raftos et al., 1999).

Taking into account typical values of [ATP]~1.65 mM, [DPG]~4.5 mM, $K_{DPG} = 1.2$ mM, $N/V \sim 2$ mM, the mean values of parameters $\gamma \sim 0.5$ and $\tau \sim 40\ \text{min}^{-1}$ from Table 5 one can estimate (using Eqs. (8) and (9)) the association constant of Mg^{2+} with Band 3 as $K_B \sim 0.07$ mM and the rate of magnesium influx as $v_{\max} \sim 0.01\ \text{mM min}^{-1}$, correspondingly. The estimated value of $v_{\max}$ is close to the known range from 35 to 500 μM h^{-1} (depending on a donor) in human erythrocytes (Flatman, 1991). As for K_B, in the literature there are no reports on its direct measurements, but our estimation of K_B is in agreement with known values of the apparent Mg^{2+} dissociation constant (from 0.01 to 0.1 mM) that reflects experiments on enrichment of Mg^{2+} at the inner erythrocyte membrane (Gunther, 2007).

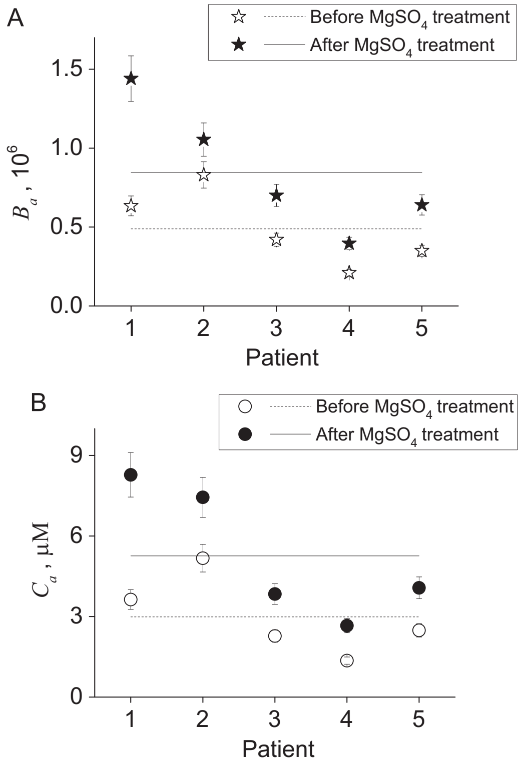

Fig. 3. *In situ* data on B_a (A) and C_a (B) of patients before (empty dots) and after (filled dots) $MgSO_4$ treatment (4 day for patients 1 and 2; and 3 days for patients 3, 4, and 5). Dash and solid lines indicate corresponding mean values (among patients). For each donor, standard errors of B_a and C_a were evaluated using 30 time points of experimental kinetics of 10,000 erythrocytes measured by SFC during their sphering in the lysing solution.

Table 4
Mean and standard deviation (S.D.) of B_a and C_a of patients under $MgSO_4$ and healthy donors, as shown in Figs. 1 and 2.

	Mean B_a, 10^6	S.D. of B_a, 10^6	Mean C_a, μM	S.D. of C_a, μM
Patients before treatment	0.73 ± 0.07	0.20 ± 0.05	4.9 ± 0.4	1.2 ± 0.3
Patients after treatment	0.8 ± 0.2	0.41 ± 0.11	5.3 ± 1.1	2.5 ± 0.7
Healthy donors	0.73 ± 0.07	0.20 ± 0.05	4.9 ± 0.4	1.2 ± 0.3

4. Discussion

The influence of magnesium sulfate on the activity of Band 3 in erythrocytes is interesting from medical point of view due to a key role of Band 3 in CO_2/O_2 physiological exchange. Carbon dioxide is more readily dissolved in deoxygenated blood (the Haldane effect), facilitating its removal from the body after the oxygen has been released to tissues undergoing metabolism. In erythrocytes, carbon dioxide binds to hemoglobin and forms carbaminohemoglobin (Guyton and Hall, 2006) that decreases hemoglobin's affinity for oxygen (the Bohr effect). Conversely, when the carbon dioxide levels in the blood decrease (in lung), carbon dioxide is released from hemoglobin, increasing the oxygen affinity of the

