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Protein Kinases and Protein Phosphatases as Participants in Signal Transduction of Erythrocytes

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1. Introduction

Signal transduction is defined as the transfer of a signal starting from a primary messenger with a ligand that binds to specific receptors on the cell membrane. The signal then reaches the effector molecule(s) through a cascade involving various protein kinases and/or protein phosphatases as well as other molecules such as adaptor proteins, anchoring proteins, amplifier proteins, etc. Although erythrocytes are nucleus-free cells, they use elements of cell signal pathways that help them to maintain membrane integrity, ion transport, and metabolism. Aging, some "stress" conditions, and various diseases also may generate cell signals into erythrocytes (Minetti и Low, 1997; Antonelou et al. 2010; Berzosa et al. 2011; Pantaleo et al. 2010). Molecules from both erythrocyte membranes and from cytosol may take part in signal transduction. Membrane components involved in signal transduction include receptors, heterotrimeric G-proteins, adaptor and anchoring proteins, and some proteins of the Ras-superfamily. The erythrocyte intracellular signaling cascade is divided into protein kinases and phosphatases, second messengers and small GTPases, which could further be defined as cell surface and intracellular activities (Pasini et al. 2006). In general, the mechanisms of signal transduction in erythrocytes can be summarized in the following groups: 1) specific mechanisms triggering changes in the activity of erythrocyte enzymes, 2) specific mechanisms inducing changes in the activity of transport systems, 3) signal transduction related to membrane association/dissociation of cytoskeletal and integral proteins, 4) "stressful" conditions may induce activation of specific cellular signals into erythrocytes. Phosphorylation of the erythrocyte proteins by protein kinases and phosphatases is the key mechanism for controlling erythrocyte functions.

2. Binding of extracellular ligands to receptors on erythrocytes membrane initiates various processes of phosphorylation of erythrocyte proteins

2.1 Thyroid stimulating hormone

Thyroid stimulating hormone (TSH) binds to a specific TSH receptor (TSHR) which activates adenylate cyclase and increases cAMP levels in thyroid cells. Recent studies have

reported that TSHR and Na/K-ATPase are localized on the membranes of both erythrocytes and erythrocyte ghosts. TSHR responds to TSH treatment by increasing intracellular cAMP levels from two to tenfold. The authors suggest a novel cell signalling pathway, potentially active in local circulatory control (Balzan et al. 2009).

2.2 Parathyroid hormone

Parathyroid hormone (PTH) operates with G-protein dependent receptors and has been shown to decrease erythrocyte deformability in a Ca²⁺-dependent manner (Bogin et al. 1986). In pseudohypoparathyroidism, PTH resistance results from impairment caused by a deficiency of G α -signaling cascade due to a methylation defect of the GNAS gene and decrease in erythrocyte G α activity (Zazo et al. 2011).

2.3 Insulin

Insulin stimulates erythrocyte glycolysis, Na⁺/H⁺-antiport and Na⁺/K⁺-ATPase (Rizvi et al. 1994), as well as membrane-associated NO-synthase (Bhattacharaya et al. 2001). It has been shown that insulin uses signaling pathways involving both ζ -PKC and phosphatidylinositol 3 kinase (PI3K), which leads to the activation of Na⁺/H⁺-antiports (Sauvage et al. 2000). Insulin is also used in the MAPK-signaling cascade of phosphorylation and activation of protein NHE1, responsible for Na⁺/H⁺ - antiport (Sartori et al. 1999).

Participation of C-peptide in insulin signaling pathways has also been discussed, including involvement of G-protein and Ca-dependent phosphatase. It has been reported that insulin restores the activity of Na⁺/K⁺-ATPase (De La Tour et al. 1998). The decrease in Na⁺/K⁺ adenosine triphosphatase (ATPase) in erythrocytes of type 1 diabetes is thought to play a role in the development of long-term complication. Infusion of insulin may restore this enzyme activity in red cells (Djemli-Shipkolye et al. 2000).

A reduced erythrocyte insulin receptor binding and tyrosine kinase activity was measured in hypertensive subjects with hyperinsulinemia (Corry et al. 2002). Erythrocytes from normal individuals showed increased pH and increased sodium influx (NHE1) after insulin stimulation. In contrast, insulin had no effect on NHE1 activity of erythrocyte from obese individuals (Kaloyianni et al. 2001). Insulin activation of insulin receptor kinase in erythrocytes is not altered in non-insulin-dependent diabetes and not influenced by hyperglycemia (Klein et al. 2000).

2.4 Insulin-like growth factor I

Erythrocytes possess receptors for insulin-like growth factor I (IGF-I). Binding to these receptors is dependent on cell age (Polychronakos et al. 1983). Acromegalic patients with higher plasma IGF-I and insulin levels presented lower IGF-I specific binding and affinity than normal adults. Growth hormone (GH)-deficient children showed higher IGF-I binding without significant affinity alterations than normal prepubertal children (el-Andere et al. 1995).

2.5 Leptin

The specific binding of leptin on erythrocytes is established by Scatchard analysis (Tsuda, 2006). NHE1 (Na⁺/H⁺-exchanger) activity increases in the presence of leptin but significantly

less in the obese than in the control group. Since NHE1 activity is associated with insulin resistance and hypertension, the activation of this antiport by leptin may represent a link between adipose tissue hypertrophy and cardiovascular complication of obesity (Konstantinou-Tegou et al. 2001). It is possible to assume that leptin, similarly to insulin, might be able to activate NHE1 through MAPK activation (Sartori et al. 1999; Bianchini et al. 1991).

2.6 Adrenaline, Noradrenaline and DOPA

The β -2-adrenergic receptor coupled to the G-protein binds catecholamines and activates adenylate cyclase in human erythrocytes (Horga et al. 2000). The functional beta-receptor response depends to a large extent on Ca^{2+} concentrations (Horga et al. 2000). According to Muravyov et al. (2010) a crosstalk between adenylate cyclase signaling pathway and Ca^{2+} regulatory mechanism exists. The potent beta-adrenergic agonist, isoproterenol (2 μM), epinephrine (10 μM) and norepinephrine (10 μM) stimulated the cAMP-dependent protein kinase in erythrocyte membranes, 38 +/- 7%, 31 +/- 6%, and 30 +/- 6%, respectively (Tsukamoto and Sonenberg, 1979). Micromolar concentration of noradrenaline (1 μM) increases the ^{32}P intake in band 2 with 70%, and with 40% in band 3 (Nelson et al. 1979).

Adrenaline and noradrenaline are both found to stimulate the erythrocyte Na^+/H^+ -antiport (Perry et al. 1991; Paajaste and Nikinmar, 1991). Adrenaline stimulates Na^+/H^+ -antiport through activation of NHE1 transport system with participation of PKC, since the effect has been reported to potentiate in the presence of phorbol ester (PKC activator), and being inhibited from calphostin (PKC inhibitor), respectively (Bourikas et al. 2003).

A specific erythrocyte DOPA transport protein was found who is also capable to transport choline. Its functions are regulated by insulin (Azoui et al. 1996).

Several catecholamines (phenylephrine, dobutamine and dopamine) inhibit the Cl^- -removal-activated Ca^{2+} entry into erythrocytes, thus preventing increase of cytosolic Ca^{2+} activity, subsequent cell shrinkage and activation of erythrocyte scramblase. The catecholamines thus counteract erythrocyte phosphatidylserine exposure and subsequent clearance of erythrocytes from circulating blood (Lang et al. 2005).

Exposure of RBCs to adrenaline resulted in a concentration-dependent increase in RBC filterability and authors supposed that adrenergic agonists may improve passage of erythrocytes through microvasculature (Muravyov et al. 2010). Rasmussen et al (1975) reported that the same low doses of adrenaline and isoproterenol induce a decrease of erythrocyte deformability.

2.7 Adenosine

Adenosine binds to the erythrocyte adenosine type1 receptors (A1AR) and adenosine type 2 receptors (A2AR), (Lu et al. 2004; Zhang et al. 2011). A1ARs are functionally coupled with pertussis toxin-sensitive G proteins and inhibit the activity of adenylate cyclase. A1ARs bind to erythrocyte membrane cytoskeletal protein 4.1G, which can inhibit A1-receptor action (Lu et al. 2004). A1ARs activation can also trigger the release intercellular Ca^{2+} (Lu et al. 2004). Increased adenosine levels promoted sickling, hemolysis and damage to multiple tissues in SCD transgenic mice and promoted sickling of human erythrocytes. (Zhang et al. 2011).

2.8 Prostaglandins (PG)

Binding of PGE₂ to receptors coupled to the G-protein activates phospholipase C which in turn catalyzes phospholipid turnover (Minetti et al. 1997) and/or stimulates a Ca²⁺-dependent K⁺ channel in human erythrocytes and alters cell volume and filterability (Li et al. 1996). The PGE₁ receptor coupled to the G-protein activates adenylate cyclase in human erythrocytes and increases erythrocyte deformability (Dutta-Roy et al. 1991). Prostacyclin (PGI) binding to human erythrocyte receptors stimulates cAMP synthesis and ATP release (Sprague et al. 2008).

2.9 Thyroid and steroid hormones

Lipophilic hormones take part in signal transduction also with "non-genomic" effects using signals starting from the plasma membrane (Falkenstein et al. 2000). Their role in erythrocyte signal transduction pathways and their activities have not been fully explored. Similar results have been reported for thyroid hormones (Angel et al. 1989; Botta and Farias, 1985), and for estrogens (Gonçalves et al. 2001). Effect of thyroid hormones is probably related to calmodulin-dependent activation of erythrocyte membrane ATPase (Lawrence et al. 1993), that is inhibited in the presence of retinoic acid (Smith et al. 1989).

In vitro beta-estradiol 10⁻⁵ M decreased erythrocyte aggregation in blood samples of postmenopausal women undergoing hormone therapy, which could prevent high blood viscosity and, consequently, cardiovascular events (Gonçalves et al. 2001).

