Erythrocyte Signal Transduction Pathways, their Oxygenation Dependence and Functional Significance

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Key Words

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Abstract

Erythrocytes play a key role in human and vertebrate metabolism. Tissue O₂ supply is regulated by both hemoglobin (Hb)-O₂ affinity and erythrocyte rheology, a key determinant of tissue perfusion. Oxygenation-deoxygenation transitions of Hb may lead to reorganization of the cytoskeleton and signalling pathways activation/deactivation in an O₂-dependent manner. Deoxygenated Hb binds to the cytoplasmic domain of the anion exchanger band 3, which is anchored to the cytoskeleton, and is considered a major mechanism underlying the oxygenation-dependence of several erythrocyte functions. This work discusses the multiple modes of Hb-cytoskeleton interactions. In addition, it reviews the effects of Mg²⁺, 2,3-diphosphoglycerate, NO, shear stress and Ca²⁺, all factors accompanying the oxygenation-deoxygenation cycle in circulating red cells. Due to

the extensive literature on the subject, the data discussed here, pertain mainly to human erythrocytes whose O₂ affinity is modulated by 2,3-diphosphoglycerate, ectothermic vertebrate erythrocytes that use ATP, and to bird erythrocytes that use inositol pentaphosphate.

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Introduction

Erythrocytes play a key role in human and vertebrate metabolism. Apart from transporting O₂ to the tissues to support oxidative phosphorylation, they interact with and transport a range of other substances [protons, CO₂, chloride ions, organic phosphates and nitric oxide (NO)] that modulate tissue O₂ supply, either directly, through perturbation of the equilibrium between the 'tense' and 'relaxed' states of hemoglobin (Hb) molecules, or indirectly, by influencing cellular metabolism, erythrocyte rheology and tissue (e.g. capillary) perfusion. Moreover, oxygenation-dependent reactions of hormones, enzymes and factors from plasma and cytosolic compartments, with components of erythrocyte membranes may alter

erythrocyte properties and metabolism, and Hb itself may act as a transducer regulating cellular functions in an O₂-dependent manner through its interaction with cytoskeletal proteins.

Erythrocytes are equipped with a number of hormone and cytokine receptors [1]. Growth factor and cytokine receptors control apoptosis in nucleated cells [2] and are suggested as possible cell volume sensors [3, 4]. Apoptosis is characterized by cell shrinkage due to impaired cell volume regulation [5]. In human red cells, Ca²⁺-permeable cation channels involved in apoptosis are inhibited by erythropoietin [6]. Cytokines regulate Ca²⁺-activated K⁺ channels [7, 8], which also contribute to apoptosis in erythrocytes [9].

This communication reviews the possible coupling between blood oxygenation-deoxygenation transitions on the one hand and the metabolic reactions and physical properties of red blood cells (RBCs) on the other. Despite extensive data describing O₂-dependence of erythrocyte functions, such as membrane ion transport [10], the mechanical properties of whole RBCs or erythrocyte membranes [11-13], metabolic processes [14], the mechanisms responsible for numerous oxygenation effects remain to be established. Moreover, the oxygenation-deoxygenation cycle is not the exclusive factor determining O₂-dependent changes in cellular functions of the circulating erythrocytes *in vivo*; but alterations in other factors such as 2,3-diphosphoglycerate (DPG) and Mg²⁺ levels, the release of NO, and shear stress are also of physiological importance.

We review the possible signalling mechanisms triggering cytoskeletal reorganization to safeguard erythrocyte integrity under shear stress conditions. The erythrocyte signalling pathways have been summarized earlier [1]. Here, we examine the involvement of components of signalling pathways, which seem to be unique to circulating erythrocytes. We will first pay attention to Hb oxygenation-deoxygenation cycles. Second, we consider changes in Mg²⁺ and DPG levels that accompany blood oxygenation-deoxygenation transitions. Third, we discuss possible implications of the NO/cyclic GMP (cGMP)-dependent pathways. Finally, we address some aspects of shear stress-induced and Ca²⁺-dependent signalling. The data discussed pertain mainly to human RBCs whose O₂ affinity is modulated by the organophosphate DPG, but also to RBCs of fish and other ectotherm vertebrates that use ATP -together with guanosine triphosphate (GTP) in many fish species- and to bird RBCs that use inositol pentaphosphate [15].

Oxygenation-deoxygenation cycle and erythrocyte properties

O₂ sensing

Current concepts regarding the nature of O₂ sensor(s) in mammalian cells have been extensively reviewed [16]. Recently, a protein termed PASKIN has been identified as a potential O₂ sensor in these cells [17]. This protein contains a PAS domain resembling the O₂ sensing, heme-bearing PAS domain of bacterial O₂ sensors, and a serine/threonine kinase region that can provide coupling of O₂ sensing and signalling. In erythrocytes, possible candidates for O₂ sensing include Hb (especially its membrane-bound fraction), hypothetical heme-containing proteins, reactive O₂ species and changes in redox status [10, 18-20].