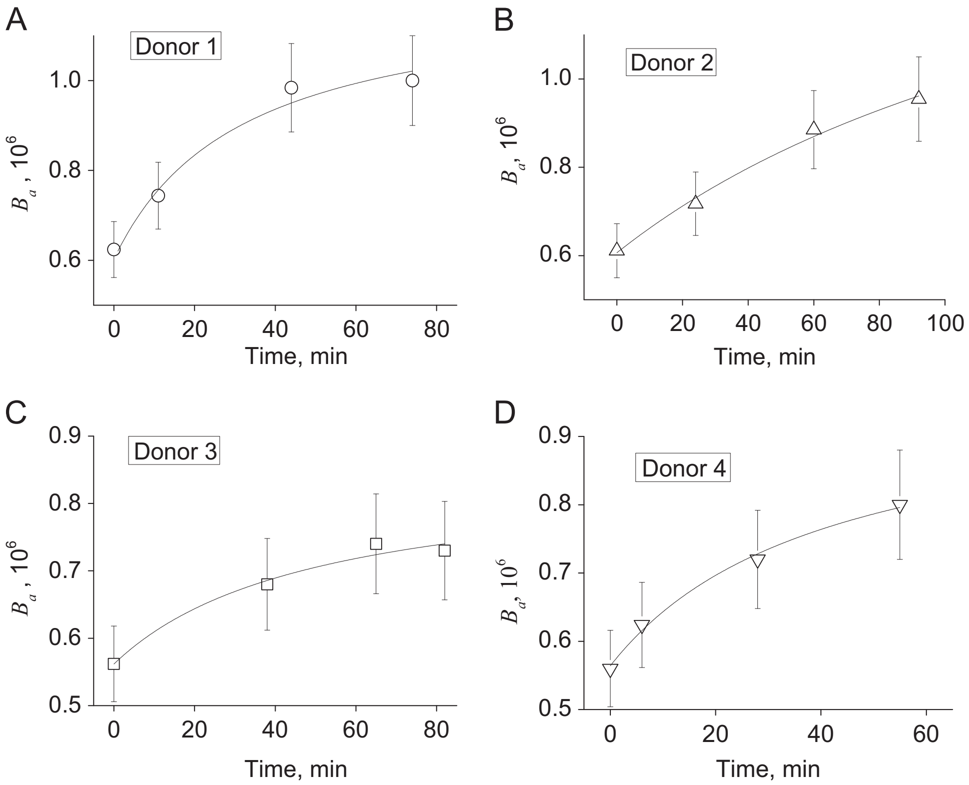

Fig. 4. *In vitro* kinetics of Band 3 activation by $MgSO_4$ in blood of 4 healthy donors (A–C): dots – experiment, solid line – theoretical best fit with model Eq. (7). These donors are not ones from Table 2 and/or 3.

hemoglobin. Major amount ($>$ 90%) of carbon dioxide inside and outside erythrocytes in blood is converted to anions HCO_3^- (the enzyme carbonic anhydrase accelerates this reaction) (Swietach et al., 2010), which penetrate through erythrocyte's membrane by HCO_3^-/Cl^- transmembrane exchange via Band 3. Because the transmembrane permeability of HCO_3^- in erythrocytes is much less (Chernyshev et al., 2008) than the permeability of such neutral molecules as CO_2 and O_2, therefore, the rate of Band 3 functioning in erythrocytes limits CO_2/O_2 physiological exchange rate. The estimated rate of HCO_3^-/Cl^- exchange (Chernyshev et al., 2008) by erythrocytes of blood passed through the human lung corresponds to the rate of oxygen consumption of a human body (Loer et al., 1997). That is particularly important for prenatal medicine. In the literature, there are only a few publications related to the influence of magnesium sulfate on the activity of Band 3 from the viewpoint of hypoxia, and the molecular mechanisms of the influence is unclear. It was reported (Zylinska et al., 2002) that in the case of hypoxia the amount of active Band 3 per erythrocyte of asphyxiated neonates was decreased in comparison with healthy newborns. *In situ*, it was published (Gulczynska et al., 2006; Szemraj et al., 2005) that magnesium sulfate treatment significantly increases the activity Band 3 of asphyxiated newborns. *In vitro*, the increasing of Band 3 anion transport by elevation of intracellular Mg^{2+} was reported (Teti et al., 2002), however only for SO_4^{2-} ions.