Results from X-ray diffraction studies revealed that cortisol and estradiol bind into the erythrocyte membrane bilayer, and exert opposite effect over Na⁺/K⁺-ATPase activity: cortisol diminishes its activity by 24%, but estradiol increases it by 18% (Golden et al. 1999). Other published *in vitro* studies showed that aldosterone stimulates Na⁺/K⁺-ATPase activity in human erythrocyte membranes (Hamlyn and Duffy, 1978). One possible explanation is that the incorporation leads to conformational changes and reorganization in the active center of the enzyme molecule of Na⁺/K⁺-ATPase. In addition to the delayed genomic steroid actions, increasing evidence for rapid, nongenomic steroid effects has been demonstrated for virtually all groups of steroids, and transmission by so far hypothetical specific membrane receptors is very likely. Nongenomic effects on cellular function involve conventional second messenger cascades (Falkenstein et al., 2000). Plasma selenium as well as plasma and erythrocyte glutathione peroxidase activity increase with estrogen during the menstrual cycle. The mechanism is still unknown (Ha and Smith, 2003).

2.10 Cytokines

Biochemical evidence is provided for the presence of endothelin (ET) receptor subtype B in sickle and normal red cells. It was found that ET-1, PAF (Platelet Activating Factor), RANTES and IL-10 induce a significant increase in red cell density. These data suggest that activation of the Gardos channel is functionally coupled to receptors as C-X-C (PAF), C-C (RANTES) and ET receptors type B and the cell volume regulation or erythrocyte hydration state might be altered by activation of the Gardos channel by cytokine *in vivo* (Rivera et al. 2002).

Human red cells bind specifically IL-8 (a neutrophil activating chemokine) with IL-8RA and IL-8RB receptors. Red cell absorption of IL-8 may function to limit stimulation of leucocytes

by IL-8 released into blood (Darbonne et al. 1991). IL-8 released after acute myocardial infarction is mainly bound to erythrocytes (de Winter et al. 1997).

ICAM-4 belongs to the intercellular adhesion molecules and is an erythrocyte membrane component. ICAM-4 interacts specifically with platelet alphaIIb beta 3 integrin. RBCs are considered passively entrapped in fibrin polymers during thrombosis through ICAM-4 (Hermand et al. 2003).

2.11 Thrombin

It is assumed that thrombin induces a signal that stimulates the formation of cAMP and PGE1 via activation of Gs-dependent adenylate cyclase and Ca²⁺-independent PKC in erythrocyte progenitors. Signaling pathway is inhibited by amiloride and by PKC inhibitors such as GF-109203X, Go 6976 and staurosporine (Haslauer et al. 1998). However, whether or not these signals are also valid for mature erythrocytes has not been studied yet.

2.12 Lactoferrin

Lactoferrin (Lf) is a metal-binding glycoprotein with antioxidative (Cohen et al., 1992), anti-inflammatory, immunomodulatory (Legrand et al., 2004), anticarcinogenic (Thotathil and Jameson, 2007) anti-bacterial (Weinberg, 2007), antiviral (Mistry et al., 2007), antiatherogenic (Kajikawa et al. 1994), and antithrombotic properties (Levy-Toledano et al., 1995).

Our previous studies showed that Lf binding with erythrocyte membrane receptors (Taleva et al., 1999) results in stimulation of glycolysis, antioxidative protection (Maneva et al., 2003) and activation of Na⁺/K⁺-ATPase activity (Maneva et al., 2007). Lf-receptor interaction might intervene in short-term effects of regulation, involving processes of changes in association, phosphorylation and oxidation of the membrane proteins.

Lf (10-50 nM) decreased the ATP content from 9 to 57% depending on the concentration used. There is a negative correlation found between the concentration of added Lf and formed ATP: $y = 2.550 - 0.0076.x$, $r = - 0.993$, $p < 0.001$, $n = 5$ (Figure 1). The reason for the decrease of ATP in the presence of Lf could be the processes of phosphorylation and/or ion transport activated by Lf. Lf is an activator of protein phosphorylation (Maekawa et al. 2002; Curran et al. 2006) and an activator of ion transport (Sun et al. 1991). Such a decrease in ATP content was found by other authors (Assouline-Cohen and Beitner, 1999; Boadu & Sager, 2000) due to stimulation of vital processes of erythrocytes.

Lf could stimulate glycolysis by interfering with phosphorylation processes: 1) There is evidence that insulin activates the Na⁺/H⁺ - antiport in erythrocytes by PI3K-dependent signaling pathway (Sauvage et al. 2000) leading to activation of glycolysis (Madshus, 1988). Like insulin, Lf also activates Na⁺/H⁺ - antiport (Sun et al. 1991) and stimulates the lactate formation (Maneva et al. 2003). Therefore, it could be suggested that Lf uses the same PI3K - dependent mechanism for glycolysis activation (Boivin, 1988); 2) Phosphorylation of tyrosin residues in band 3 (Boivin, 1988) leads to dissociation of the complex with glycolytic enzymes, leading to their activation (Low et al. 1993). Lf could also stimulate the glycolytic enzymes by activating Src-kinase-dependent phosphorylation of band 3. There is data showing that Lf is an activator of tyrosine phosphorylation by Src-kinases (Takayama & Mizumachi, 2001).

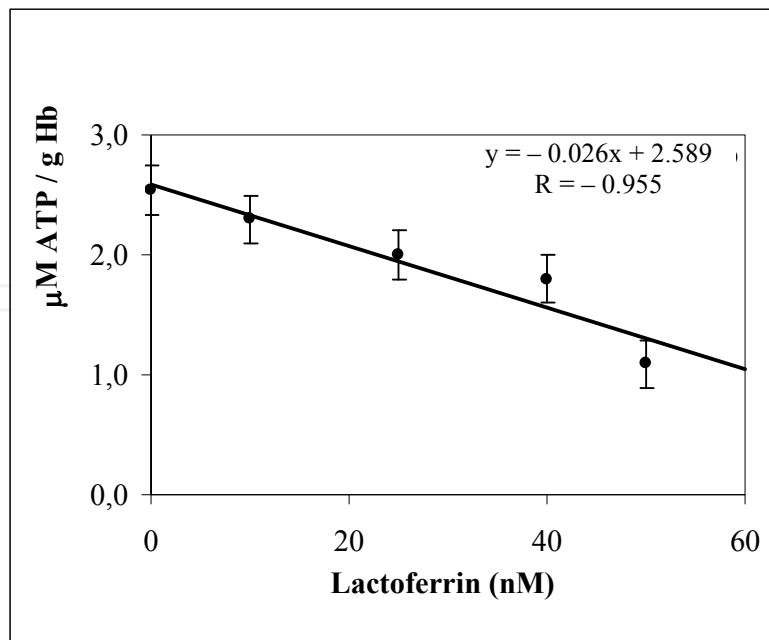


Fig. 1. Correlation and regression analysis of the relation between the concentration of Lf and content in erythrocyte ATP (unpublished data).

Sigma-Aldrich test kit has been used in this experiment. The method is based on using a reaction from oxidative phosphorylation (OP) in glycolysis where ATP is produced. OP is two-stage process involving the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 3-phosphoglycerate kinase. The output products of OP are 3-phosphoglycerate, NADH⁺ and ATP. The test uses GAPDH in reverse direction: a conversion of 1,3 bisphosphoglycerate into glyceraldehyde-3-phosphate, where the oxidation of NADH⁺ to NAD⁺ is directly proportional to the amounts of the ATP produced. The results are presented in µmols ATP/g.Hb.

Several possible mechanisms could be discussed in regard to how Lf binding to the receptors on the erythrocyte membrane might lead to Na⁺/K⁺-ATPase activation (Maneva et al. 2007) via changes in levels of phosphorylation of membrane and cytosolic proteins: 1) Changes in the levels of phosphorylation of tyrosine residues in erythrocyte membrane modulates not only the activities of band 3 (Brunati et al. 2000), but also the activity of α-subunit of Na⁺/K⁺-ATPase (Done et al. 2002). α-subunit of Na⁺/K⁺-ATPase is phosphorylated by tyrosine kinases, as the target of phosphorylation is known to be the 537 tyrosine residue in the molecule. This phosphorylation is proved to be a prerequisite for the participation of Na⁺/K-ATPase in signal transduction in experiments with renal cells (Done et al. 2002). Lf stimulates tyrosine phosphorylation of proteins (Takayama & Mizumachi, 2001). 2) It is known that Lf activates casein kinase 2 (CK2), and is phosphorylated by the latter (Maekawa et al. 2002). CK2 phosphorylates and activates a wide spectrum of erythrocyte membrane proteins associated in multiprotein complexes with Na⁺/K⁺-ATPase (Wei and Tao, 1993); 3) Non-receptor tyrosine kinase Src takes part in signal pathway that regulates Na-pump activity in other cell types (Haas et al. 2002). Lf is also able to activate Src (Takayama and Mizumashi, 2001). 4) Stimulating effect of Lf could be indirect as Lf activates Na⁺/H⁺ antiport (Sun et al. 1991). By this mechanism erythrocytes are loaded with Na⁺ thus activating Na⁺/K⁺-ATPase which exports Na⁺ out of the erythrocyte cell.

2.13 Polyamines

Membrane receptors for polyamines were proved to exist on erythrocyte membranes (Moulinoux et al. 1984). After binding with their receptors, polyamines enhance the protein-protein interactions among the cytoskeletal proteins and membrane lipid bilayer (Bratton, 1994; Farmer et al. 1985). Polyamine also inhibits transbilayer movement of plasma membrane phospholipids in the erythrocyte ghost (Bratton, 1994).

Polyamines are CK2 protein kinase activators (Leroy et al. 1997). It was found that positively charged betaine and polyamines inhibit the activity of membrane Na⁺/K⁺-ATPase in erythrocytes. It has been demonstrated that the effect of polyamines is based on the competitive inhibition mechanism (Kanbak et al. 2001). A similar mechanism is most likely responsible for the poor ion transport in cancer patients whose tumor cells secrete polyamines (Villano et al. 2001). Raised erythrocyte polyamine levels are estimated in patients with diabetes type 1 and in non-insulin dependent diabetes mellitus with great vessel disease and albuminuria (Seghieri et al. 1997).