Additionally, the recently discovered new members of the globin family may act as O₂ sensors. These are: a) neuroglobins (Ngb) that are expressed primarily in vertebrate cerebral neurons and retinae [21], b) cytoglobins (Cygb, also termed histoglobins) that appear to be tissue-specific, occur in all tissues of higher vertebrates and have nuclear location [22, 23], and c) globin X that appears to occur in fish and amphibians but not in higher vertebrates [24]. Both Ngb and Cygb are up-regulated under hypoxic conditions [25, 26]. Ngb, which appears to be an endogenous neuroprotective factor in focal cerebral ischemia, may have a role in sensing and responding to neuronal hypoxia [27]. The surface structural changes that have been observed in Ngb upon O₂ and carbon monoxide (CO) ligation markedly affect its interaction with other proteins. Such changes may be important for an O₂-sensing function [28].

Interactions between Hb and the RBC membrane

Adult vertebrate Hb (HbA) is a tetrameric molecule consisting of two α and two β chains (Fig. 1). During oxygenation, Hb undergoes a transition from the low-affinity ("tense") structure to the high-oxygenated ("relaxed") structure that has a lower capacity for binding CO₂, protons, chloride ions and organophosphates. The effectors that stabilize the tense structure of Hb (and thus decrease its O₂ affinity) bind at well-defined sites. Anionic DPG binds to positively-charged amino acid residues (Val-1 of one β chain, and His-2, Lys-82 and His-143 of the two β chains) located in the entrance of the central cavity of deoxyHb (Fig. 1). Crystallography studies indicate that inositol pentaphosphate binds to two additional residues (Arg-135 and His-139) of chicken β chains [29].

Fig. 1. Diagrammatic illustration of the interactions between Hb, cdB3, glycolytic enzymes (GE), divalent cations (Mg^{2+}) and organic phosphates (ATP/DPG) in red blood cells. As indicated by the dotted lines, deoxyHb and GE compete for binding anionic cdB3 [14, 31], and divalent cations reduce the reaction of deoxyHb with cdB3 [42] and with ATP and DPG [59, 121]. The stereochemical implications are that ATP and DPG will bind to GE in competition with cdB3 and that these reactions will also be antagonized by cations (modified after [42]).

The Hb conformational transitions, thus, are accompanied by changes in such parameters as PO_2 , PCO_2 , pH, and in the levels of inorganic and organic anions, which in turn affect the concentrations of free inorganic cations such as Mg^{2+} . The effects of these factors on ion transport systems in the erythrocyte membrane have recently been reviewed [10, 30].

Hb is known to interact with the anion exchanger band 3, which constitutes an integral protein of the erythrocyte membrane (Fig. 1). Hb binds to the N-terminal cytoplasmic domain of band 3 protein (cdB3), with deoxyHb having a higher affinity for cdB3 than oxyHb [31]. As DPG, cdB3 carries several negatively-charged amino acid residues and the two bind at the same site in the central cavity, between the β -chains of Hb [31]. It may thus be expected that the anion transport function of band 3 is influenced by its reaction with Hb. Accordingly, incubation of human erythrocyte ghosts with Hb and Mg^{2+} increases $^{32}P_i$ uptake mediated by the anion exchanger [32]. However, data on the influence of O_2 tension on the anion exchanger are controversial. For instance, comparison of the kinetics of band 3-mediated Cl^- -transport in fish (carp, rainbow trout, eel and cod) and human erythrocytes did not reveal a significant difference between oxygenated and deoxygenated RBCs in the species studied [33]. Nor was O_2 -dependence of the anion exchanger revealed in crocodilian [34] and chicken [35] RBCs. On the other hand, there are recent reports that in normal human red cells, oxygenation stimulates the anion exchanger [35, 36].

CdB3 also binds to a range of glycolytic enzymes [14]. Competition between deoxyHb and these enzymes for binding with cdB3 suggests an O_2 -dependent regulation

of glucose metabolism (Fig. 1). Thus, upon Hb deoxygenation, glycolysis accelerates [37–40]. Indeed, hypoxia induces release of ATP from RBCs due to the activation of glycolysis following deoxyHb binding to cdB3 [41].

