Biorheology 51 (2014) 135–145
DOI 10.3233/BIR-140664
IOS Press

Signaling pathways regulating red blood cell aggregation

Alexei Muravyov * and Irina Tikhomirova
Department of Medicine and Biology, State Pedagogical University, Yaroslavl, Russia

Received 23 January 2014
Accepted in revised form 29 May 2014

Abstract. The exposure of red blood cells (RBC) to some hormones (epinephrine, insulin and glucagon) and agonists of α- and β-adrenergic receptors (phenylephrine, clonidine and isoproterenol) may modify RBC aggregation (RBCA). Prostaglandin E_1 (PGE_1) significantly decreased RBCA, and PGE_2 had a similar but lesser effect. Adenylyl cyclase (AC) stimulator forskolin added to RBC suspension, caused a decrease of RBCA. More marked lowering of RBCA occurred after RBC treatment by dB-cAMP. Phosphodiesterase (PDE) inhibitors markedly reduced RBCA. Ca^{2+} influx stimulated by A23187 was accompanied by an increase of RBCA. The blocking of Ca^{2+} entry into the RBC by verapamil or the chelation of Ca^{2+} by EGTA led to a significant RBCA decrease. Lesser changes of aggregation were found after RBC incubation with protein kinase C stimulator phorbol 12-myristate 13-acetate (PMA). A significant inhibitory effect of tyrosine protein kinase (TPK) activator cisplatin on RBCA was revealed, while selective TPK inhibitor, lavendustin, eliminated the above mentioned effect. Taken together, the data demonstrate that changes in RBCA are connected with activation of different intracellular signaling pathways. We suggest that alterations in RBCA are mainly associated with the crosstalk between the adenylyl cyclase-cAMP system and Ca^{2+} control mechanisms.

Keywords: Erythrocyte aggregation, adenylyl cyclase, phosphodiesterases, intracellular signaling pathways, intracellular calcium, protein kinases

1. Introduction: Red blood cell aggregation and cell signaling

The erythrocyte or red blood cell (RBC) has often been regarded as simply a vehicle for the circulation of hemoglobin and transport of oxygen. However, it has become evident that this cell also participates in the regulation of vascular caliber in microcirculation [3,48] through effects of its microrheological properties, namely aggregation and deformability [6,12]. Alteration of red blood cell aggregation (RBCA) has been observed in many physiological and pathophysiological states [7,34,38,60]. The reversible aggregation of red blood cells affects the low shear blood viscosity and microvascular flow dynamics, and can be markedly enhanced in several diseases [7,37,57]. Likewise, the red blood cell aggregation at low flow rates increases venous vascular resistance [6,8,11]. In the past most reports dealt primarily with the ability of plasma proteins (mainly fibrinogen) and higher molecular weight polymers to promote aggregation [8,9,11,22,34,54]. Studies specifically focused on cellular factors and mechanisms of RBC aggregation began in the 1980s [37]. However, the molecular mechanisms regulating aggregate formation are not so well understood [9,22]. RBC were historically considered inert to regulatory signals from

*Address for correspondence: Prof. Alexei Muravyov, Department of Medicine and Biology, State Pedagogical University, Respublikanskaya 108, Yaroslavl 150000, Russia. Fax.: +4852 30 55 96; E-mail: alexei.47@mail.ru.

other cells. However, they are well equipped with the machinery required for intercellular communication [23,25,39,46,58]. It has been proven experimentally that the RBC membrane contains α- and β-adrenoreceptors [51,61], insulin receptors [10,67], endothelin-1 receptors [53], and cholinergic muscarinic receptors [62]. Indeed, marked changes of RBC deformability and aggregation have been shown to be mediated by an activation of cellular signaling pathways [31,35,45,53,59].

The present study was designed to explore the effects of various signaling molecules on RBCA as a basis for the understanding the control mechanisms of this cellular behaviour.

2. Materials and methods

2.1. Preparation of blood samples

Venous blood samples (15 ml) were drawn via sterile venipuncture using heparin (5 IU/ml) as anticoagulant. The study was approved by the local ethic committee at the Yaroslavl State Pedagogical University, and informed consent of all the subjects was obtained according to the recommendations of the Declaration of Helsinki.

Red blood cells (RBC) were separated from the blood by centrifugation at 1,400g for 20 min and washed 3 times with 10 mM phosphate buffered saline (PBS) (pH $=$ 7.4). The washed RBC were resuspended in PBS at a hematocrit (Hct) of approximately 40% for incubation with desired agents. After incubation, RBC suspensions were centrifuged, the supernatant was removed and cells were resuspended in autologous plasma at Hct $=$ 40% to initiate RBC aggregation.

The whole blood and red cell suspension hematocrits were determined via the microhematocrit methods (i.e., 12,000g for 7 min).