2.14 Caffeine

Caffeine binds to erythrocytes membrane proteins (Sato et al. 1990) and could induce changes in the erythrocyte protein complex's formation, thus modulating the activity of enzymes involved in signal transduction. Caffeine could interfere with the phosphorylation processes in erythrocytes as an inhibitor of erythrocyte CK2 (Lecomte et al. 1980), and PI3K (Buckley, 1977) as it was reported in other cell types. Methylxanthines are capable for inhibition of cyclin-dependent protein kinases found in both cytosol and erythrocyte membranes (Biovin, 1988).

Processes of phosphorylation-dephosphorylation of erythrocyte membrane and plasma proteins provide different levels of interaction and participate in maintaining the integrity of the erythrocyte membrane. They also exert control over important metabolic processes in erythrocytes. The literature data shows that signals in erythrocytes lead to changes in phosphorylation and association of integral membrane proteins and other intracellular proteins. Cytosolic protein kinases and protein phosphatases were found in erythrocyte membrane.

3. Protein kinases

3.1 Protein serine/threonine kinases

3.1.1 cAMP dependent protein kinases

Type 1 isoform of cAMP dependent protein kinase is localized in the membrane, while type 2 is located in the cytosol (Dreytuss et al. 1978). cAMP-protein kinases-dependent phosphorylation of isoforms of membrane Ca²⁺-ATPase was found. Isoform 1 is shown to be better substrate in comparison to isoforms 2 and 4. Isoform 1 is susceptible to degradation by calpain (thiol-dependent protease) (Guerini et al. 2003).

In all cells, increasing in cAMP are regulated by the activity of phosphodiesterases (PDEs) (Sheppard and Tsien, 1975). In erythrocytes, activation of either beta adrenergic receptors (beta (2) AR) or the prostacyclin receptor (IPR) results in increases in cAMP and ATP release

(Sprague et al. 2001). Receptor-mediated increases in cAMP are tightly regulated by distinct PDEs associated with each signaling pathway, as shown by the finding that selective inhibitors of the PDEs localized to each pathway potentiate both increases in cAMP and ATP release (Adderley et al. 2010).

Adenylyl cyclase and cAMP are components of a signal-transduction pathway relating red blood cells (RBC) deformation to ATP release from human and rabbit RBCs (Sprague et al. 2008). Exposure of RBC to catecholamines (epinephrine, phenylephrine, an agonist of α 1-adrenergic receptors, clonidine, an agonist of α 2-adrenergic receptors and isoproterenol, an agonist of β -adrenergic receptors) led to change in the RBC microrheological properties. Forskolin (10 μ M), an adenylate cyclase stimulator, increases the RBC deformability (RBCD). A somewhat more significant deformability rise appears after RBC incubation with dibutyryl-AMP. Red blood cell aggregation (RBCA) is significantly decreased under these conditions. All drugs having PDE inhibitory activity increase red cell deformability (Muravyov et al. 2009).

Ca²⁺ entry increase is accompanied by red cell aggregation rise, while adenylyl cyclase-cAMP system stimulation led to red cell deformability increase and its aggregation lowered. The crosstalk between two intracellular signaling systems is probably connected with phosphodiesterase activity. It was found that all four PDE inhibitors: IBMX, vinpocetine, rolipram, pentoxifylline decreased red cell aggregation significantly and, quite the contrary, they increased red cell deformability (Muravyov et al. 2010)

Erythrocytes are oxygen sensors and modulators of vascular tone (Ellsworth et al. 2009). It has become evident that erythrocytes participate in the regulation of vascular caliber in the microcirculation via release of the potent vasodilator, adenosine triphosphate (ATP). The regulated release of ATP from erythrocytes occurs via a defined signaling pathway and requires increases in cyclic 3',5'- adenosine monophosphate (cAMP) (Adderley et al. 2010). Heterotrimeric G protein Gi is involved in a signal transduction pathway for ATP release from erythrocytes (Olearczyk et al. 2010). Insulin inhibits human erythrocyte cAMP accumulation and ATP release. The targets of insulin action are phosphodiesterase 3 and phosphoinositide 3-kinase (Hanson et al. 2010). TSH signalling pathway is cAMP-dependent and probably it is potentially active in local circulatory control (Balzan et al. 2009).

3.1.2 cGMP-dependent protein kinases

Petrov et al. (1994) supposed that human erythrocytes possess membrane and soluble guanylate-cyclase activity stimulated by atrial natriuretic peptide III (ANP-III) and that activation of Na⁺/H⁺ exchange by this peptide is mediated by cGMP (Petrov and Lijnen, 1996). There are no reported data considering participation of protein kinases and phosphatases in the signal pathway.

3.1.3 Casein kinase 1 and 2

Membrane proteins of human erythrocytes can be phosphorylated not only by membrane casein kinase but also by cytosolic casein kinases, resembling casein kinase 1 and 2 (CK1 and CK2), respectively. CK1 and CK2 phosphorylate serine and threonine residues in target proteins (Boivin, 1988). CK2 inactivation mediated by phosphatidylinositol-4, 5-bisphosphate, a substrate for phospholipase C which catalyzes formation of lipid mediators IP3 and DAG

(Chauhan et al. 1993) has been found. It was established that CK2 is inactivated by insulin (Sommerson et al. 1987).

An increased phosphorylation of the membrane proteins, promoted by the okadaic acid (strong inhibitor of P-Ser/Thr-protein phosphatase(s)), is accompanied by a release of CK from the membrane into the cytosol. Such an intracellular translocation might provide a feedback mechanism for the regulation of the CK catalyzed phosphorylation of membrane proteins in human erythrocytes (Bordin et al. 1994).

The membrane mechanical stability of erythrocytes is exclusively regulated by phosphorylation of β -spectrin by membrane bound CK1. Increased phosphorylation of β -spectrin decreased membrane mechanical stability while decreased phosphorylation increased membrane mechanical stability (Manno et al. 1995).

CK2 isolated from erythrocyte membrane and cytosolic fractions exhibited the same subunit composition ($\alpha\alpha 1$) and the ability to utilize ATP and GTP as phosphate donors. Both kinases were found to catalyze the phosphorylation of several erythrocyte membrane cytoskeletal proteins (spectrin, ankyrin, adductin, protein 4.1 and protein 4.9). Unlike CK1, CK2 did not phosphorylate band 3. Spermine, spermidine, and putrescine stimulated to varying degrees the activities of erythrocyte CK2, whereas heparin inhibited the kinase activities (Wei and Tao, 1993).

The phosphorylation sites of calmodulin are important for its ability to activate the human erythrocyte Ca^{2+} -ATPase. Phosphorylation of mammalian calmodulin on serine/threonine residues by casein kinase 2 decreased its affinity for Ca^{2+} -ATPase by two fold. In contrast, tyrosine phosphorylation of calmodulin by the insulin-receptor kinase did not significantly alter calmodulin-stimulated Ca^{2+} -ATPase activity (Sacks et al. 1996).

The COP9 signalosome (CSN) is a multimeric complex that is conserved from yeast to man (Bech-Otschir et al., 2002). Immunoprecipitation and far-western blots reveal that CK2 and PKD are associated with CSN. The COP9 signalosome (CSN) purified from human erythrocytes possesses kinase activity that phosphorylates proteins such as c-Jun and p53 with consequence for their ubiquitin (Ub)-dependent degradation. (Uhle et al., 2003)

3.1.4 MAPK (Mitogen Activated Protein Kinases)

It is well known that signaling pathways involving MAPKs are associated with control of cell growth and proliferation, but erythrocytes are mature highly differentiated cells. There are only a few known participants in the MARK-signaling pathways so far. Immunoblot with antiMAPK antibody revealed the two erythrocyte forms of MAPK-p44 (ERK1) and p42 (ERK2). Insulin and okadaic acid (inhibitor of serine/threonine protein phosphatases) stimulate MAPK activity. Insulin enhances the erythrocyte Na^+/H^+ -exchanger through MAPK activation (Sartori et al. 1999; Bianchini et al. 1991). Membranes of human erythrocytes contain several proteins of the Ras superfamily (Ikeda et al. 1988; Damonte et al. 1990). One of them, RhoA, was detected in both cytosol and membrane fraction of the erythrocytes. Cytosolic Rho bound specifically to the cytoplasmic surface of the erythrocyte membrane. The translocation of Rho to the membrane was absolutely GTP -dependent at low Mg^{2+} concentration (Boukharov et al. 1998).

3.2 Protein tyrosine kinases

Erythrocyte membrane associated tyrosine kinase activity has been established (Zylinska et al. 2002), together with surprisingly high levels of phosphorylated tyrosine in erythrocytes (Phan-Dinh-Tuy et al. 1983).

Two tyrosine kinases that are able to phosphorylate band 3 have been found. Involvement of the Src-kinases family in regulation of erythrocyte membrane transport has been suggested. That family represents an important class of non-receptor protein kinases participating in the regulation of cell communications, proliferation, differentiation, migration and survival (Minetti et al. 2004). Subsequent two-stage phosphorylation of band-3 from Syk- and Lyn-tyrosin kinase was found. (Brunati et al. 2000).

3.3 Ca²⁺ dependent phosphorylation

Since mature RBCs lack intracellular calcium stores, elevation in intracellular calcium must stem from calcium influx. At low intracellular Ca²⁺, efflux of potassium and water predominates, leading to changes in erythrocyte rheology (Andrews et al. 2002). At higher Ca²⁺ content, activation of kinases and phosphatases was observed (Minetti et al.1996; Cohen and Gascard, 1992). Ca²⁺ ions are involved in regulation of phospholipase C, the enzyme generating inositol-1,4,5-triphosphate (InsP3), phosphatidylinositol-3-kinase (PI3K), the enzyme metabolizing InsP3 to InsP4 and in the regulation of protein kinase C (Carafoli, 1994; Carafoli, 2002).