Ten-mer cdB3 peptides corresponding to the N-terminal cytoplasmic fragments of trout, chicken and human band 3 proteins undergo only slight, physiologically-insignificant interactions with fish (trout and catfish) and amphibian (Andean frog) Hbs. In contrast, these peptides interact strongly with major bird Hbs (chicken A and D) and with human Hb. This differentiation in cdB3-Hb interaction correlates with the number of negative charges on the peptides and of positive charges at the 'organophosphate'-site of the respective Hbs [42]. Weber et al. [42] propose that differences in RBC metabolism and buffering capacity are responsible for the different cdB3-Hb interactions. Thus, in ectotherm (fish and amphibian) RBCs that exhibit high, aerobic metabolic rates and are poorly buffered, the regulation of glycolytic rates may occur via pronounced pH variations, whereas in the strongly buffered mammalian RBCs that show only small pH variations and derive energy primarily from glycolysis it appears to be a function of oxygenation-dependent Hb-cdB3 interaction. Weber et al. moreover, demonstrated that DPG inhibits cdB3-Hb interactions by competing with cdB3 for the same binding site of Hb [42].

Conformational transitions of Hb were proposed to be implicated in O_2 sensing [18], since both O_2 and CO induce similar changes in Hb conformation (favouring the R state) and exert similar effects on adrenergically stimulated Na/H exchange in trout RBCs. The binding of deoxyHb to cdB3 together with the integral involvement

of band 3 with the cytoskeleton structure implies that the cytoskeleton transmits deoxygenation-induced changes to ion transporters [18]. However, a recent report appears to provide evidence for the implication of an alternative O₂ sensor to Hb that regulates K⁺ fluxes in trout RBCs [19].

Furthermore, Hb may interact with acidic cytoplasmic fragments of other erythrocyte membrane proteins. It has been suggested to bind to glycophorin [43]. Both, the N-terminal fragment of actin and the C-terminal fragment of tubulin are negatively charged and have been shown to decrease the O₂ affinity of human Hb [44]. The oxygenation-dependence of low frequency submicron fluctuations of human red cell membranes [11] provides indirect evidence for Hb-actin interaction *in vivo* since the ATPase activity of F-actin is responsible for the stimulation of cell membrane fluctuations [45].

There are, however, observations suggesting that Hb-cdB3 interaction (generally speaking, any Hb-protein interaction involving binding of the polyanionic fragment of a protein in the DPG pocket of Hb) cannot satisfactorily account for the coupling between the oxygenation-deoxygenation cycle and function of O₂-sensitive systems. Trout RBCs possess O₂-dependent Na/H exchange [18] and K-Cl cotransport [46] and the response of trout erythrocytes to cortisol is also oxygenation-dependent [47]. However, trout Hb IV does not interact with synthetic peptides of the N-terminal fragment of trout band 3 *in vitro* [48], although it cannot be excluded that such interaction occurs *in vivo*. Besides, it cannot be excluded that fish Hbs interact with the acidic tails of actin and tubulin, as was shown with human Hb [44]. Similar evidence is provided by sheep Hb that exhibits low DPG binding and hence may not be expected to bind to negatively charged tails of proteins. Nevertheless, K-Cl cotransport in sheep RBCs has been shown to be O₂-sensitive [49].

Some findings support Hb-spectrin interaction. It was shown that spectrin can bind to Hb [50] and slightly increase the Hb-O₂ affinity [51]. The stabilizing effect of Hb on the spectrin tetramer, likewise, implies interaction between these two proteins [52]. These findings are supported, in part, by an association between Hb and the spectrin tetramer observed by Vincentelli et al. at physiological pH and ionic strength [53]. However, in this study, the Hb-O₂ affinity was not significantly affected by spectrin [53]. Furthermore, neither binding of spectrin to Hb, nor an influence of spectrin on the functional properties of Hb were observed in other studies [54, 55].

The binding constant between spectrin and oxyHb

is two orders of magnitude greater than that between spectrin and cdB3 [56]. The spectrin-Hb interaction is markedly stronger for HbA2 (the $\alpha_2\delta_2$ Hb variant found in β -thalassemia patients). A high Hb-spectrin affinity may play a role in the maintenance of the RBC membrane structural integrity. A different spectrin binding to oxy- and deoxyHb could provide a basis for oxygenation-dependent interactions between HbA or HbA2, spectrin and band 3.

The findings describing interactions between Hb and membrane components suggest that the cytoskeleton undergoes rearrangements in an O₂-dependent manner. The hypoxia-induced increase in the number of 3H-DHA (1-(4,6-propyl 3-H)-dihydroalprenolol; hydrophobic β -adrenergic antagonist) binding sites in carp red cells and the ability of 3H-DHA to bind to several nonreceptor sites led to the suggestion "that structural rearrangements occur in the red cell membrane of carp under hypoxic conditions" [57]. Indeed, in human RBCs, deoxygenation was accompanied by an increased band 3-cytoskeleton interaction [13] and by reversible dissociation of protein 4.1 from glycophorin C [58]. However, it cannot be ruled out that O₂-dependent changes in the levels of free Mg²⁺ (that complexes with erythrocyte organic phosphates and decreases their effect on Hb oxygenation [59]) and DPG can account for the effect of deoxygenation on the association of cytoskeletal proteins. The structure of the cytoskeleton, in turn, determines the organization of the signalling pathways [60], cell volume regulation [4, 61] and the mechanical properties of the membrane.