2.2. Protocols for RBC treatments

RBC suspension was divided into several aliquots and effects of linked agents were compared as follows:

(A) Epinephrine (1.0 μM); phenylehprine, alpha-1-adrenoceptor agonist (1.0 μM); clonidine, alpha-2-adrenoceptor agonist (1.0 μM); isoproterenol, non-selective beta-adrenoceptor agonist (1.0 μM); insulin (0.01 μM); glucagon (1.0 μM).
(B) Prostaglandins PGE_1, PGE_2, PGI_2 and PGF2α (0.01 μM).
(C) Adenylyl cyclase (AC) stimulator forskolin (10 μM); stable penetrating analog of cAMP (dB-cAMP, 100 μM).
(D) Phosphodiesterase inhibitors: isobutyl-methyl-xanthine (IBMX, 100 μM); vinpocetin (10 μM); cilostazol (10 μM); rolipram (10 μM).
(E) Calcium ionophore, A23187 (3 μM); EGTA, calcium chelator (1.0 mM); verapamil – Ca^{2+} channel blocker (10 μM). Ringer's solution containing 2.25 mM Ca^{2+} was used to suspend the cells for these treatments and cell shape was observed by light microscopy.
(F) Protein kinase stimulator C (PKC) – phorbol 12-myristate 13-acetate (PMA, 3 μM); PKC specific inhibitor, bisindolylmaleimide (BIM, 3.0 μM); tyrosine protein kinase (TPK) activator – cisplatin (3 nM); specific inhibitor of TPK – lavendustin (5.0 μM).

The cell incubations were performed at 37°C for 15 min. RBC incubated in phosphate buffered saline (or in Ringer's solution) without any drugs were used as controls. Stock solutions of drugs were prepared in DMSO or water. All analyses were completed within 4 h after the blood collection. Drugs and chemical compounds were purchased from Sigma.

2.3. Red blood cell aggregation

Red blood cell aggregation (RBCA) in native plasma was assessed using a Myrenne Aggregometer which provides an index of RBC aggregation facilitated by low shear. In brief, the suspension was subjected to a short period of high shear to disrupt pre-existing aggregates, following which the shear rate was abruptly reduced to 3 s^{-1} and light transmission through the suspension was integrated for 5 seconds; the resulting index, termed 'M_5' by the manufacturer and 'RBCA' herein, increased with enhanced RBC aggregation.

2.4. Statistics and data presentation

The results are presented as mean $\pm$ SEM. The differences between the mean values were evaluated using an ANOVA test. The values of $p < 0.05$ indicate statistical significance.

3. Role of extracellular ligands in signaling alteration of red blood cell aggregation

3.1. Role of some hormones

Catecholamines act as stress hormones ensuring an effective adaptation to environmental factors, by regulating oxygen transport and cell metabolism [63]. They may modify blood flow in nutritive capillaries under stress by alteration of red blood cell aggregation [41,61]. It is important to note that human RBC membranes contain both β_1- and β_2-adrenergic receptors [27,52,64]. Here, experiments were designed to evaluate the aggregation effect of adrenergic receptor agonists as extracellular ligands inducing molecular signaling pathways (Fig. 1). In the presence of 1.0 μM α_1- and α_2-receptor agonists

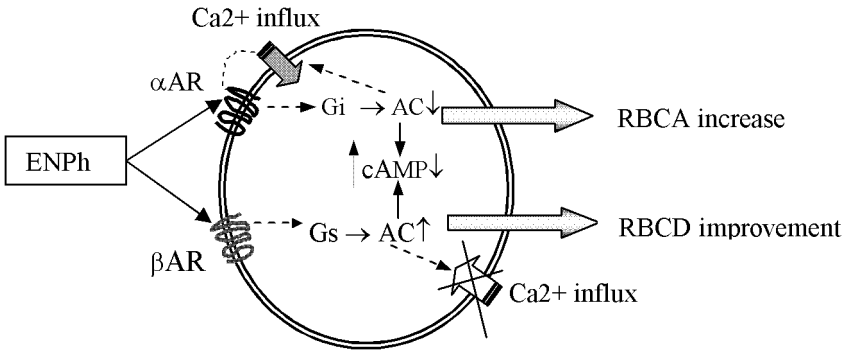

Fig. 1. Molecular signaling pathways associated with control of red blood cell microrheology. *Abbreviations:* αAR – α-adrenergic receptor; βAR – β-adrenergic receptor; ENPh – epinephrine; AC – adenylyl cyclase; Gi, Gs – heterotrimeric GTP binding proteins; RBCA – red blood cell aggregation; RBCD – red blood cell deformability.

Table 1
Effects of alpha- and beta-adrenergic agonists on red blood cell aggregation

	Control	Epinephrine	Phenylephrine	Clonidine	Isoproterenol
RBCA	7.34 ± 0.32	$9.83 \pm 0.36^*$	$11.20 \pm 0.42^*$	$11.74 \pm 0.42^*$	$6.46 \pm 0.26^*$

Notes: Control – without treatment. Data are mean $\pm$ SEM, $n = 30$. $^*p < 0.05$ versus control. RBCA – aggregation index M1; arbitrary units.

Table 2
Effects of insulin and glucagon on red blood cell aggregation ($M \pm m$, $n = 30$)

	Control	Insulin	Glucagon
RBCA	7.00 ± 0.28	3.92 ± 0.18	11.36 ± 0.35

Notes: Control – without treatment. Data are mean $\pm$ SEM, $n = 30$. $^*p < 0.05$ versus control RBCA – aggregation index M1; arbitrary units.

(epinephrine, phenylephrine and clonidine) the red blood cell aggregation was markedly increased by about 30–60% compared to controls (Table 1). Isoproterenol did not increase, but slightly decreased aggregation (Table 1). Therefore, α-adrenergic receptor stimulation has the greater effect on aggregation.

It has been reported that various rheological abnormalities are associated with diabetes, but there is little data on any direct effect of metabolic hormones on RBC aggregation. It is well known that insulin is a functional antagonist to the catecholamines [63] and RBC possess insulin receptors [24]. Therefore we tested whether red cell aggregation was altered after incubation with insulin. Indeed, RBCA was reduced by 44% under these conditions (Table 2). Glucagon, on the contrary, increased RBCA significantly by 48% (Table 2).