3.3.1 Protein kinase C (PKC)

There are four isoforms of PKC established in the erythrocyte: α , ζ , ι and μ (PKD) (Govekar and Zingle, 2001). PKC translocates from cytosol to membrane - a process shown to be initiated by phorbol esters and diacylglycerols (Cohen and Foley, 1986). PKD and CK2 are associated with COP9 signalosome (CSN) (Uhle et al., 2003).

PKC phosphorylates serine residues in band 3, band 4.1 and band 4.8 (Govekar and Zingle, 2001). It has been reported that after stimulation with phorbol ester (PMA), PKC- α translocates towards the erythrocyte membrane (Govekar and Zingde, 2001), where it phosphorylates serine residues in band 2, and 4.1 and band 4.9 which in turn leads to thorough rearrangement of the cytoskeleton membrane network (Giraud et al. 1988; Ceolotto et al. 1998). PKC phosphorylates membrane Na⁺/K⁺-ATPase (Wright et al. 1993) and the carboxyl terminus of the plasma membrane Ca²⁺-ATPase in human erythrocytes (Wang et al. 1991; Wright et al.1993; Smallwood et al.1988) and the effect of phosphorylation on the activity of the both enzymes depends on the isoenzyme form of protein kinase C.

The interactions between membrane, peripheral and cytoskeleton proteins are responsible for the maintenance of erythrocyte deformability and some of these interactions are modulated by PKC activity. Correlation was established between cytoskeleton proteins, PKC activity, band 3 phosphorylation degrees and erythrocyte deformability (de Oliveira et al. 2008). PKC participates in the processes of phosphorylation of band 3 that regulates its activity as a transport protein (Ceolotto et al. 1998). Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion (Klarl et al. 2006).

In erythrocytes, lead acetate stimulates the phosphorylation of membrane cytoskeletal proteins by a mechanism dependent on protein kinase C. Since levels of calcium or diacylglycerols did not increase, it appears that lead may activate the enzyme by a direct interaction (Belloni-Olivi et al. 1996).

Calpain is a Ca^{2+} -dependent thiol protease that translocates towards the erythrocytes membrane and activates with increase of intracellular calcium concentration (Glasser et al. 1994). Calmodulin binds proteins as calpain substrates (Wang et al. 1989). It is known that calpain causes transition from Ca^{2+} -dependent PKC form to Ca^{2+} -independent one, followed by decrease in its activity (Saïdo et al. 1994).

3.3.2 Calmodulin (CaM)

The increase in cellular Ca^{2+} results in reversible formation of Ca^{2+} /CaM complex that binds to a number of enzymes modulating their activity (Carafoli, 2002).

Calmodulin is a ubiquitous protein whose activity is regulated through phosphorylation and specific Ca^{2+} -binding (Benaim and Villalobo, 2002). Ca^{2+} -ATPase interacts with the carboxy-terminal half of calmodulin, which is the region that contains the majority of the phosphorylation sites in calmodulin (Bzdega and Kosk-Kosicka, 1992). CaM is phosphorylated by serine/threonine kinases such as CK2 and PKA (Benaim and Villalobo, 2002). Phosphorylation of a tyrosine residue in CaM (Tyr99) increases the affinity of its binding to the target proteins thus increasing their activity. A similar stimulating effect of CaM has been reported for Ca^{2+} -ATPase (Kosk-Kosicka et al. 1990), myosin light chain kinase (MLCK), Ca-CaM dependent kinase (CaM kinase II) and Ca-CaM dependent protein phosphatase 2B (calcineurin) (Corti et al. 1999). Elevated intracellular Ca^{2+} , in association with the Ca^{2+} -binding protein, calmodulin, stimulates erythrocytes phosphofructokinase (PFK) activity. This activation involves the detachment of the enzyme from erythrocyte membranes, which has been described as an important mechanism of glycolysis regulation on these cells (Zancan and Sola-Penna, 2005).

CaM is an inhibitor of PI3P-kinase (Villalonga et al. 2002), Na^+/H^+ antiport (Yingst et al. 1992) and of erythrocyte Na^+/K^+ -ATPase (Yingst et al. 1992). The latter effect could be exerted via inducing of signal pathway involving CaM-dependent kinase that activates membrane phospholipase A2(PLA2), which in turn inhibits Na-pump (Okafor et al. 1997).

4. Protein phosphatases

Erythrocyte phosphatases that dephosphorylate phosphoserine, phosphothreonine and phosphotyrosine residues in different proteins were found. Cytoplasmic phosphatases, low-molecular-weight acid phosphatases, and neutral membrane-associated tyrosine phosphatases have been reported (Clari et al. 1987; Graham et al. 1974).

Protein phosphatases originated from two ancestor genes, one serving as the prototype for the phosphotyrosine phosphatases family (the PTP family), and the other for phosphoserine/phosphothreonine phosphatases (the PPP and PPM family). The PPP family groups the PP1, PP2, PP2B, PP4, PP5, PP6 and PP7 enzymes, whereas PP2C and bacterial enzymes like SpoII or PrpC belong to the PPM family (Batford, 1996; Kiener et al. 1987).

Band 3 is a target for tyrosine phosphatases SHP-1 and SHP-2 (Bordin et al. 2002) and PTP1B activities (Zipser and Kosower, 1996). Ca^{2+} promotes erythrocyte band 3 phosphorylation via dissociation of phosphotyrosine phosphatase from band 3 (Zipser et al. 2002)

SHP-2 is an essential soluble protein tyrosine phosphatase (PTP) containing two spectrin homologous domains - SH2. SHR-2 domain participates in multiple signaling pathways of growth factors (GF) and cytokines, and plays an important role in the release of signals from the cell surface to the nucleus. SH2 is the anchor (docking) place where the protein tyrosine kinases, e.g. Src family are attracted to and attached to, thus facilitating phosphorylation-dephosphorylation cycle. SHR-2 is located in the erythrocytes cytosol and it is translocated to the erythrocyte membrane when there is increased tyrosine phosphorylation of the transmembrane protein band 3, induced by PTP inhibitors such as pervanadate, and N-ethylmaleimide. Band 3 is both anchoring protein and substrate for the SHP-2 (Bordin et al. 2002). SHP-1 and SHP-2 ensure dephosphorylation of band 3 in different conditions: SHP-2 through interaction of its SH2 domain(s) to p-tyr protein is regulated by the band 3 tyr-phosphorylation level; SHP-1 dephosphorylates tyr-8, tyr-21 and tyr-904 and may be involved by simple membrane rearrangement (Bragadin et al. 2007).

Protein phosphatase-1 α (PP1 α) is a selective substrate of peroxynitrite activated src family kinase frg and tyrosine phosphorylation of PP1 α correspond to inhibition of its enzyme activity. The final effect of peroxynitrite is the amplification of tyrosine dependent signaling, a finding of general interest in nitrite oxide related pathophysiology (Mallozzi et al. 2005)

PTP1B is localized at the erythrocyte membrane associated with band 3. It is activated by Mg^{2+} and inhibited by Mn^{2+} and vanadate ions (VO_3^-), (Zipser and Kosower, 1996). PTP1B, unlike the other enzymes examined, was quantitatively conserved during erythrocyte aging (Minetti et al. 2004) and erythrocytes may undergo in vivo activation of the Ca^{2+} -dependent calpain system that proteolytically regulates PTP1B activity (Ciana et al. 2004).

The activities of Na^+/H^+ exchanger and Na-K-2Cl cotransporter in rat erythrocytes are regulated by protein phosphatases PP1 and PP2 and stimulated when protein dephosphorylation is inhibited (Ivanova et al. 2006).

5. Control over glycolytic enzymes

Glycolytic erythrocyte enzymes that form a complex with band 3 are under regulation by the processes of phosphorylation and oxidation (Campanella et al. 2005), and pyruvate kinase (PK) - by phosphorylation/dephosphorylation (Kiner and Westhead, 1980). Binding of phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase to band 3 lead to their reversible inhibition (Low et al. 1993), which is neutralized by tyrosine kinase phosphorylation of band 3 resulting in enzymes activation. (Harrison et al. 1991). p72^{syk} and p56/53^{lyn} tyrosine kinases are also involved in band 3 phosphorylation (Hubert et al. 2000), and phosphorylation/dephosphorylation cycle is maintained by protein phosphatases PTP1B and SHP-2 (Bordin et al. 2002; Zipser and Kosower, 1996; Minetti et al. 2004).

PK turns to an inactive form after phosphorylation by cAMP-dependent protein kinase, though it is unclear why since erythrocytes do not undergo gluconeogenesis. PK is activated by erythrocyte protein phosphatases (Kiener and Westhead, 1980).

CaM induces dimerisation of phosphofructokinase (PFK) and physiological levels of concentration of intracellular Ca^{2+} stimulates its catalytic activity (Marinho-Carvalho et al., 2006). However, results obtained from other authors showed that Ca^{2+} /calmodulin protein kinase (CaM-kinase) phosphorylation inhibits phosphofructokinase (PFK) in sheep heart (Mahrenholz et al. 1991)

Ferricyanide activates glycolysis in erythrocytes in two ways. Ferricyanide is the only known non-physiological extracellular agent who induces signal transduction leading to activation of cytoplasmic protein tyrosin kinase that phosphorylates enzyme-binding site in band 3, which in turn leads to release and activation of glycolytic enzymes GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), PFK (phosphofructokinase) and aldolase (Low et al. 1990). It is proved that ferricyanide is a stimulator of glycolysis as end acceptor of electrons produced in glyceraldehyde-3-phosphate dehydrogenase reaction. Thus, ferricyanide is capable of recovering the level of oxidated NAD^+ which is important for maintaining the rate of glycolysis (Orringer and Roer, 1979). There are experiment-based evidence for the existence of erythrocyte e^- transport transmembrane chain related to transferrin receptor (Orringer and Roer, 1979; Goldenberg et al. 1990), that is known to reduce $\text{K}_3\text{Fe}(\text{CN})_6$ and removes protons from inside the cell through the cell membrane (Low et al. 1987). Our own experiments showed that $\text{K}_3\text{Fe}(\text{CN})_6$ inhibits $^{59}\text{FeLF}$ binding to erythrocytes (Maneva et al. 2003) as most probably Lf and $\text{K}_3\text{Fe}(\text{CN})_6$ compete for one and the same electron formed in oxidative phosphorylation in glycolysis; Lf is the physiological activator of the signal pathway used by ferricyanide that leads to tyrosine phosphorylation of band 3 and consequent glycolysis activation.