Our results demonstrate, for the first time, that HCO_3^-/Cl^- exchange rate via Band 3 is increased by elevation of intracellular Mg^{2+} during $MgSO_4$ treatment of erythrocytes. In order to quantitatively characterize the functional activity of Band 3 we applied a method of scanning flow cytometry (Chernyshev et al., 2008) in real-time experiments.

Our *in situ* measurements demonstrated a significant positive day-to-day trend of the number (per cell) and concentration of Band 3 in blood of patients with placental insufficiency, which were under $MgSO_4$ treatment in the participating hospital. Surprisingly, a positive day-to-day trend had been observed also for the concentration of erythrocytes in blood of some patients that leads to the increase of possible anti-hypoxic effect of $MgSO_4$ for them due to elevation of Band 3 concentration in blood. Both the number of Band 3 per cell and the concentration of Band 3 in blood of all patients were relatively low at admission, but increased during MgSO4 treatment and approached the range of healthy donors after three-four days of the treatment.

Table 5
Obtained parameters of the model from *in vitro* experimental data. These donors are not ones from Tables 2 and/or 3.

Donor	γ	τ, min	B_0, 10^6
1	0.51 ± 0.07	20 ± 8	1.20 ± 0.16
2	0.36 ± 0.13	70 ± 20	1.8 ± 0.4
3	0.66 ± 0.10	40 ± 15	0.84 ± 0.12
4	0.58 ± 0.06	30 ± 10	0.96 ± 0.10

Our *in vitro* measurements may partly explain the molecular mechanisms of $MgSO_4$ action in erythrocytes. For a quantitative analysis of the obtained experimental data *in vitro*, we introduced a molecular kinetic model, which describes Band 3 activation by its complexation with free intracellular Mg^{2+} taking into account intracellular magnesium buffering and Michaelis–Menten kinetics of magnesium transport through erythrocytes membrane. In the model, we assume that 4 parameters of erythrocytes ($v_{\max}$, B_0, [ATP], [DPG]) are not changed significantly during the measurements. From this point of view, a healthy individual and a patient may differ in some of these parameters or/and $\left[Mg_{in}^{2+}\right]$ that affects the obtained values of B_a, γ, τ and B_0. A good agreement between theoretical and experimental curves supports the model. The values of parameters obtained by a standard fitting procedure from *in vitro* experimental data are in agreement with known literature data. Using this model, we for the first time estimated the association constant of Mg^{2+} with Band 3 as $K_B \sim 0.07$ mM.

Results of this work partly clarify the molecular mechanisms of $MgSO_4$ action in human erythrocytes. The method developed allows one to obtain the following characteristics of erythrocytes, which are important to quantitatively estimate the perspectives of $MgSO_4$ treatment for a particular patient: 1) the fraction of initially active Band 3; 2) the maximum number of Band 3, which can be activated; 3) the characteristic time of Band 3 activation. It should be helpful particularly in prenatal medicine also for early detection of pathologies associated with the risk of fetal hypoxia.

Acknowledgments

The *in situ* work was supported by Russian Foundation for Basic Research (Grants numbers 16-04-01283 and 16-34-00476), whereas Russian Science Foundation (Grant number 14-15-00155) supported *in vitro* research. D.I. Strokotov also acknowledge the support by the Stipend of the President of Russian Federation for young scientists.

References

Abad, C., Teppa-Garran, A., Proverbio, T., Pinero, S., Proverbio, F., Marin, R., 2005. Effect of magnesium sulfate on the calcium-stimulated adenosine triphosphatase activity and lipid peroxidation of red blood cell membranes from preeclamptic women. Biochem. Pharmacol. 70, 1634–1641.

Abad, C., Carrasco, M.J., Piñero, S., Delgado, E., Chiarello, D.I., Teppa-Garrán, A., Proverbio, T., Proverbio, F., Marín, R., 2010. Effect of magnesium sulfate on the osmotic fragility and lipid peroxidation of intact red blood cells from pregnant women with severe preeclampsia. Hypertens. Pregnancy 29, 38–53.

Achilles, W., Cumme, G.A., Hoppe, H., 1972. 2, 3-Diphosphoglycerate: acid dissociation and complex formation with magnesium, potassium, and sodium. Acta Biol. Med. Ger. 29, 531–538.