3.2. Role of prostaglandins

It has been shown that prostaglandins have a marked effect on RBC microrheology including their aggregation [17,41,45,49,59]. PGE_1 activates adenylyl cyclase [58] which may be one mechanism altering RBD rheology [59]. Prostacyclin (PGI_2) has been shown to increase filterability of normal and rigidified human red blood cells *in vitro* [31]. The activation of the RBC prostacyclin receptor (IPR) results in an increase of cAMP content.

We found that RBCA was decreased after the cells were incubated with PGE_1 or PGE_2, or with PGI_2 (Table 3). The other species of the prostaglandin family – PGF2α on the contrary intensified the red cell aggregation. In presence of PGF2α RBCA increased by 73% (Table 3). PGF2α is known as a calcium cell entry stimulator [47]. Therefore in this case the aggregation rise may be associated with Ca^{2+} influx. Thus, the results demonstrate that prostaglandins of the E-family and prostacyclin significantly decrease red cell aggregation, while PGF2α enhances it.

4. Role of intracellular signaling pathway in alteration of red blood cell aggregation

4.1. Adenylyl cyclase-cAMP-G-protein system

Recently it has been shown that alterations in red cell microrheology may be connected with activation of various intracellular signaling pathways [18,31,36,45,59]. Changes in red cell aggregation have

Table 3
Effects of prostaglandins on red blood cell aggregation

	Control	PGE1	PGE2	PGI2	PGF2α
RBCA	7.20 ± 0.26	$3.68 \pm 0.18^*$	$5.81 \pm 0.48^*$	$4.08 \pm 0.36^*$	$13.20 \pm 0.63^*$

Notes: Control – without treatment. Data are mean $\pm$ SEM from 24 experiments. $^*p < 0.05$ versus control. RBCA – aggregation index M1; arbitrary units.

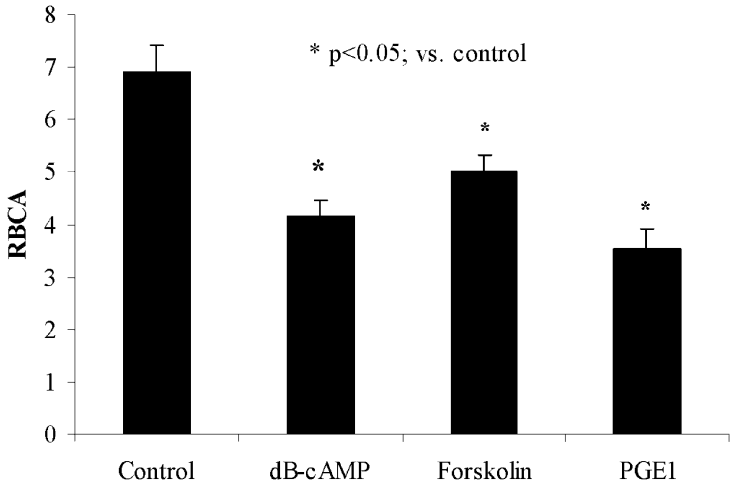

Fig. 2. Effects of cAMP modulators (dB-cAMP, forskolin and prostaglandin E_1, PGE1) on red blood cell aggregation. Data are mean $\pm$ SEM from 24 experiments. $^*p < 0.05$ versus control. RBCA – aggregation index M1.

been associated with a depression of the adenylyl cyclase – cAMP system. It was shown that the direct stimulation of Gi-protein activity with mastaparan-7 was accompanied by significant increase in aggregation [41,42]. On the other hand, elevation of cAMP can lead to cytosolic Ca^{2+} decrease via an inhibition of its influx [17]. Indeed a rise in intracellular Ca^{2+} content was prevented if RBC were incubated with phosphodiesterase inhibitors or a permeable cAMP analog [64]. It is known that both α_2- and β-adrenergic receptors are coupled to the G-protein – adenylate cyclase system in human RBC [16, 27]. The diterpene forskolin has been reported to activate adenylate cyclase [40]. Here, RBC incubation with forskolin caused a decrease of RBCA by 20% (Fig. 2). Adenylyl cyclase stimulation and cAMP level rise in red cells may be achieved by using the stable analog of cAMP (dB-cAMP) or prostaglandin E_1(PGE_1) [45]. It was found that the red blood cell aggregation was significantly decreased under either of these conditions (Fig. 2).

4.2. *Phosphodiesterase* (*PDE*) *activity*

The intracellular cAMP level is regulated by phosphodiesterases (PDEs) [2,4,19,21,36]. To study their role in red cell aggregation, RBC were incubated with: nonselective PDE inhibitor isobutyl-methyl-xanthine (IBMX); PDE_1 inhibitor vinpocetine; PDE_3 inhibitor cilostazol; PDE_4 inhibitor rolipram. All of these drugs reduced red cell aggregation, with the PDE_1 inhibitor vinpocetine having the greatest effect (Table 4). Overall, RBCA reduction averaged 25% under these treatments. These data suggest

Table 4
Effects of phosphodiesterase inhibitors on red blood cell aggregation

	Control	IBMX	Vinpocetin	Cilostazol	Rolipram
RBCA	7.60 ± 0.33	$5.92 \pm 0.19^*$	$4.90 \pm 0.25^*$	$6.28 \pm 0.32^*$	$6.60 \pm 0.29^*$

Notes: Control – without treatment. Data are mean $\pm$ SEM from 20 experiments. $^*p < 0.05$ versus control. RBCA – aggregation index M1; arbitrary units.