6. Modulation of components of erythrocyte membrane skeleton

In terms of proteins, the RBC membrane is a complex network of transporters, cytoskeletal molecules, and membrane-bound enzymes. The ability of transmembrane receptor proteins to change their cytoskeleton associations in response to ligand binding looks like a key mechanism for cell signaling through erythrocyte membrane. The erythrocyte membrane skeleton has 3 main components: spectrin molecules forming tetramers $\alpha_2\beta_2$, short actin oligomers containing 12-15 monomers, and band 4.1. A variety of membrane-associated enzymes, including several kinases (protein kinase A, protein kinase C, cdc kinase, casein kinase 1) are thought to regulate interactions within the network through induction of phosphorylation, methylation, myristoylation, palmytoylation, or farnesylation (Pasini et al. 2010).

Spectrin binding to actin is initiated from adductin and is significantly inhibited in the presence of Ca^{2+} and CaM. CaM is able to form weak bonds with spectrine and in the presence of Ca^{2+} could influence binding of protein 4.1 to actin, and probably spectrin phosphorylation as well (Manno et al. 1995).

Band 4.1 is mainly involved in membrane skeleton organization due to its ability to initiates spectrin/actin association. Band 4.1 binds to the membrane in at least two sites: one high affinity site localized on glycoporphins, and another low-affinity site associated with band 3. Phosphorylation of Band 4.1 by PKC was found to modulate its binding to band 3. Most probably, this increases the flexibility of the membrane in response to mechanical stress as was reported for some other models where the elevated levels of membrane

phosphorylation made cells more deformable and more resistant to mechanical stress (Danilov and Cohen, 1989). Association of band 4.1 with glycophorin is important for the maintenance of cell shape and is regulated by polyphosphoinositide co-factor. Indeed, when a change in erythrocyte shape was found, a change in the phosphoinositide content was also found (Danilov et al. 1990). Band 4.1 phosphorylation regulates the assembling of spectrin, actin, and band 4.1 as phosphorylation diminish affinity of band 4.1 to spectrin. Band 4.1 and band 4.9 could be phosphorylated in different sites by PKC and by cAMP-dependent protein kinases (Horne et al. 1990).

Cytoskeletal protein band 4.1G binds to the third intercellular loop of the A1 adenosine receptor and inhibits receptor action. (Lu et al., 2004)

7. Modulation of band 3

The two most important integral proteins in human erythrocyte membranes are glycophorin A and band 3. Band 3 is multifunctional protein whose N- and C-ends are localized on the cytoplasmic surface of the membrane and crosses the cell membrane 14 times. The C-terminal end is 52 kD domain (residues from 360 to 919) of band 3 and is responsible for anion transport through the membrane, while 40 kD from N-terminal end of the cytosolic domain plays a critical role in the binding of bilayer to spectrin-based skeletal net. Cytoplasmic domain of band 3 serves as a center of membrane organization, interacting with proteins, such as ankyrin, protein 4.1 and 4.4, hemoglobin, some glycolytic enzymes, tyrosine phosphatases, tyrosine kinase p72 (*syk*), and Na⁺/K⁺ - ATPase. There are about 1 million copies of band 3 per cell and they are presented as dimeric and tetrameric forms. Approximately 40-60% of band 3 is associated with spectrin-stabilizing cytoskeleton (van den Akker et al. 2010).

Band 3 (AE1) is a member of the family of anion-transporting proteins that maintain Cl⁻/HCO₃⁻ exchange. It refers mainly to erythrocytes and plays a role in CO₂ transport among the tissues and lungs (Saldanha et al. 2007). Erythrocyte PKC phosphorylates serine residues in band 3 (Govekar and Zingle, 2001).

Band 3 modifications normally occur during physiological red blood cell (RBC) senescence and some pathological condition in humans (Santos-Silva et al. 1998). Band 3-tyrosine phosphorylation might be induced in "stress" conditions: "oxidative stress", inhibition of the phosphoprotein phosphatase activity by vanadate or by *thiol group blockers* (diamide, N-ethylmaleimide, etc.), protein tyrosine kinase (PTK) activation from hypertonic NaCl solution, or by intracellular increase of Ca²⁺ levels (Hecht & Zick, 1992). Erythrocyte thiol status is an intrinsic regulator of phosphotyrosine residues levels in band 3 by oxidation/reduction of band 3-associated phosphotyrosine protein phosphatases (PTP) (Zipser et al. 1997). It is considered that the increase in phosphorylation induced by Ca²⁺ ions, and including significant inhibition of PTP activity by dissociation of PTPase from band 3 may also play an important part in signal transduction pathways in some pathophysiological conditions accompanied with increased levels of intracellular Ca²⁺ (Minetti and Low, 1997).

Abnormal band 3 tyrosine phosphorylation has been observed in a number of red cell disorders (Terra et al. 1998). Hyper-phosphorylated band 3 showed a manifest tendency to cluster, indicating a change in its interactions with the cytoskeletal network. Irreversible

band 3 tyrosine phosphorylation leads to membrane vesiculation in G6PD deficient red cells. Syk kinase inhibition largely prevents red cell membrane lysis and vesiculation, strongly suggesting a functional role of band 3 tyrosine phosphorylation in the red cell membrane destabilization (Pantaleo et al. 2011; Bordin et al. 2005).

Based on similarity in oligosaccharide components of band 3 and Lf (Ando et al. 1996), a competition for binding sites could be assumed (Beppu et al. 2000; Eda et al. 1996) together with restructuring of already established complexes. Our experiments revealed that Lf activates erythrocyte glycolysis (Maneva et al. 2003). This fact might be explained with some conformational changes leading to the release of glycolytic enzymes from their complex with band 3. Studies of the same investigators show that Lf, and erythrocyte band 3 occupy the same places on monocyte leukemic cell line THP-1 (Eda et al. 1996). Lf could also activate tyrosine phosphorylation of band 3 since Lf is already proved to be able to increase tyrosine phosphorylation of membrane proteins (Tanata et al. 1998).

8. Changes in phosphorylation in oxidative stress

The alteration of red cell thiol status affects the cell phosphotyrosine status and oxidative stress involves inhibition of PTP. Erythrocyte thiol alkylation by N-ethylmaleimide results in irreversible PTP inhibition and irreversible phosphorylation (Zipser et al. 1997).

Oxidation of erythrocyte membrane by diamide leads to formation of disulfide bonds and following conformational changes in band 3. Most probably those changes lead to the opening of cryptic sites that become accessible for the binding of anti-band 3 antibodies (Turrini et al. 1994). Macrophages recognize oxidatively damaged autologous erythrocytes, and cell surface fibronectin of macrophages enhances the recognition (Beppu et al. 1991). Ca^{2+} signaling including Ca^{2+} influx, calmodulin activation, and myosin light chain phosphorylation are involved in the fibronectin stimulation of the recognition of macrophages for oxidized erythrocytes (Beppu et al. 2000).

Pervanadate, N-ethylmaleimide and diamide strongly increase the two-stage phosphorylation of tyrosine residues of band 3 by Syk-kinase and Src-family (probably Lyn-kinase). An intriguing fact is that there are different mechanisms by which osmotic and oxidative stress activate PTK Syk: oxidative stress leads to autophosphorylation, but osmotic one-to SH-2 domain phosphorylation. It was demonstrated that the same agents strongly enhance interaction between SHP-2 and band 3, which take part simultaneously with the translocation of this phosphatase from cytosol to erythrocyte membrane. These events are most likely mediated by Src-phosphorylation, since both translocation of SHP-2 and phosphatase interaction with the erythrocyte membrane are prevented by PP2, a specific Src inhibitor. SHP-2 binds to band 3 via its SH2 region(s). Authors suggest that the attraction of cytosolic SHP-2 to band 3 precedes the next dephosphorylation of the transmembrane protein (Bordin et al. 2002).

Peroxynitrite (ONOO \cdot) is a product of the reaction of nitric oxide and superoxide anion. It is able to nitrate protein tyrosine. If this modification occurs on phosphotyrosine kinase, substrates can down-regulate cell signaling. ONOO \cdot at low concentrations is a stimulator of both band 3 tyrosine phosphorylation and erythrocyte glycolysis, but higher concentrations ONOO \cdot induces cross-linking of membrane proteins, inhibition of band 3 phosphorylation,

nitration of tyrosines in cytosolic domain of band 3, and irreversible inhibition of lactate production (Mallozi et al. 1997).

Deoxygenation and increase in intracellular Mg^{2+} content induce phosphorylation of tyrosine residues in human erythrocytes (Barbul et al. 1999).

Increasing in erythrocyte volume is combined with stimulation in the activities of the two PTK- p72 (syk) and p56 (lyn) that phosphorylate band 3 (Musch et al. 1999).

Oxidation of methionine residues in CaM prevents its activating effect on membrane Ca^{2+} -ATPase (Gaop et al. 2001).

The erythrocyte has a pool of flavonic compounds, which are considered a buffer, maintaining the antioxidative activity of the erythrocyte (Fiorani et al., 2003) Quercetin and resveratrol (piceatannol) take part in the regulation of the phosphorylation of band 3 in the presence of the physiological oxidant peroxynitrite. Quercetin decreases the Syk activity and particularly prevents mediated by the free radical peroxynitrite (ONOO \cdot) phosphotyrosine phosphatase inhibition. Resveratrol (whose analogue is piceatannol) has another mechanism of action – it enables the mediated by peroxynitrite stimulation of tyrosine phosphorylation by another phosphotyrosine protein kinase – Lyn (Maccaglia et al., 2003).