Hb oxygenation and signalling pathways

Oxygenation-deoxygenation transitions appear to evoke a cascade of tyrosine and serine/threonine phosphorylation reactions transmitted from the O₂ sensor to the executive mechanisms. At least two subsequent tyrosine phosphorylation steps were shown to participate in O₂ signalling in trout red cells [62]. Vanadate, an inhibitor of protein tyrosine phosphatase, activates K-Cl cotransport in a N₂ atmosphere [62]. Furthermore, N-ethylmaleimide (NEM) a well known activator of K-Cl cotransport [63, 64] also inhibits protein tyrosine phosphatase [65, 66] and activates K-Cl cotransport in trout [67] and equine RBCs [68] under low O₂ and deoxygenation conditions, respectively. Interestingly, oxygenation also has an activating effect on K-Cl cotransport and this is most pronounced at Hb-O₂ saturation above 70% [62, 68]. This tallies with the observations that (as judged from the intrinsic "Adair" association constants for binding of the successive O₂

molecules) the major conformational changes in human and tench (fish) Hbs occur late in the oxygenation process [69, 70].

In trout RBCs, genistein, an inhibitor of protein tyrosine kinase, completely inhibits both oxygenation- and vanadate-activated K-Cl cotransport but does not influence NEM-activated K-Cl cotransport [62]. Calyculin A, an inhibitor of serine/threonine protein phosphatase, abolishes activation of K-Cl cotransport by oxygenation both in trout [62] and equine erythrocytes [68]. Thus oxygenation is likely to inhibit tyrosine phosphatase, and/or to activate tyrosine kinase resulting in subsequent activation of serine/threonine phosphatase.

Little is known about the effects of O₂ tension per se on protein kinase and phosphatase activities in erythrocytes. Protein tyrosine kinase activity proved to be insensitive to deoxygenation in normal human RBCs [71], whereas in sickle cells deoxygenation leads to inhibition of protein tyrosine phosphatase and activation of Syk tyrosine kinase [72]. On the other hand, in normal human erythrocytes, deoxygenation was shown to increase tyrosine phosphorylation of band 3, apparently via stimulation of tyrosine kinase activity [13]. Serine/threonine phosphorylation also appears to be O₂-sensitive. Thus, dephosphorylation of serine/threonine residues of membrane proteins in normal human RBCs under deoxygenation conditions appears to be due to activation of serine/threonine phosphatase(s) rather than inhibition of kinase(s) [73], as reported for the effect of oxygenation in trout [67] and equine erythrocytes [68].

The response of the Na/H exchanger in trout [18] and cell membrane fluctuations in human RBCs [12] to adrenergic stimulation is more pronounced in deoxygenated cells. However, production of cyclic AMP (cAMP) after hormonal stimulation was similar in oxygenated and deoxygenated RBCs [18]. This suggests that in trout erythrocytes, adenylyl cyclase activity is not affected by oxygenation. In contrast, in carp RBCs, hypoxia was shown to increase both the apparent number of β -antagonist binding sites [57] and accumulation of cAMP after adrenergic stimulation [74]. The β -Na/H exchanger (NHE) of trout RBCs is directly phosphorylated by cAMP-dependent protein kinase [75], which is bound to actin microfilaments and microtubules [60]. In other cell types, transduction of the signal through the cAMP-dependent pathway can be interrupted by changes in the cytoskeleton [76, 77]. Besides, mammalian NHE1 serves as an anchoring protein for the actin cytoskeleton although this isoform is insensitive to cAMP [78]. Hence, an O₂-dependent association of Hb with

the cytoskeleton is likely to affect protein kinase A anchoring and/or a direct interaction between the cytoskeleton and Na/H exchanger.

Adrenergic activation of the erythrocyte Na/H exchanger plays a significant role in the acute response of some fish species to hypoxia. Adrenergic stimulation of RBCs increases their Hb-O₂ affinity via the Bohr effect (since the proton export alkalinizes the cells), and by concomitant RBC swelling resulting from uptake of Na, Cl and osmotically-obliged water, which dilutes cellular ATP and Hb concentrations [74, 79]. Fish RBCs remain swollen under hypoxia since K-Cl cotransport, which is normally implicated in regulatory volume decrease (RVD), is both deactivated and activated by low and high O₂ tension, respectively [80, 81]. Jensen [82] has recently reviewed the oxygenation dependence of RBC pH and its implications for blood gas transport.