Table 5
Effects of Ca^{2+} entry blocker verapamil, calcium ionophore A23187 and Ca^{2+} chelator EGTA on red blood ell aggregation

Parameters	Control	Verapamil	A23187	EGTA
RBCA	7.18 ± 0.28	5.88 ± 0.25	12.78 ± 0.54	4.16 ± 0.20

Notes: Control – without treatment. Data are mean $\pm$ SEM from 30 experiments. $^*p < 0.05$ versus control. RBCA – aggregation index M1; arbitrary units.

that PDEs are a part of the cell regulatory system "the cell membrane receptor – G-protein – AC-cAMP complex" and that they are involved in regulation of RBC microrheology.

4.3. Role of Ca^{2+} in red blood cell aggregation

The measurement of total red cell calcium concentration has yielded values between 5 and 50 nM [42,50,51], while only a few percent of the total is in an ionized form [66]. We previously noted that RBC aggregation was decreased significantly after red cell incubation with Ca^{2+} chelator EGTA [42]. On the other hand, RBC treatment with stimulators of Ca^{2+} influx as well as the inhibition of Ca^{2+} efflux resulted in a significant increase of their aggregation [41,42]. Here blocking, Ca^{2+} entry into RBC using verapamil or EGTA led to a significant decrease in RBCA (Table 5). Conversely, stimulation of Ca^{2+} influx with A23187 caused a significant increase of red blood cell aggregation (Table 5). The changes in RBC Ca^{2+} and cAMP content do not represent isolated linear signaling pathways, but ones that interact at multiple levels to form an effective signaling network.

4.4. Protein kinases

Proteomic analysis has shown that the red cell membrane contains several kinases and phosphatases [5,30,39,46]. *In vitro* studies have shown that phosphorylation can alter membrane-skeletal protein function [35], while drugs affecting phosphorylation may change RBC microrheological properties [23,45]. However, our understanding of the regulation of the mechanical function of intact red cell membranes by phosphorylation of membrane skeletal proteins is incomplete. When the stimulator (dB-cAMP) of protein kinase A was added to RBC suspension, the RBCA was significantly decreased (Fig. 3). Little changes of aggregation was induced after RBC incubation with protein kinase stimulator C – phorbol 12-myristate 13-acetate (PMA) (Fig. 3). The slight increase induced was lost in the presence of the PKC specific inhibitor, bisindolylmaleimide (BIM) (Fig. 4(A)). After the activation of Lyn protein tyrosine kinase (TPK) of the Src family by cisplatin [55] a significant RBCA reduction was observed (Fig. 3) and a specific inhibitor of TPK lavendustin (5.0 μM) [28] eliminated this effect (Fig. 4(B)).

5. Conclusions

The above studies indicate that red cell microrheological properties connected with oxygen transport efficiency may be regulated through activation of cellular molecular signaling pathways. Likely

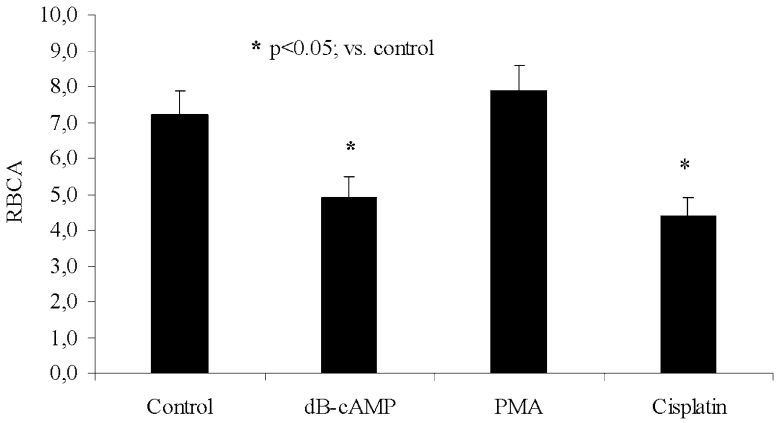

Fig. 3. Effects of stable cAMP analog (dBu-cAMP), phorbol 12-myristate 13-acetate (PMA) and cisplatin on red blood cell aggregation. Data are mean ± SEM from 20 experiments. $^{*}p < 0.05$ versus control. RBCA – aggregation index M1.

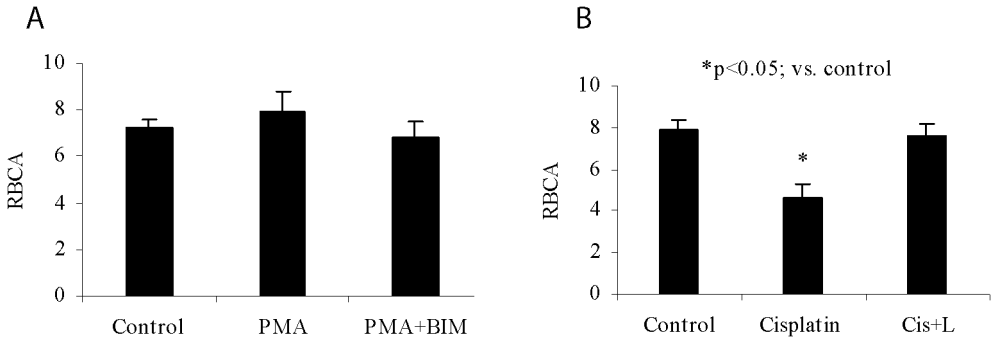

Fig. 4. Changes in red blood cell aggregation after treatment with: (A) phorbol 12-myristate 13-acetate (PMA), PKC specific inhibitor bisindolylmaleimide (BIM); (B) cisplatin (Cis), Cis plus tyrosine protein kinase selective inhibitor – lavendustin (L). Data are mean ± SEM from 24 experiments. $^{*}p < 0.05$ versus control. RBCA – aggregation index M1.