Pleiotropic effects of resveratrol include antioxidant activity and inhibition of cyclooxygenase with decrease of PGE(2) formation. In erythrocytes, oxidation and PGE(2) activate Ca^{2+} -permeable cation channels. The Ca^{2+} -entry leads to activation of Ca^{2+} -sensitive K^+ channels with subsequent cell shrinkage and cell membrane scrambling with phosphatidylserine (PS) exposure at the erythrocyte surface. Cell shrinkage and phosphatidylserine exposure are hallmarks of suicidal erythrocyte death or eryptosis. Eryptotic cells adhere to the vascular wall, thus compromising microcirculation, and are cleared from circulating blood, thus leading to anemia. Resveratrol is a potent inhibitor of suicidal erythrocyte death during energy depletion, oxidative stress, and isoosmotic cell shrinkage. The nutrient could thus counteract anemia and impairment of microcirculation under conditions with excessive eryptosis (Qadri et al. 2009). However, in some cases, flavonoids have been suggested to work as prooxidants (Kitagawa et al. 2004)

9. Eryptosis (Apoptosis)

Two signaling pathways converge to trigger apoptosis: (1) formation of PGE2 leads to activation of Ca^{2+} -permeable cation channels; (2) the PLA2 (Phospholipase A 2) mediated release of PAF (platelet-activating factor) activates a sphingomyelinase, leading to formation of ceramide. Increased cytosolic Ca^{2+} activity and enhanced ceramide levels lead to membrane scrambling with subsequent PS exposure. Ca^{2+} -activated Ca^{2+} -sensitive K^+ channels leading to cellular KCl loss and cell shrinkage. Ca^{2+} stimulates the protease calpain, resulting in degradation of cytoskeleton (Foller et al. 2008; Lang et al. 2010).

Most triggers of eryptosis, except oxidative stress, are effective without activation of caspases. The involvement of Fas/caspase 8/caspase 3-dependent signaling in erythrocytes leads to PS externalization, a central feature of erythrophagocytosis and erythrocyte biology. The oxidatively stressed red cell recapitulated apoptotic events, including translocation of Fas into rafts, formation of a Fas-associated complex, and activation of caspases 8 and 3. The ROS (Radical Oxygen Species) scavenger N-acetylcysteine inhibits eryptosis (Mandal et al. 2005).

Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion (Klarl et al. 2006).

Eryptosis is stimulated in a wide variety of diseases including sepsis, haemolytic uremic syndrome, malaria, sickle-cell anemia, beta-thalassemia, glucose-6-phosphate dehydrogenase (G6PD)-deficiency, phosphate depletion, iron deficiency, and Wilson's disease. Excessive eryptosis is observed in erythrocytes lacking the cGMP-dependent protein kinase-1 (cGK1) or cAMP activated protein kinase (AMPK). Moreover, eryptosis is elicited by osmotic shock, oxidative stress, and energy depletion, as well as a wide variety of endogenous mediators and xenobiotics. Inhibitors of eryptosis include erythropoietin, nitric oxide NO, catecholamines, and high concentrations of urea (Lang et al. 2006).

10. Effect of modulators of phosphorylation on activity of erythrocyte glycolysis and sodium pump

Modulation of cellular signals from other agents is a leading pharmaceutical approach for developing a therapeutic strategy for many diseases. Of significant practical importance is to address the right questions and to find the appropriate answers in regard to whether certain agents would exert synergic or opposite effects over key cellular functions.

Erythrocyte membranes mediate the relation between ion transport and glycolysis. Na⁺/K⁺-ATPase is not only transport system but also participates in ouabain - initiated signal transduction (Haas et al. 2002; Xie and Cai, 2003; Mohammadi et al.2001), and initiated cell signals in cardiac myocytes that are independent from changes of intracellular concentrations of Na⁺ and Ca²⁺ (Liu et al. 2000). It was demonstrated that glyceraldehyde-3-phosphate-dehydrogenase interacts with cytoplasmic surface of Na⁺/K⁺-ATPase, and that interaction is shown to be inhibited by ouabain (Fossel and Solomon, 1978). The α -subunit of Na⁺/K⁺-ATPase and band 3 form a heterodimeric structure (Martin and Sachs, 1992). Similar structural integration allows shared control of both glycolysis and ion transport, and possible interference of modulators of ion transport in the control of glycolysis might be suggested (Fossel and Solomon, 1978). To verify our hypothesis we studied the effect of the same modulators of phosphorylation on both glycolysis (by measuring the amounts of its final product lactate) and Na⁺/K⁺-ATPase activity.

10.1 Methods

10.1.1 Isolation of the erythrocytes

Heparinized fresh drawn blood from healthy donors was centrifuged at 2,000 ×g for 5 min at 4°C and the pellet was resuspended in 4 volumes of phosphate buffered saline (PBS) pH 7.4. After three washes at 1,800, 1,500, 1,300 ×g, the erythrocytes were isolated by density separation (Cohen et al., 1976). The erythrocyte fraction was resuspended in PBS pH 7.4 to obtain the same concentration as in the fresh blood. Cell concentration was counted in Burker's camera by a Standard KF2 microscope (Carl Zeiss, Jena, Germany). The suspension did not contain other cell species.

10.1.2 Preparation of the erythrocyte membranes

Five milliliters of packed RBCs were mixed with 15 ml cold PBS (0.144 g/l KH₂PO₄·7H₂O, 9.0 g/l NaCl, and 0.795 g/l Na₂HPO₄·7H₂O), pH 7.4, and centrifuged at 5,900 ×g for 10 min

at 4°C. The supernatant was discarded, and cells were washed with 15 ml cold PBS, centrifuged as above, and resuspended in 5 ml of 5 mM Na₂HPO₄, pH 8.0, for hypotonic cell lysis. Lysed cells were centrifuged at 25,000 ×g for 15 min at 4°C, after which the supernatant was gently aspirated and discarded. The RBC membrane pellet was repeatedly washed (4–5 times) with 5 mM Na₂HPO₄ until the pellet appeared white (indicating removal of Hb), and membranes were used for further experiments. The protein content of hemoglobin-free pellets was determined according to Bradford (1976), with human serum albumin as a calibrator. Samples were diluted to protein contents of 1.5 g/L.

10.1.3 Hemoglobin measurement

The hemoglobin (Hb) concentration of the erythrocyte suspension was determined according to Beutler (1975).

10.1.4 Cell treatment

The 50 µl erythrocyte suspension (2×10⁷ cells/ml) was incubated 30 min at 25°C. Samples were performed in quadruplicates. In order to estimate the effect of cell signal modulators, samples were incubated either in the presence or in the absence of the agents.

10.1.5 Chemicals

All chemicals were purchased from Sigma-Aldrich Co, St Louis, MO USA. The mentioned below concentrations were chosen according the producer's prescription and data existing in the literature about their optimal effect: Go6976 (50 nM) and Go6983 (50 nM) – inhibitors of protein serine kinase PKC. Go6976 is an inhibitor of the classic isoforms of PKC and PKD, while Go6983 inhibits only the classic isoforms. Both inhibitors were used simultaneously to exclude the effect of PKD; Caffeine (20 mM) was used as a phosphodiesterase inhibitor; Okadaic acid (20 nM) and Calyculin A (50 nM) as inhibitors of serine/treonine protein phosphatases; N-(6-Aminoethyl)-5-chloro-1-naphthalene-sulfonamide (W-7) (30 µM) as a calmodulin antagonist.

10.1.6 Determination of lactate content in erythrocytes

After centrifugation for 10 min at 2,000 ×g erythrocytes were resuspended in 0.4 ml 10% trichloroacetic acid (TCA). For obtaining of total precipitation the samples were cooled on ice 10 min and then centrifuged again at the same conditions. 0.1 ml from the supernatant was used further according the prescription of the test kit reagent obtained from "SIGMA Diagnostics" (St. Louis, USA). The method was based on the reaction of pyruvate oxidation in the presence of NADH and lactate dehydrogenase. To estimate lactate, the reaction was carried out with excess NAD⁺ and the OD of NADH was measured at 340 nm.

50 µl of erythrocyte suspension (2×10⁷ cells/ml) was incubated for 30 min at 25°C in the presence (samples) or absence (control) of the chosen agents. After 10 min of centrifugation at 2,000 ×g, erythrocytes were resuspended in 0.4 ml of 10% TCA. For total sedimentation, erythrocytes were cooled down for 10 min and the centrifugation step was repeated at the same conditions. 0.1 ml from the supernatant was added to a preliminary prepared solution of NAD⁺ in 2.0 ml glycine buffer, 4.0 ml ddH₂O and 0.1 ml lactate dehydrogenase. Instead of

supernatant, 0.1 ml 10% TCA was added as a blank. After 30 min of incubation at room temperature, the extinction was read at OD=340 nm. Results were calculated using a standart curve and equation according to kit instruction. Results were presented in $\mu\text{g/ml}$.

10.1.7 Erythrocyte membrane ATPase activity

Here, a method based on enzyme kinetics was used for evaluation of ATPase activity based on the amounts of generatated NAD^+ in glycolytic reactions. The following reactions were explored: 1) conversion of phosphoenolpyruvate (PEP) to pyruvate and ATP by pyruvate kinase; 2) interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+ catalyzed by lactate dehydrogenase (Vásárhelyi et al., 1997).