Antonov, S., Johnson, J.W., 1999. Permeant ion regulation of N-methyl-daspartate receptor channel block by Mg2. Proc. Natl. Acad. Sci. USA 96, 14571–14576.

Ariza, A.C., Bobadillab, N., Fernandez, C., Munoz-Fuentes, R.M., Larrea, F., Halhali, A., 2005. Effects of magnesium sulfate on lipid peroxidation and blood pressure regulators in preeclampsia. Clin. Biochem. 38, 128–133.

Bara, M., Guiet-Bara, A., Durlach, J., 1993. Regulation of sodium and potassium pathways by magnesium in cell membranes. Magnes. Res. 6, 167–177.

Barbul, A., Zipser, Y., Nachles, A., Korenstein, R., 1999. Deoxygenation and elevation of intracellular magnesium induce tyrosine phosphorylation of band 3 in human erythrocytes. FEBS Lett. 455, 87–91.

Beydemir, S., Ciftci, M., Ozmen, I., Okuroglu, M.E.B., Ozdemir, H., Kufrevioglu, O.I., 2000. Effects of some medical drugs on enzyme activities of carbonic anhydrase from human erythrocytes in vitro and from rat erythrocytes in vivo. Pharmacol. Res. 42, 187–191.

Blitz, M., Blitz, S., Hughes, R., Diner, B., Beasley, R., Knopp, J., Rowe, B.H., 2005. Aerosolized magnesium sulfate for acute asthma: a systematic review. Chest 128, 337–344.

Bock, J.L., Crull, G.B., Wishnia, A., Springer, C.S., 1991. 25 Mg NMR studies of magnesium binding to erythrocyte constituents. J. Inorg. Biochem. 44, 79–87.

Brunati, A.M., Bordin, L., Clari, G., Moret, V., 1996. The lyn-catalyzed tyr phosphorylation of the transmembrane band-3 protein of human erythrocytes. Eur. J. Biochem. 240, 394–399.

Chanson, A., Feillet-Coudray, C., Gueux, E., Coudray, C., Rambeau, M., Mazur, A., Wolf, F.I., Rayssiguier, Y., 2005. Evaluation of magnesium fluxes in rat erythrocytes using a stable isotope of magnesium. Front. Biosci. 10, 1720–1726.

Chernyshev, A.V., Tarasov, P.A., Semianov, K.A., Nekrasov, V.M., Hoekstra, A.G., Maltsev, V.P., 2008. Erythrocyte lysis in isotonic solution of ammonium chloride: Theoretical modeling and experimental verification. J. Theor. Biol. 251, 93–107.

Deering, S.H., Stagg, A.R., Spong, C.Y., Abubakar, K., Pezzullo, J.C., Ghidini, A., 2005. Antenatal magnesium treatment and neonatal illness severity as measured by the Score for Neonatal Acute Physiology (SNAP). J. Matern. Fetal Neonatal. Med. 17, 151–155.

Doyle, L.W., Crowther, C.A., Middleton, P., Marret, S., 2009. Antenatal magnesium sulfate and neurologic outcome in preterm infants: a systematic review. Obstet. Gynecol. 113, 1327–1333.

Duley, L., Neilson, J.P., 1999. Magnesium sulphate and pre-eclampsia. Trial needed to see whether it's as valuable in pre-eclampsia as in eclampsia. Br. Med. J. 319, 3–4.

Elimian, A., Verma, R., Ogburn, P., Wiencek, V., Spitzer, A., Quirk, J.G., 2002. Magnesium sulfate and neonatal outcomes of preterm neonates. J. Matern. Fetal Neonatal. Med. 12, 118–122.

Euser, A.G., Cipolla, M.J., 2009. Magnesium sulfate for the treatment of eclampsia: a brief review. Stroke 40, 1169–1175.

Fawcett, W.J., Haxby, E.J., Male, D.A., 1999. Magnesium: physiology and pharmacology. Br. J. Anaesth. 83, 302–320.

Ferru, E., Giger, K., Pantaleo, A., Campanella, E., Grey, J., Ritchie, K., Vono, R., Turrini, F., Low, P.S., 2011. Regulation of membrane-cytoskeletal interactions by tyrosine phosphorylation of erythrocyte band 3. Blood 117, 5998–6006.