In trout, cortisol decreased the viscosity of deoxygenated but not oxygenated blood [47]. Trout RBCs possess microtubules and a certain architecture of this cytoskeletal structure was shown to be necessary for short-term non-genomic action of corticosterone in vascular smooth muscle cells [83].

The role of reactive O₂ species (ROS) in intracellular signalling received significant attention [84]. RBCs act as a ROS sink and therefore are subjected to a constant flow of Hb auto-oxidation whereby about 3% of human Hb is oxidized to metHb each day [85]. In trout erythrocytes, ROS were shown to activate K-Cl cotransport at low O₂ tension [20]. Furthermore, at high O₂ levels, scavenging of ROS inhibited K-Cl cotransport [20] and activated adrenergically stimulated Na/H exchange [86], suggesting that ROS are mediators of O₂-induced signalling. On the other hand, in normal human RBCs, the oxidants' effect on the activity of the Gardos channel is O₂-dependent: activation of the Gardos channel by 1-chloro-2,4-dinitrobenzene (CDNB) and by phenazine methasulphate (PMS) was more effective in oxygenated and deoxygenated cells, respectively [87].

DPG appears to interfere with the phosphoinositide cascade, since it can be considered as a structural analog of inositol triphosphate (IP₃) and the activities of both inositol triphosphate phosphatase and inositol triphosphate kinase are inhibited by DPG [88]. The structural similarity between 2,3-DPG and IP₃, together with an apparent involvement of the phosphoinositide turnover pathway in swelling-induced signalling in skate [89], human [90] and sheep [91] RBCs imply that deoxyHb is likely to interact with IP₃ and phosphatidylinositol-4,5-bisphosphate (PIP₂), and thus affect cell volume regulation. In erythrocytes,

Fig. 2. Putative interactions between Hb (or alternative O₂ sensors) and the cellular structures and functions implicated in cell volume regulation.

polyphosphoinositides are involved in the regulation of cell shape [92], activity of Ca²⁺-ATPase [93] and K-Cl cotransport [91], and in the redistribution of membrane phospholipids [94].

Hb oxygenation and volume regulation

Membrane ion transport has been thoroughly investigated for various erythrocyte functions that are regulated by O₂ tension [10, 30]. K-Cl and Na-K-2Cl cotransporters as well as the Na/H exchanger are known to be O₂-dependent. In addition, some ion channels, as well as amino acid and glucose transporters appear to be O₂-sensitive [10, 36]. However, data on the Cl/HCO₃ exchanger (see above) and Na/K pump are controversial. Drew et al. [36] found no O₂-sensitivity of the Na/K pump in human and chicken red cells. On the other hand, Bogdanova et al. [95] have shown that hypoxia inhibited the Na/K pump in mouse erythrocytes. The processes implicated in cell volume sensing and regulation have been extensively reviewed [3, 4, 96, 97]. It is postulated that signals triggered by cell volume alterations are received by volume sensors which, in turn, induce signalling cascades leading to activation of ion transporters responsible for regulatory volume increase and decrease (RVI and RVD, see Fig. 2). In vertebrate RBCs, oxygenation stimulates swelling-activated ion transport (K-Cl cotransport), whereas deoxygenation evokes shrinkage-activated transport (Na-K-2Cl cotransport and Na/H exchange) [10]. Furthermore, deoxygenated trout [81] and carp RBCs [98] are characterized by incomplete RVD after hypotonic swelling whereas oxygenated cells completely recover their volume. Specific organization

of actin in the cytoskeleton appears to be necessary for the generation and transduction of signals triggered by cell volume alterations [4, 61] (Fig. 2). In rat, involvement of the cytoskeleton in cell volume regulation has been suggested since denaturation of spectrin (after heating erythrocytes to 49°C) obliterated the responses of K-Cl and Na-K-2Cl cotransporters to cell volume alterations [99].

Integrins, growth factor and cytokine receptors, and heterotrimeric GTP-binding proteins (G proteins) are considered as putative volume sensors [3, 4, 100] (Fig. 2). Heterotrimeric G proteins, growth factor and cytokine receptors are present in human red cells [1]. There is increasing evidence for a role of integrins as volume sensors in other cell types, although the presence of integrins in mature human erythrocytes remains unclear [101]. Hypotonic swelling of rat hepatocytes leads to integrin-dependent activation of Src tyrosine kinase [102]. In lymphocytes, p56lck, a Src-like tyrosine kinase, is responsible for swelling-induced activation of chloride channels [103]. Recently, the mechanism of activation of Src tyrosine kinases via direct interaction with integrins has been reported [104].

Hb participates in volume sensing via its non-specific effect on macromolecular crowding since the effect of Hb was mimicked by albumin [105, 106] (Fig. 2). According to this hypothesis, concentration or dilution of Hb during cell volume perturbation may change the activity of protein kinases and phosphatases that are involved in volume regulation.