Samples (50 μl) with protein concentration 1.5 g/l of erythrocytes membranes were added to 450 μl of the following solution with final concentration per liter: 100 mmol of NaCl, 20 mmol of KCl, 2.5 mmol of MgCl_2 , 0.5 mmol of EGTA, 50 mmol of Tris-HCl, pH7.4, 1.0 mmol of ATP, 1.0 mmol of phosphoenolpyruvate, 0.16 mmol of NADH, 5 kU of pyruvate kinase, 12 kU of lactate dehydrogenase (all purchased from Sigma). Finally, the tested modulators were added in the samples in above mentioned concentrations. After 5 min, 5 μl of 10 mmol/l ouabain was added to inhibit the ouabain-sensitive ATPase activity. The change in absorbance was measured at OD=340 nm by a twin test (i.e., combination of two assays in one cuvette); Rate A (i.e., slope of total ATPase activity), 80–280 s; Rate B (i.e., slope of ouabain-resistant ATPase activity), 400–600 s. The difference between the two slopes was proportional to the Na^+/K^+ -ATPase activity (Vásárhelyi *et al.* 1997). One unit of ATPase activity (1U) equals to one μmol oxidized NADH for 1 min. Calculations are based on the fact that NADH solution with concentration 1 mg/ml has 0.80 extinction at OD=340 nm ($E_{340} = 0.80$) (Vásárhelyi et al. 1997).

10.2 Results

PKC inhibitors (Go6876 and Go6983), protein phosphatase inhibitors (OA and calyculin A), caffeine, and W-7 increased reliable lactate formation. Calyculin A inhibits reliable Na^+/K^+ -ATPase (Table 1 and 2). From all studied modulators only Go6976 and Go6983, which are PKCs inhibitors, stimulated reliably Na^+/K^+ -ATPase, with 194% and 84%, respectively (Table 1 and 2; Figure 2 and 3).

Agents ($n = 6$)	Lactate $\mu\text{mol/g.Hb}$ $\bar{x} \pm \text{SD}$	p
No agents (control)	5.15 \pm 0.19	-
Go6976	8.23 \pm 0.63	<0.001
Go6983	8.21 \pm 3.24	<0.05
Caffeine	8.07 \pm 0.65	<0.001
OA	7.84 \pm 1.45	<0.001
Calyculin A	7.23 \pm 0.37	<0.001
W-7	11.12 \pm 0.30	<0.001

Table 1. Effect of modulators of phosphorylation on lactate formation

10.3 Discussion

10.3.1 Effect of PKC inhibitors

Four isoforms of PKC in erythrocytes have been found: α , ζ , ι and μ (PKD). Erythrocyte PKC phosphorylates serine residues in band 3, band 4.1 and band 4.8 (Govekar and Zingle, 2001). Go6976 and Go6983 are used to exclude the involvement of PKD in cell signals. Go6976 inhibits classical isoforms of PKC and PKD, but Go6983 inhibits classical isoforms only. The absence of differences in the effects of Go6976 and Go6983 on the lactate formation (Table 1) excludes the involvement of PKD in the control of glycolysis and may indicate involvement of classical PKC α as a negative regulator of glycolysis. It was demonstrated that α PKC translocated from the interior of erythrocytes to the membrane as a result of various stimuli (Govekar and Zingle, 2001).

Go6976 (inhibitor of classic isoforms and PKD), and also PKD "switch-off" inhibitor Go6983, reliably activate ATPase activity. This presents PKC as a negative regulator of Na⁺/K⁺-ATPase. The effect of stimulation with Go6976 is almost twice higher than stimulation with Go6983 (Table 2 and Fig.3). This probably suggests a specific function of PKD as an inhibitor of Na⁺/K⁺-ATPase. PKD has a catalytic domain, which shows more similarity to CaM-dependent kinases than to PKC. PKD efficiently phosphorylated synthetic substrates of Ca²⁺/CaM -dependent kinase II, but does not catalyze phosphorylation of substrates

Agents (<i>n</i> = 6)	Na ⁺ /K ⁺ ATPase [U/g. Protein]	p
No agents (control)	4.44 ± 0.98	-
Go6976	13.04 ± 2.11	<0.001
Go6983	8.18 ± 3.24	<0.05
Caffeine	5.68 ± 1.57	>0.1
OA	5.18 ± 0.61	>0.1
Calyculin A	2.71 ± 0.98	<0.02
W-7	4.81 ± 0.86	>0.1

Table 2. Effect of modulators of phosphorylation on Na⁺/K⁺-ATPase

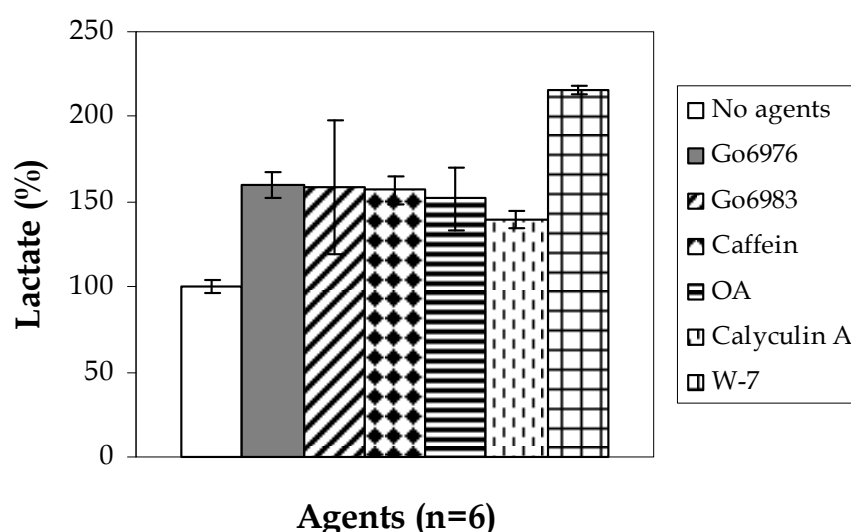


Fig. 2. Effect of modulators of phosphorylation on lactate formation (control=100%).

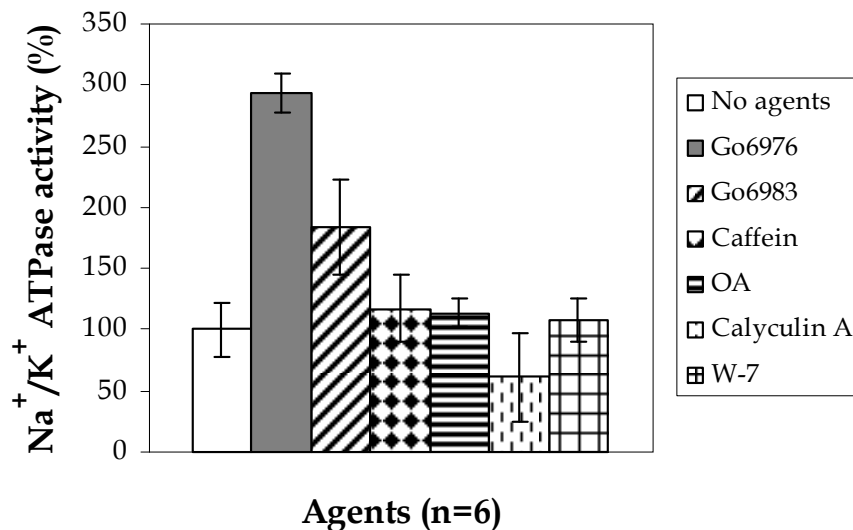


Fig. 3. Effect of modulators of phosphorylation on Na⁺/K⁺-ATPase (control=100 %)

typical of PKC (Ron et al., 1999). CaM inhibits the sodium pump in erythrocytes (Okafor et al. 1997; Yingst et al. 1992), and because of similarities with the effects of CaM, PKD participation in erythrocyte phosphorylation could be assumed, leading to a decrease in activity of Na⁺/K⁺-ATPase. It is also proven an inhibitory effect of PKD on Na⁺/H⁺ - antiport in other cell types (Haworth and Sinnet-Smithh, 1999). It could be speculated that due to the antiport inhibition by PKD, the activity of Na⁺/K⁺-ATPase is being reduced.

10.3.2 Effect of caffeine

The mechanisms by which caffeine stimulates erythrocyte glycolysis (Table 1 and Fig.2) may include modulation of erythrocyte protein phosphorylation (Boivin, 1988) or changes in the conformation of the erythrocyte membrane proteins that facilitate the access of enzymes phosphorylating band 3 (Sato et al. 1990). It is known that methylxanthines are able to inhibit cyclic nucleotide-dependent protein kinases present in the cytosol and the erythrocyte membrane (Boivin, 1988). This inhibition may be due to competition for ATP (Boivin, 1988) and may be involved in the regulation of PK, whose active form is the dephosphorylated one (Kiener and Weatherhead, 1980; Garrillo et al., 2000; Nakashima et al., 1982). Caffeine can exert an indirect effect on lactate formation in erythrocytes through stimulation of Na⁺/K⁺-ATPase (Gusev et al. 2000), but our results showed that caffeine has no reliable effect on the activity of erythrocyte Na⁺/K⁺-ATPase (Table 2 and Fig 3). The identified differences may be due to the fact that in that study, erythrocytes from *Rana temporaria* were examined (Gusev et al, 2000).

10.3.3 Effect of protein phosphatases inhibitors

Okadaic acid (OA) is an inhibitor of serine/treonine phosphatases (Bordin et al. 1993). The incentive effect of OA on glycolysis (Table 1 and Fig.2) may be indirect, as a consequence of activation of MAPK-dependent way that increases Na⁺/H⁺-antiport (Sartori et al. 1999). It is known that one of the mechanisms for glycolysis stimulation is the activation of proton export through this antiport (Madshus, 1988). An interesting fact is that OA and various

growth factors (GF), e.g. TGF and EGF, exert a stimulatory effect on Na^+/H^+ -antiport (NHE-1) through phosphorylation of identical serine residues in its molecule (Sardet et al. 1991). Calyculin A and OA are likely to cause stimulation of glycolysis by using pathways involving protein phosphatase type II (PP2), Table 1 and Fig 2. Protein phosphatase type I (PP1) is highly sensitive to calyculin A (CalA), but not to OA. Protein phosphatase type II (PP2) is highly sensitive to both inhibitors, CalA and OA (Bize et al. 1999). OA has no effect on erythrocyte sodium pump (Table 2 and Fig.3), which probably means that phosphatase type II (PP2) is not involved in cellular signals engaged with the control of Na^+/K^+ -ATPase, but Calyculin A inhibits reliably (with about 40%) the pump activity, which may be due to the involvement of PP1 in cell signals leading to activation of Na^+/K^+ -ATPase (Table 2 and Fig.3).