Flatman, P.W., 1991. Mechanisms of magnesium transport. Annu. Rev. Physiol. 53, 259–271.

Gill, P.E., Murray, W., 1978. Algorithms for the solution of the nonlinear least-squares problem. SIAM J. Numer. Anal. 15, 977–992.

Grubbs, R.D., 2002. Intracellular magnesium and magnesium buffering. BioMetals 15, 251–259.

Gulczynska, E., Gadzinowski, J., Wilczynski, J., Zylinska, L., 2006. Prenatal MgSO4 treatment modifies the erythrocyte band 3 in preterm neonates. Pharmacol. Res. 53, 347–352.

Gunther, T., 2007. Total and free Mg^{2+} contents in erythrocytes: a simple but still undisclosed cell model. Magnes Res 20, 161–167.

Gupta, R.K., Benovic, J.L., Rose, Z.B., 1978. The determination of the free magnesium level in the human red blood cell by 31P NMR. J. Biol. Chem. 253, 6172–6176.

Guyton, A.C., Hall, J.E., 2006. Textbook of Medical Physiology. Elsevier Saunders, Philadelphia.

Harrison, M.L., Isaacson, C.C., Burg, D.L., Geahlen, R.L., Low, P.S., 1994. Phosphorylation of human erythrocyte band 3 by endogenous p72syk. J. Biol. Chem. 269, 955–959.

Hoffmann, J.J., van den Broek, N.M., Curvers, J., 2012. Reference intervals of extended erythrocyte and reticulocyte parameters. Clin. Chem. Lab. Med. 50, 941–948.

Idama, T.O., Lindow, S.W., 1998. Magnesium sulphate: a review of clinical pharmacology applied to obstetrics. Br. J. Obstet. Gynaecol. 105, 260–268.

Kummerow, D., Hamann, J., Browning, J.A., Wilkins, R., Ellory, J.C., Bernhardt, I., 2000. Variations of intracellular pH in human erythrocytes via $K^+(Na^+)/H^+$ exchange under low ionic strength conditions. J. Membr. Biol. 176, 207–216.

Loer, S.A., Scheeren, T.W., Tarnow, J., 1997. How much oxygen does the human lung consume? Anesthesiology 86, 532–537.

Maltsev, V.P., 2000. Scanning flow cytometry for individual particle analysis. Rev. Sci. Instrum. 71, 243–255.

McLean, R.M., 1994. Magnesium and its therapeutic uses: a review. Am. J. Med. 96, 63–76.

Merciris, P., Hardy-Dessources, M.D., Sauvage, M., Giraud, F., 1998. Involvement of deoxygenation-induced increase in tyrosine kinase activity in sickle cell dehydration. Pflug. Arch. 436, 315–322.

Michelet-Habchi, C., Barberet, P., Dutta, R., Moretto, P., Guiet-Bara, A., Bara, M., 2003. Elemental maps in human allantochorial placental vessels cells. 2. $MgCl_2$ and $MgSO_4$ effects. Magnes Res 16, 171–175.

Minetti, G., Ciana, A., Balduini, C., 2004. Differential sorting of tyrosine kinases and phosphotyrosine phosphatases acting on band 3 during vesiculation of human erythrocytes. Biochem. J. 377, 489–497.

Minetti, G., Seppi, C., Ciana, A., Balduini, C., Low, P.S., Brovelli, A., 1998. Characterization of the hypertonically induced tyrosine phosphorylation of erythrocyte band 3. Biochem. J. 335, 305–311.

Mittendorf, R., Roizen, N.J., Pryde, P.G., 2004. The risks of extending the controversial use of tocolytic magnesium sulfate for the purpose of neuroprotection in preterm labor. J. Perinatol. 24, 465–466.

Mittendorf, R., Dambrosia, J., Pryde, P.G., Lee, K.S., Gianopoulos, J.G., Besinger, R.E., Tomich, P.G., 2002. Association between the use of antenatal magnesium sulfate in preterm labor and adverse health outcomes in infants. Am. J. Obstet. Gynecol. 186, 1111–1118.

Murata, Y., Itakura, A., Matsuzawa, K., Okumura, A., Wakai, K., Mizutani, S., 2005. Possible antenatal and perinatal related factors in development of cystic periventricular leukomalacia. Brain Dev. 27, 17–21.