However, the volume-dependence of K-Cl cotransport on HbA and S in human erythrocyte ghosts

suggests an alternative mechanism of participation of Hb in cell volume regulation [107]. K-Cl cotransport in white ghosts of human erythrocytes is volume- and pH-independent [107]. However, re-incorporation of Hb A and S into white ghosts restores the volume dependence of K-Cl cotransport but not its pH dependence. Moreover, the effect of Hb is type specific: in hypertonic medium, both Hb A and Hb S decrease K-Cl cotransport, while in hypotonic medium, Hb A decreases whereas Hb S increases K-Cl cotransport activity. However, in contrast to the observations of Vitoux et al. [107], Sachs and Martin [108] found that K-Cl cotransport in white ghosts of human erythrocytes retains its volume dependence. Recently, deoxygenation was shown to decrease K-Cl cotransport activity only in pink ghosts (i.e. those retaining about 5–10% of the whole cell Hb) although under oxygenation, the level of K-Cl cotransport activity is similar in pink and white ghosts [109].

The activity of K-Cl cotransport is significantly increased in cells containing Hb variants where negatively-charged Glu residues at the β -6 or β -7 position are substituted with neutral or positively-charged residues [110]. Amino acids at positions β -6 and β -7 are part of Hb's DPG-binding pocket and the reduced negativity may increase the interaction between Hb and negatively charged fragments of membrane proteins. Cells containing Hb variants where amino acids at positions other than β -6 or β -7 are substituted for positively charged residues did not exhibit increased K-Cl cotransport activity [110]. However, it was recently shown that Hb variants containing an additional positive charge (β 121 Glu replaced by Lys) are associated with increased K-Cl cotransport [111].

In this regard, sheep RBCs are of particular interest, since sheep Hb has a low DPG-binding affinity and therefore is not expected to exhibit significant binding of cytoplasmic elements that are structurally similar to DPG, suggesting that these cells may not undergo significant O_2 -dependent cytoskeletal reorganization. Unlike the complete inactivation and volume- and pH-insensitivity of K-Cl cotransport in trout [81] and equine [68] RBCs under deoxygenation, K-Cl cotransport in deoxygenated sheep erythrocytes is active and volume- and pH-sensitive [49]. Nevertheless, oxygenation of sheep RBCs affects K-Cl cotransport by increasing both its rate and the degree of its volume- and pH-dependence [49]. It is noteworthy that K-Cl cotransport in sheep erythrocytes seems to be controlled by two signals of cell swelling, a reduction in macromolecular crowding and a putative mechanical signal [112].

It would be interesting to study K-Cl cotransport in RBCs containing Hb variants that have negative charges close to the DPG pocket, such as sheep Hb. Adult sheep RBCs have low DPG concentrations [113] and sheep Hb binds DPG only slightly (when in concentrated solution) [114]. Incorporation of variant Hbs in human erythrocyte ghosts [107, 109] may provide a tool to study the effects of Hb structure on K-Cl cotransport regulation. Thus, ghost incorporation of Hbs that do or do not bind DPG could help to distinguish between effects of Hb via interaction of membrane proteins with the DPG pocket in the Hb molecule from those due to interactions of membrane proteins with alternative site(s) to the DPG pocket.

Hb oxygenation and mechanical properties of erythrocytes

The main factors determining red cell deformability are (i) cell shape (a function of cell membrane surface-to-volume ratio), (ii) rheological properties of the intracellular fluid, and (iii) rheological properties of the cell membrane [115] (See Fig. 6 below).

Information on whole cell rheological behaviour as a function of the oxygenation status is not unequivocal. No difference between oxygenated and deoxygenated cells has been detected with regard to viscosity in trout RBCs [47] and deformability in human RBCs [58]. On the other hand, decreased filterability has been observed in deoxygenated human RBCs [12]. Only one report appears to exist on O_2 -dependence of RBC aggregation [116], where in the presence of CO_2 RBC aggregation was lower both under oxygenation and deoxygenation conditions, and in the absence of CO_2 , RBC aggregation was lower in oxygenated cells. This effect of CO_2 appears to improve blood flow in metabolically active tissues [116].

The mean cell volume was found to be similar in oxygenated and deoxygenated trout and equine RBCs [47, 117], whereas membrane rigidity proved to be dependent on oxygenation in human cells [11, 13]. These findings suggest that if O_2 tension were to control deformability of the whole cell, it would involve modulation of the membrane mechanical properties rather than changes in cell volume. However, O_2 -dependent changes of RBC aggregation are partly due to changes in cell volume [116].