regulatory pathways causing rheological responses in RBC include: extracellular ligands (hormones, prostaglandins), membrane-receptors coupled with Gs-protein (e.g. β-adrenergic receptor), adenylyl cyclase (AC), cAMP, protein kinase A (PKA) and phosphorylation of membrane proteins (e.g. band 3, band 4.1) (see Fig. 5).

Spotlighting the important molecular loci at which cAMP and Ca^{2+} signaling pathways converge in non-excitable cells may help us to further understand and appreciate the complexity and specificity of stimulus–response coupling in RBC. A site of the cross-talk between Ca^{2+} and cAMP signaling systems RBC may be PDEs. For example, the constitutive type 4 phosphodiesterase activity rapidly hydrolyzes cAMP, while Ca^{2+} inhibition of AC may also lead to lower cAMP. Thus high phosphodiesterase activity may work coordinately with AC to regulate membrane-delimited cAMP concentration, which is important for control of cell–cell apposition [43]. Moreover, previous studies indicate that the activation of AC and increase in cAMP lead to decreased Ca^{2+} influx and reduction in red cell aggregation [41]. Similar results were found after blocking Ca^{2+} entry into RBC or inhibition of PDE activity.

Therefore we can propose that there are two molecular mechanisms for the short-term regulation of RBCA. The first one can be activated under physiological or pathological conditions (stress, exercise, diseases) in order to the increase the postcapillary flow resistance, increase the capillary filtration pres-

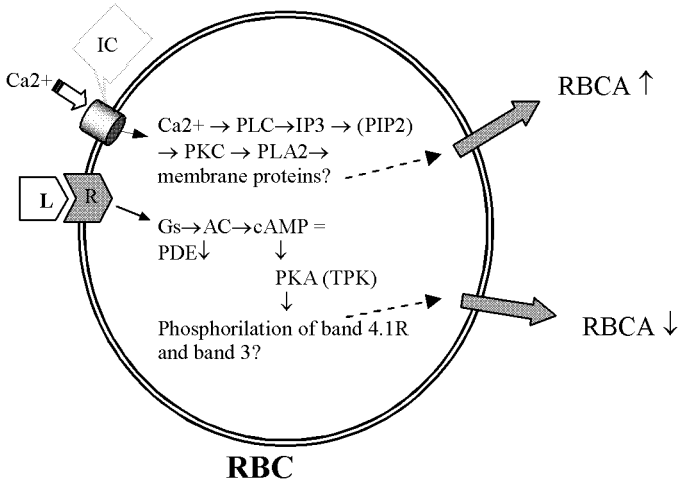

Fig. 5. Summary of signaling pathways modulating red blood cell aggregation. *Abbreviations*: RBCA – red blood cell aggregation; IC – ionic channel; PLC – phospholipase C; PLA_2 – phospholipase A_2; IP_3 – inositol trisphosphate; PIP_2 – phosphatidylinositol 4,5-bisphosphate; L – ligand; R – membrane receptor; PDE – phosphodiesterase; Gs – G-protein; AC – adenylyl cyclase; PKA – protein kinase A; TPK – tyrosine protein kinase.

sure and make delivery of oxygen and substrates to the tissue more effective [12,48]. This control mechanism is connected with Ca^{2+} signaling pathway activation. The second molecular control mechanism of aggregation is associated with an activation of the AC-cAMP-PKA system. A decrease of RBCA can lead to the postcapillary flow resistance reduction. This results in a decrease of capillary hydrostatic pressure and an activation of reabsorption. This could be important for stimulation of the recovery processes after exercise, stress etc.

The mechanism by which these molecular pathways influence RBC aggregation may be linked to integrin membrane receptors expressed by the cells [13,25,27,54]. The existence of a single molecule interaction between fibrinogen and an unknown receptor on the RBC membrane has been demonstrated by force spectroscopy measurements using an atomic force microscope [14]. Genetic sequencing data indicates that one of the units of the fibrinogen receptor on the RBC membrane is a product of the expression of the beta-3 integrin gene [13]. Thus there is some evidence of specific red blood cell membrane binding of ligands during the aggregation process. CD47 has also been proposed as a mediator for specific fibrinogen binding [56] and is able to associate with Rh complex forming a link to the band 3 complex by binding to protein 4.2 or to associate with several integrins and thereby to induce their activation [56].

The physiological and pathological significance of short-term changes in red cell aggregation may also connected to effects on leukocyte adhesion. It is known that increasing red cell aggregation correlated with an increase in slow-flowing leukocytes near the vessel wall and more efficient leukocyte adhesion [1,33]. Thus modulation of red blood cell aggregation may influence immune function by influencing recruitment of leukocytes to inflamed tissue.