Poole, J., 2000. Red cell antigens on band 3 and glycophorin A. Blood Rev. 14, 31–43.

Puceat, M., Roche, S., Vassort, G., 1998. Src family tyrosine kinase regulates intracellular pH in cardiomyocytes. J. Cell Biol. 141, 1637–1646.

Raftos, J.E., Lew, V.L., Flatman, P.W., 1999. Refinement and evaluation of a model of Mg^{2+} buffering in human red cells. Eur. J. Biochem. 263, 635–645.

Shvalov, A.N., Soini, J.T., Chernyshev, A.V., Tarasov, P.A., Soini, E., Maltsev, V.P., 1999. Light-scattering properties of individual erythrocytes. Appl. Opt. 38, 230–235.

Strokotov, D.I., Moskalensky, A.E., Nekrasov, V.M., Maltsev, V.P., 2011. Polarized light-scattering profile-advanced characterization of nonspherical particles with the scanning flow cytometry. Cytom. A 79A, 570–579.

Sugiyama, A., Xue, Y., Hagihara, A., Saitoh, M., Hashimoto, K., 1996. Characterization of magnesium sulfate as an antiarrhythmic agent. J. Cardiovasc. Pharmacol. Ther. 1, 243–254.

Swaminathan, R., 2003. Magnesium metabolism and its disorders. Clin. Biochem. Rev. 24, 47–66.

Swietach, P., Tiffert, T., Mauritz, J.M.A., Seear, Rachel, Esposito, Alessandro, Kaminski, C.F., Lew, V.L., Vaughan-Jones, R.D., 2010. Hydrogen ion dynamics in human red blood cells. J. Physiol. 588, 4995–5014.

Szemraj, J., Sobolewska, B., Gulczynska, E., Wilczynski, J., Zylinska, L., 2005. Magnesium sulfate effect on erythrocyte membranes of asphyxiated newborns. Clin. Biochem. 38, 457–464.

Teti, D., Venza, I., Crupi, M., Busa, M., Loddo, S., Romano, L., 2002. Anion transport in normal erythrocytes, sickle red cells, and ghosts in relation to hemoglobins and magnesium. Arch. Biochem. Biophys. 403, 149–154.

Ulger, Z., Ariogul, S., Cankurtaran, M., Halil, M., Yavuz, B.B., Orhan, B., Kavas, G.O., Aribal, P., Canlar, S., Dede, D.S., Ozkayar, N., Akyol, O., 2010. Intra-erythrocyte magnesium levels and their clinical implications in geriatric outpatients. J. Nutr. Health Aging 14, 810–814.

Vasseur, C., Piau, J.P., Bursaux, E., 1987. Cation dependence of the phosphorylation of specific residues in red cell membrane protein band 3. Biochim. Biophys. Acta 899, 1–8.

von Ruckmann, B., Schubert, D., 2002. The complex of band 3 protein of the human erythrocyte membrane and glyceraldehyde-3-phosphate dehydrogenase: stoichiometry and competition by aldolase. Biochim. Biophys. Acta 1559, 43–55.

Weber, R.E., Voelter, W., 2004. 'Novel' factors that regulate oxygen binding in vertebrate hemoglobins. Micron 35, 45–46.

Weber, R.E., Voelter, W., Fago, A., Echner, H., Campanella, E., Low, P.S., 2004. Modulation of red cell glycolysis: interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R454–R464.

Wheeler, K.A., 1993. Computer software reviews. J. Am. Chem. Soc. 115, 3396 3396.

Yeagle, P.L, 2005. The Structure of Biological Membranes. CRC Press LLC, USA.

Zhang, D., Kiyatkin, A., Bolin, J.T., Low, P.S., 2000. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. Blood 96, 2925–2933.

Zipser, Y., Kosower, N.S., 1996. Phosphotyrosine phosphatase associated with band 3 protein in the human erythrocyte membrane. Biochem. J. 314, 881–887.

Zylinska, L., Sobolewska, B., Gulczynska, E., Ochedalski, T., Soszynski, M., 2002. Protein kinases activities in erythrocyte membranes of asphyxiated newborns. Clin. Biochem. 35, 93–98.