Indeed, deoxygenation was accompanied by elevation of band 3 tyrosine phosphorylation, increased band 3-cytoskeleton interaction and increased rigidity of the membrane in human RBCs [13]. The increased rigidity was proposed to be due to changes in free Mg^{2+} and to a

Fig. 3. Diagram illustrating oxygenation-dependent changes in cytoskeletal properties mediated via changes in the free Mg^{2+} and 2,3-DPG levels. Effects of Mg^{2+} and 2,3-DPG on the cytoskeleton are also mediated via protein kinases and phosphatases [146, 147] (Panel A). The free concentration of Mg^{2+} ions increases under low O_2 saturation by binding of 2,3-DPG to deoxy Hb. Mg^{2+} binds directly to spectrin (shaded circles) [126] (Panel B) and increases the stability of the erythrocyte membrane [125]. 2,3-DPG (whose free concentration increases upon Hb oxygenation) binds directly to spectrin, band 3 and protein 4.1 (open triangles) [143, 145] (Panel C) and decreases the interactions between cytoskeletal proteins [144, 145].

possible effect of deoxyHb-cdB3 binding on tyrosine kinase activity. Tuvia et al. [11] showed that Mg^{2+} changes during an oxygenation-deoxygenation cycle may be responsible for O_2 -dependent modulation of membrane fluctuations, which, in turn, determine membrane plasticity. Membrane fluctuations of RBCs appear to be associated with the ATPase activity of F-actin [45]. Binding of Hb to actin [44] could also contribute to the dependence of membrane properties on oxygenation. In this context, divalent cations significantly promote Hb-membrane binding [32, 118] that itself leads to rigidification of the membrane and decreases filterability of human RBCs [118].

Furthermore, both tyrosine [13, 119] and serine/threonine phosphorylation [120] of the cytoskeletal proteins are obviously implicated in the regulation of membrane mechanical properties. In turn, tyrosine [13, 62] and serine/threonine phosphorylation [73] appear to be regulated by O_2 tension. Taken together these observations provide indirect evidence for the dependence of membrane mechanical properties on oxygenation.

Mg^{2+} and DPG

DeoxyHb and Mg^{2+} both bind to DPG with the result that the free Mg^{2+} levels are high at low O_2 tension (low O_2 saturation) and low at high O_2 concentrations (Fig. 3A). Likewise, free DPG levels are low at low O_2 tensions

and vice versa. Moreover, binding of Mg^{2+} to DPG and nucleoside triphosphates like ATP increases Hb- O_2 affinity [59]. Mg^{2+} ions, similarly, decrease ATP and GTP binding to carp (fish) Hb and the interaction between human Hb and cdb3 [42, 121]. Thus, Mg^{2+} increases O_2 affinity by reducing DPG, ATP and cdb3 binding to Hb.

The role of Mg^{2+} in regulating intracellular processes in mammalian cells has recently been discussed [122]. The effects of Mg^{2+} are thought to be mediated by protein kinases and phosphatases [123]. Mg^{2+} is necessary for the association of actin with tropomyosin [124]. Also, Mg^{2+} was shown to increase erythrocyte membrane stability [125]. It is noteworthy that spectrin has binding sites for both Mg^{2+} and Ca^{2+} [126] (Fig 3B). As for DPG levels in mammals, there are significant inter-species differences [127, 128] and regulation of DPG metabolism has been analyzed mathematically [129-131]. DPG is a major modulator of Hb- O_2 affinity. However, its role as modulator of the cytoskeleton is not yet completely understood and conflicting results concerning the involvement of DPG in membrane function have been reported. Waugh [132] holds that under physiological conditions, DPG is unlikely to affect erythrocyte membrane properties. However, Mentzer et al. [133] found that elevated DPG levels decrease the membrane stability of normal human erythrocytes. Chasis and Mohandas [134] showed that DPG also decreases membrane deformability. It should be emphasized that membrane stability and deformability are distinct

properties that are independently regulated by cytoskeletal associations. Erythrocyte deformability is the same for RBCs with low DPG levels and their corresponding controls, whereas DPG-enriched erythrocytes are less deformable [135]. On the other hand, *in vivo* administration of pentoxifylline for improvement of blood rheology increases deformability of erythrocytes and DPG levels [136, 137]. Likewise, KBT-3022, an anti-platelet agent, improves deformability and increases the contents of ATP and DPG in erythrocytes [138], supporting the idea that these two nucleotides are responsible for the improved RBC deformability.

There is evidence suggesting that DPG may participate in the regulation of Na-K-2Cl cotransport. This transporter's activity varies inversely with the DPG levels and directly with free intracellular Mg^{2+} [123, 139]. Mairbaur and Hoffman [123] suggested that protein kinases and phosphatases that are targeted to the Na-K-2Cl cotransporter are themselves regulated by free Mg^{2+} , which, in turn, is titrated by the DPG levels. In this context, increased mean cell Hb concentration is a major cause of decreased deformability of DPG-enriched erythrocytes [135]. However, regulation of K-Cl cotransport, which in contrast to Na-K-Cl cotransport, is inhibited by free Mg^{2+} [140, 141] is independent of the oxygenation-induced fluctuations of free Mg^{2+} [10, 49].