10.3.4 Effect of W-7

As calmodulin antagonist W-7 restores reduced physiological ability of erythrocytes to change their shape when loaded with Ca^{2+} (Murakami et al. 1986) and also exerts a vasodilator effect (Bereseviz, 1989). The beneficial effect of W-7 on erythrocyte rheology may be due to its stimulatory effect on glycolysis and improvement of erythrocyte bioenergetics (Table 1 and Fig.2). The stimulatory effect of W-7 on glycolysis may be due to blocking the inhibitory effect of calmodulin on Na^+/H^+ -antiport (Yingst et al. 1992), or to decrease in the processes of phosphorylation/dephosphorylation, regulated by Ca^{2+} -calmodulin (Benaim and Villalobo, 2002, Corti et al. 1999). W-7 increases by 8% the activity of Na^+/K^+ -ATPase but the effect lacks statistical significance (Table 2 and Fig.3) in the absence of Ca^{2+} . There is evidence in the literature for an inhibitory effect of calmodulin on erythrocyte Na^+/K^+ -ATPase, which occurs at $2 \mu\text{M}$ Ca^{2+} in the incubation medium (Yingst et al. 1992). The difference with our results could be explained probably with these specific experimental conditions. In our study, no extra amounts of calcium were added into incubation medium. Erythrocytes have about 80 nM intracellular Ca^{2+} (Astarie et al. 1992). Perhaps the inhibitory effect of calmodulin (resp. stimulation with W-7) on the sodium pump acts as a regulatory mechanism when an increase in intracellular calcium content is presented, and primarily affects Ca^{2+} -binding capacity of calmodulin (Astarie et al. 1992).

10.4 Conclusions

Processes of phosphorylation-dephosphorylation of erythrocyte membrane and plasma proteins provide different levels of interaction, and also participate in maintaining the integrity of erythrocyte membrane and exerting control over important metabolic processes in erythrocytes. Summary of the existing data in literature shows they are more comprehensive in terms of the effect of primary messengers that bind to membrane receptors on erythrocytes and the consequent biological effects of the ligand-receptor interaction. Down-stream signaling pathways, though, where a major role in phosphorylation processes is played by protein kinases and protein phosphatases, remain less clear. Expansion of our knowledge and better understanding of signal transduction in erythrocytes will enable possible control to be exerted over their impact in maintenance of vascular tone, procoagulant activity and antioxidant status, erythrocyte aging, senescence, eryptosis (apoptosis), and erythrophagocytosis. Our results demonstrate that erythrocytes

use different cell signals for regulation of glycolysis and activity of the sodium pump. They also reveal that different classes of PKC most likely taking part in regulation of glycolysis and Na^+/K^+ -ATPase. Protein phosphatase inhibitors have opposite effects in the control of glycolysis and sodium pump, which indicates involvement of different protein phosphatases in cellular signal transduction that controls ion transport and cell energetics. Caffeine and W-7 have a reliable effect on the stimulation of glycolysis, but not on sodium pump.

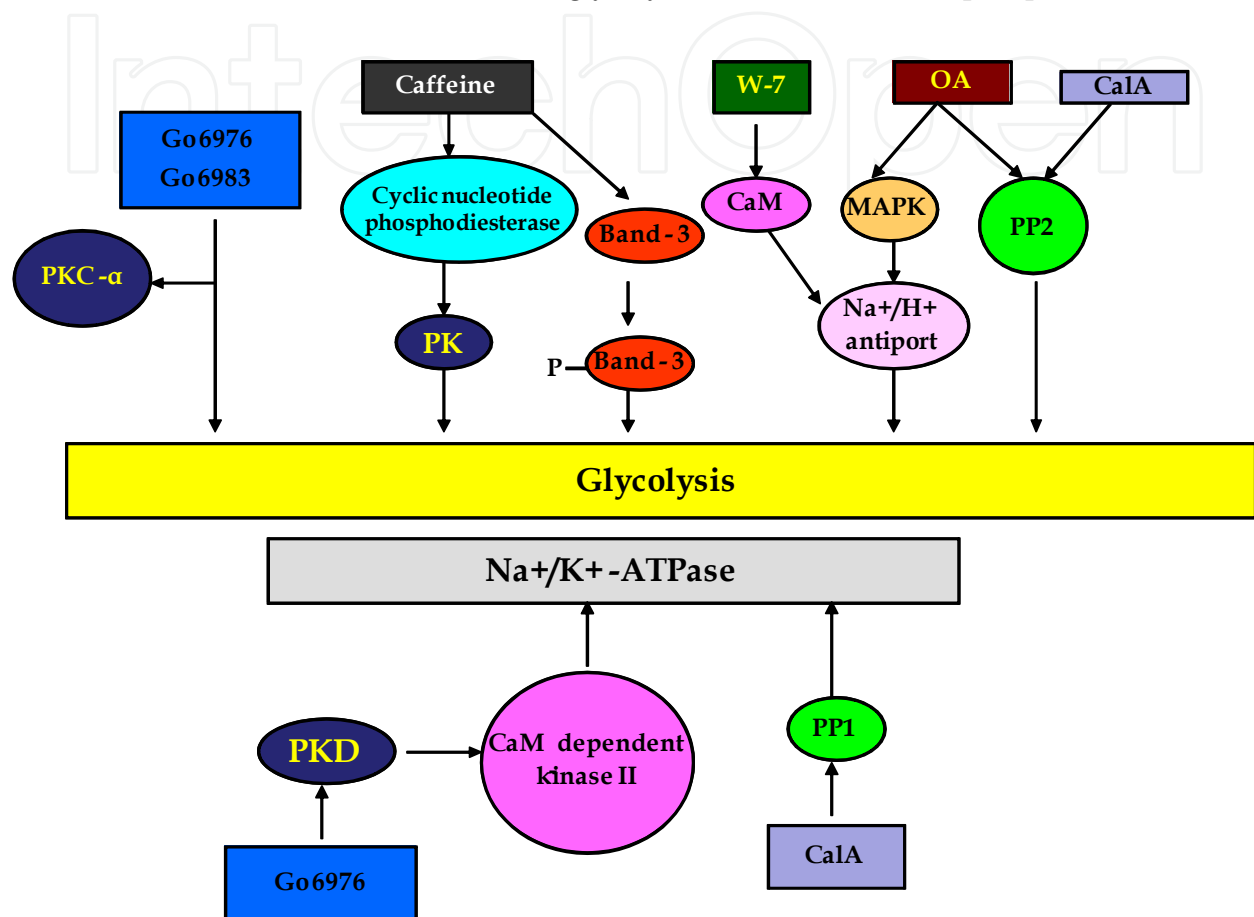


Fig. 4. Erythrocytes use different processes of phosphorylation in regulation of glycolysis and sodium pump.

(A). Glycolysis. Go6976 and Go6983 are inhibitors of PKC and stimulators of glycolysis. The target for this effect is PKC- α , which is probably a negative regulator of glycolysis. Caffeine increases the formation of lactate as: a) an activator of phosphodiesterase and inhibitor of c.AMP-dependent phosphorylation processes that keeps pyruvate kinase in dephosphorylated active form; b) induces conformational changes or activates phosphorylation of band 3. W-7 as a CaM antagonist blocks its inhibitory effect on Na^+/H^+ -antiport, which activates glycolysis. Okadaic acid (OA) stimulates glycolysis as an inhibitor of protein phosphatase PP2, which is probably a negative regulator of glycolysis, and as a stimulator of MAPK-dependent phosphorylation of Na^+/H^+ -antiport. Calyculin A (CaA) activates glycolysis as PP2 inhibitor that turns the proteins involved in glycolysis to their active dephosphorylated form. (B) Na^+/K^+ -ATPase. PKD is likely a negative regulator of the sodium pump using CaM-dependent pathway since its specific inhibitor Go6976 significantly increases the activity of Na^+/K^+ -ATPase. Cal A inhibits the activity of the pump reliably, which may be due to the involvement of PP1 in cell signals leading to activation of Na^+/K^+ -ATPase.

11. Acknowledgments

The authors would like to thank Dr. Ilian Radichev (Staff Scientist at Sanford Research/USD), for his valuable comments, and to acknowledge the helpful assistance of Jacob Ellefson (Research Associate at Sanford Research/USD) on improving the language quality of this work.

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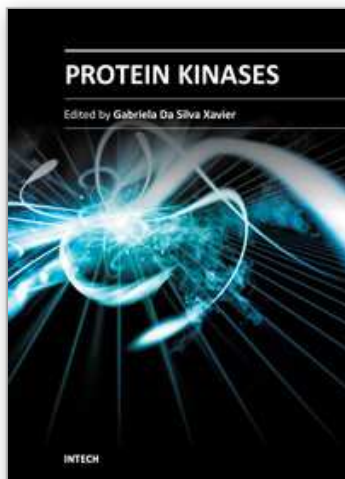
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Protein Kinases

Edited by Dr. Gabriela Da Silva Xavier

ISBN 978-953-51-0640-1

Hard cover, 484 pages

Publisher InTech

Published online 05, June, 2012

Published in print edition June, 2012

Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ana Maneva and Lilia Maneva-Radicheva (2012). Protein Kinases and Protein Phosphatases as Participants in Signal Transduction of Erythrocytes, Protein Kinases, Dr. Gabriela Da Silva Xavier (Ed.), ISBN: 978-953-51-0640-1, InTech, Available from: <http://www.intechopen.com/books/protein-kinases/protein-kinases-and-protein-phosphatases-as-key-participants-in-erythrocyte-signal-transduction>

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