Acknowledgements

Work was supported grants of RFBR Nos 12-04-00550-a and 14-04-01703-a.

References

[1] K.B. Abbitt and G.B. Nash, Rheological properties of the blood influencing selectin-mediated adhesion of flowing leukocytes, *Am. J. Physiol. Heart Circ. Physiol.* **285** (2003), H229–H240.

[2] S.P. Adderley, R.S. Sprague, A.H. Stephenson and M.S. Hanson, Regulation of cAMP by phosphodiesterases in erythrocytes, *Pharmacol. Rep.* **62** (2010), 475–482.

[3] S.P. Adderley, M. Sridharan, E.A. Bowles, A.H. Stephenson, M.L. Ellsworth and R.S. Sprague, Protein kinases A and C regulate receptor-mediated increases in cAMP in rabbit erythrocytes, *Am. J. Physiol. Heart Circ. Physiol.* **298** (2010), H587–H593.

[4] S.P. Adderley, K.M. Thuet, M. Sridharan, E.A. Bowles, A.H. Stephenson, M.L. Ellsworth et al., Identification of cytosolic phosphodiesterases in the erythrocyte: a possible role for PDE5, *Med. Sci. Monit.* **17** (2011), CR241–CR247.

[5] D.A. Andrews, L. Yang and P.S. Low, Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells, *Blood* **100** (2002), 3392–3399.

[6] O.K. Baskurt and H.J. Meiselman, Blood rheology and hemodynamics, *Semin. Thromb. Hemost.* **9** (2003), 435–450.

[7] O.K. Baskurt and H.J. Meiselman, Erythrocyte aggregation: Basic aspects and clinical importance, *Clin. Hemorheol. Microcirc.* **53** (2013), 23–37.

[8] H. Bäumler, B. Neu, E. Donath and H. Kiesewetter, Basic phenomena of red blood cell rouleaux formation, *Biorheology* **36** (1999), 439–442.

[9] R. Ben-Ami, G. Barshtein, T. Mardi, V. Deutch, O. Elkayam, S. Yedgar et al., A synergistic effect of albumin and fibrinogen on immunoglobulin-induced red blood cell aggregation, *Am. J. Physiol. Heart Circ. Physiol.* **285** (2003), H2663–H2669.

[10] S. Bhattacharya, P.S. Chakaborty, R.S. Basu, N.N. Kahn and A.K. Sinha, Purification and properties of insulin-activated nitric oxide synthase from human erythrocyte membranes, *Arch. Physiol. Biochem.* **109** (2001), 441–449.

[11] J.J. Bishop, P.R. Nance, A.S. Popel, M. Intaglietta and P.C. Johnson, Effect of erythrocyte aggregation on velocity profiles in venules, *Am. J. Physiol. Heart Circ. Physiol.* **280** (2001), H222–H236.

[12] M. Cabel, H.J. Meiselman, A.S. Popel and P.C. Johnson, Contribution of red blood cell aggregation to venous vascular resistance in skeletal muscle, *Am. J. Physiol. Heart Circ. Physiol.* **272** (1997), H1020–H1032.

[13] F.A. Carvalho, S. Connell and G. Miltenberger-Miltenyi, Atomic force microscopy-based molecular recognition of a fibrinogen receptor on human erythrocytes, *ACS Nano.* **4**(8) (2010), 4609–4620.

[14] F.A. Carvalho and N.C. Santos, Atomic force microscopy-based force spectroscopy – biological and biomedical applications, *IUBMB Life* **64** (2012), 465–472.

[15] K. Chen and A.S. Popel, Nitric oxide production pathways in erythrocytes and plasma, *Biorheology* **46** (2009), 107–119.

[16] G. Cheng, J. Zielonka, B.P. Dranka, D. McAllister, A.C. Mackinnon, J. Joseph et al., Mitochondria-targeted drugs synergize with 2-deoxyglucose to trigger breast cancer cell death, *Cancer Res.* **72** (2012), 2634–2644.

[17] G. Ciuffetti, E. Sokola, R. Lombardini, L. Pasqualini, M. Pirro and E. Mannarino, The influence of iloprost on blood rheology and tissue perfusion in patients with intermittent claudication, *Kardiol. Pol.* **59** (2003), 197–204.

[18] J.R. Creighton, N. Masada, D.M. Cooper and T. Stevens, Coordinate regulation of membrane cAMP by Ca^{2+}-inhibited adenylyl cyclase and phosphodiesterase activities, *Am. J. Physiol. Lung Cell Mol. Physiol.* **284** (2003), L100–L107.

[19] D.L. Dawson, Q. Zheng, S.A. Worthy, B. Charles and D.V. Bradley, Jr., Failure of pentoxifylline or cilostazol to improve blood and plasma viscosity, fibrinogen, and erythrocyte deformability in claudication, *Angiology* **53** (2002), 509–520.

[20] S. De Oliveira, V. Vitorino de Almeida, A. Calado, H.S. Rosario and C. Saldanha, Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane, *Biochimica et Biophysica Acta* **1818** (2012), 481–490.

[21] C.K. Derian, R.J. Santulli, P.E. Rao, H.F. Solomon and J.A. Barrett, Inhibition of hemostatic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators, *J. Immunol.* **154** (1995), 308–317.

[22] T. Fabry, Mechanism of erythrocyte aggregation and sedimentation, *Blood* **70**(5) (1987), 1572–1576.

[23] M. Grau, S. Pauly, J. Ali, K. Walpurgis, M. Thevis, W. Bloch et al., RBC-NOS-dependent S-nitrosylation of cytoskeletal proteins improves RBC deformability, *PLoS One* **8**(2) (2013), e56759.

[24] M.S. Hanson, A.H. Stephenson, E.A. Bowles and R.S. Sprague, Insulin inhibits human erythrocyte cAMP accumulation and ATP release: role of phosphodiesterase 3 and phosphoinositide 3-kinase, *Exp. Biol. Med. (Maywood)* **235**(2) (2010), 256–262.

[25] P.S. Hines, Q. Zen, S.N. Burney, D.A. Shea, K.I. Ataga, E.P. Orringer et al., Novel epinephrine and cyclic cAMP – mediated action on BCAM/Lu – dependent sickle (SS) RBC adhesion, *Blood* **101** (2003), 3281–3287.

[26] J.F. Hoffman, W. Joiner and K. Nehrke, The hSK4 (KCNN4) isoform is the Ca^{2+}-activated K^{+} channel (Gardos channel) in human red blood cells, *Proc. Nat. Acad. Sci. USA* **100** (2003), 7366–7371.

[27] J.F. Horga, J. Gisbert, J.C. De Agustin, M. Hernández and P. Zapater, A beta-2-adrenergic receptor activates adenylate cyclase in human erythrocyte membranes at physiological calcium plasma concentrations, *Blood Cells Mol. Dis.* **26** (2000), 223–228.

[28] D.E. Hu and T.P. Fan, Suppression of VEGF-induced angiogenesis by the protein tyrosine kinase inhibitor, lavendustin A, *Br. J. Pharmacol.* **114** (1995), 262–268.
[29] K. Kaiserova, B. Lakatos, E. Peterajova, J. Orlicky and L. Varecka, Investigation of properties of the Ca^{2+} influx and of the Ca^{2+}-activated K^+ efflux (Gardos effect) in vanadate-treated and ATP-depleted human red blood cells, *Gen. Physiol. Biophys.* **21** (2002), 429–442.
[30] B.A. Klarl, P.A. Lang and D.S. Kempe, Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion, *Am. J. Physiol. Cell Physiol.* **290** (2006), C244–C253.
[31] I.B. Kovacs and J. O'Grady, Prostacyclin increases filterability of normal and rigidified human red blood cells *in vitro*, *Agents Actions* **14** (1984), 306–310.
[32] F. Lang, C. Birka, S. Myssina, K.S. Lang, P.A. Lang, V. Tanneur et al., Erythrocyte ion channels in regulation of apoptosis, *Adv. Exp. Med. Biol.* **559** (2004), 211–217.
[33] H.H. Lipowsky, Microvascular rheology and hemodynamics, *Microcirculation* **12** (2005), 5–15.
[34] N. Maeda and T. Shiga, Opposite effect of albumin on erythrocyte aggregation induced by immunoglobulin G and fibrinogen, *Biochim. Biophys. Acta* **855** (1986), 127–135.
[35] S. Manno, Y. Takakuwa and N. Mohandas, Modulation of erythrocyte membrane mechanical function by protein 4.1 phosphorylation, *J. Biol. Chem.* **280** (2005), 7581–7587.
[36] J.M. Manns, K.J. Brenna, R.W. Colman and S.B. Sheth, Differential regulation of human platelet responses by cGMP inhibited and stimulated cAMP phosphodiesterases, *Thromb. Haemost.* **87** (2002), 873–879.
[37] H.J. Meiselman, RBC aggregation: 45 years being curious, *Biorheology* **46** (2009), 1–19.
[38] H.J. Meiselman, B. Neu, M.W. Rampling and O.K. Baskurt, RBC aggregation: laboratory data and models, *Indian J. Exp. Biol.* **45** (2007), 9–17.
[39] G. Minetti, A. Ciana and C. Balduini, Differential sorting of tyrosine kinases and phosphotyrosine phosphatases acting on band 3 during vesiculation of human erythrocytes, *Biochem. J.* **377** (2004), 489–497.
[40] S.A. Morris and J.P. Bilezikian, Evidence that forskolin activates turkey erythrocyte adenylyl cyclase through a noncatalytic site, *Arch. Biochem. Biophys.* **220** (1983), 628–636.
[41] A.V. Muravyov, S.V. Bulaeva, A.A. Maimistova and I.A. Tikhomirova, Extra- and intracellular signaling pathways under red blood cell aggregation and deformability changes, *Clin. Hemorheol. Microcirc.* **43** (2009), 223–232.
[42] A.V. Muravyov and I.A. Tikhomirova, Role Ca^{2+} in mechanisms of the red blood cell microrheological changes, in: *Calcium Signaling*, Md. Shahidul Islam, ed., Springer Science + Business Media, Dordrecht, 2012, pp. 1017–1038.
[43] A.V. Muravyov, V.V. Yakusevich, A.A. Maimistova, F.A. Chuchkanov and S.V. Bulaeva, Hemorheological efficiency of drugs, targeting on intracellular phosphodiesterase activity: *in vitro* study, *Clin. Hemorheol. Microcirc.* **24** (2007), 19–23.
[44] J.J. Olearczyk, A.H. Stephenson, A.J. Lonigro and R.S. Sprague, NO inhibits signal transduction pathway for ATP release from erythrocytes via its action on heterotrimeric G protein G_i, *Am. J. Physiol. Heart Circ. Physiol.* **287** (2004), H748–H754.
[45] K. Oonishi, N. Sakashita and N. Uysaka, Regulation of red blood cell filterability by Ca^{2+} influx and cAMP-mediated signaling pathways, *Am. J. Physiol. Cell Physiol.* **273** (1997), C1828–C1834.
[46] E.M. Pasini, M. Kirkegaard, D. Salerno, P. Mortensen, M. Mann and A.W. Thomas, Deep coverage mouse red blood cell proteome. A first comparison with the human red blood cell, *Mol. Cell Proteomics* **7** (2008), 1317–1330.
[47] J.G. Pitter, G. Szanda, M.R. Duchen and A. Spät, Prostaglandin $F_{2\alpha}$ potentiates the calcium dependent activation of mitochondrial metabolism in luteal cells, *Cell Calcium.* **37** (2005), 35–44.
[48] A.S. Popel and P.C. Johnson, Microcirculation and hemorheology, *Ann. Rev. Fluid Mech.* **37** (2005), 43–69.
[49] H. Rasmussen, W. Lake and J.E. Alien, The effect of catecholamines and prostaglandins upon human and rat erythrocytes, *Biochim. Biophys. Acta* **411** (1975), 63–73.
[50] P.J. Romero and E.A. Romero, The role of calcium metabolism in human red blood cell ageing: a proposal, *Blood Cells Mol. Dis.* **25** (1999), 9–19.
[51] P.J. Romero, V. Salas and C. Hernández, Calcium pump phosphoenzyme from young and old human red cells, *Cell Biol. Int.* **26** (2002), 945-949.
[52] G. Sager and S. Jacobsen, Effect of plasma on human erythrocyte betaadrenergic receptors, *Biochem. Pharmacol.* **34** (1985), 3767–3771.
[53] K. Sakashita, T. Oonishi, N. Ishioka and N. Uyesaka, Endothelin-1 improves the impaired filterability of red blood cells through the activation of protein kinase C, *Jpn J. Physiol.* **49** (1999), 113–120.
[54] C. Saldanha, Fibrinogen interaction with the red blood cell membrane, *Clin. Hemorheol. Microcirc.* **53** (2013), 39–44.
[55] R.A. Singh and A. Sodhi, Expression and activation of Lyn in macrophages treated *in vitro* with cisplatin: regulation by kinases, phosphatases and Ca^{2+}/calmodulin, *Biochim. Biophys. Acta* **21** (1998), 171–179.
[56] D. Soto-Pantoja, E.V. Stein, N.M. Rogers, M. Sharifi-Sanjani, J.S. Isenberg and D.D. Roberts, Therapeutic opportunities for targeting the ubiquitous cell surface receptor CD47, *Expert Opin. Ther. Targets* **17** (2013), 89–93.
[57] M.I. Spengler, M.J. Svetaz, M.B. Leroux, S.M. Bertoluzzo, P. Carrara, F. Van Isseldyk et al., Erythrocyte aggregation in patients with systemic lupus erythematosus, *Clin. Hemorheol. Microcirc.* **47** (2011), 279–285.

[58] R. Sprague, E. Bowles, M. Stumpf, G. Ricketts, A. Freidman, W.H. Hou et al., Rabbit erythrocytes possess adenylyl cyclase type II that is activated by the heterotrimeric G proteins Gs and Gi, *Pharmacol. Rep.* **57** (2005), 222–228.
[59] D. Starzyk, R. Korbut and R.J. Gryglewski, Effects of nitric oxide and prostacyclin on deformability and aggregability of red blood cells of rats *ex vivo* and *in vitro*, *J. Physiol. Pharmacol.* **50** (1999), 629–637.
[60] J. Stuart and G.B. Nash, Red cell deformability and haematological disorders, *Blood Rev.* **4** (1990), 141–147.
[61] J. Sundquist, S. Blas, J.E. Hogan, F.B. Davis and P.J. Davis, The alpha 1-adrenergic receptor in human erythrocyte membranes mediated interaction *in vitro* of epinephrine and thyroid hormone at the membrane Ca(2+)-ATPase, *Cell Signal* **47** (1992), 95–99.
[62] L.C. Tang, E. Schoomaker and W.P. Wiesmann, Cholinergic agonists stimulate calcium uptake and cGMP formation in human erythrocytes, *Biochim. Biophys. Acta* **772** (1984), 235–238.
[63] J. Tepperman and H. Tepperman, *Metabolic and Endocrine Physiology and Introductory Text*, Year Book Publishers, Chicago/London, 1987.
[64] S. Tuvia, A. Moses, N. Gulayev, S. Levin and R. Korenstein, Beta-adrenergic agonists regulate cell membrane fluctuations of human erythrocytes, *J. Physiol.* **516** (1999), 781–787.
[65] P. Ulker, L. Sati, C. Celik-Ozenci, H.J. Meiselman and O.K. Baskurt, Mechanical stimulation of nitric oxide synthesizing mechanisms in erythrocytes, *Biorheology* **46** (2009), 121–132.
[66] J.S. Wiley and K.E. McCulloch, Calcium ions, drug action and the red cell membrane, *Pharmacol. Ther.* **18** (1982), 271–292.
[67] P. Zancan and M. Sola-Penna, Calcium influx: a possible role for insulin modulation of intracellular distribution and activity of 6-phosphofructo-1-kinase in human erythrocytes, *Mol. Genet. Metab.* **86** (2005), 392–400.