Concerning DPG-mediated mechanisms modulating membrane properties, spectrin has binding sites for both ATP [142] and DPG [143] (Fig. 3C), which suggests that Hb oxygenation may increase the formation of spectrin-DPG and spectrin-ATP complexes. The possible physiological effects of such interactions have not been determined. The resulting changes in spectrin's flexibility would be of particular interest, since DPG is known to inhibit interactions of cytoskeletal proteins [144, 145].

A few data are available concerning the direct effects of DPG on protein kinases and phosphatases. For instance, erythrocyte casein kinases are either inhibited or stimulated by DPG, depending on casein and monovalent salt concentrations [146]. DPG stimulates both the rate and the extent of phosphorylation of protein 4.1 by protein kinase C [147], whereas phosphorylation of protein 4.1 by cytosolic casein kinase and cAMP-dependent protein kinase are inhibited by DPG. Protein 4.1-band 3 and ankyrin-band 3 interactions (Fig. 3B-C) are important for modulation of erythrocyte membrane stability and deformability [148]. Interestingly, activation of protein kinase C [149] and cAMP-dependent protein kinase [150] partially improve RBC filterability.

Possible implications of NO

NO acts principally through activation of soluble guanylyl cyclase, leading to increased cGMP levels, which, in turn, activate cGMP-dependent protein kinase (protein kinase G or PKG) (Fig. 4). Vascular endothelium serves as an important source of NO. Interestingly, RBCs themselves may regulate NO production by endothelial cells via release of ATP from RBCs in response to hypoxia and mechanical deformation [151, 152]. At high PO_2 , NO appears to bind to Hb with high affinity, whereas at low PO_2 , NO is released from Hb to improve blood flow in precapillary resistance arterioles [153]. Recently, it was shown that band 3 may provide a route for exporting NO from RBCs [154]. The recent discovery that cdB3 drastically decreases the O_2 affinity of S-nitrosated Hb indicates that an intracellular S-nitroso-Hb-band 3 complex may play a role in targeting NO and that it may be linked to Heinz body formation [155]. Moreover, erythrocytes have their own NO synthase [156], although it is supposed to be inactive [157]. However, a more recent report does support the existence of active NO synthase in human red cells [158]. Soluble guanylyl cyclase, the next signalling component in the NO/cGMP/PKG pathway (Fig. 4), is present in human RBCs [159].

Both in erythrocytes and in vascular smooth muscle cells, the activation of K-Cl cotransport by NO is inhibited by genistein and calyculin [140], suggesting that NO triggers a cascade of phosphorylation/dephosphorylation reactions. In smooth muscle cells, NO was shown to activate phosphotyrosine phosphatase-1B [160] and protein tyrosine phosphatase containing 2 SH2 domains (SHP-2) [161] via a cGMP-dependent pathway. In erythrocytes, the stimulating action of nitrovasodilators on K-Cl cotransport also appears to require cGMP-dependent protein kinase activity [140] (Fig. 4). Human erythrocytes possess SHP-2 tyrosine phosphatase [66] and seem to have the protein tyrosine phosphatase 1B type [162]. These tyrosine phosphatases are associated with band 3 (see above).

NO activates Na/H exchange in human RBCs [159] and K-Cl cotransport in sheep erythrocytes [140, 163]. In both cases cGMP-dependent pathways were involved (Fig. 4). The erythrocyte shape may also be affected by NO [164]. NO can improve the rheological properties of both erythrocyte membrane [165] and whole cells [158, 166]. However, NO decreases rat RBC deformability in sepsis [167]. Activation of the cGMP-dependent pathway by atrial natriuretic peptide also increases filterability of RBCs [168].

Ca^{2+} is a well-known signalling ion and the interactions between NO- and Ca^{2+} -dependent signalling are briefly discussed below.

Shear stress-induced and Ca^{2+} -dependent mechanisms

It is in the microcirculation where erythrocytes undergo maximal mechanical load. Mechanical stress activates multiple signalling pathways and reorganization of the cytoskeleton in order to maintain the integrity of the cellular plasma membrane [169]. Multiple signalling mechanisms activated by shear stress in endothelial cells have been reviewed [170]. In endothelial cells, the role of integrins [171] and heterotrimeric G proteins [172] in shear stress-induced signalling is well established. Heterotrimeric G proteins of the G_i subtype [173], adenylyl cyclase and protein kinase A [174], as well as the cystic fibrosis transmembrane conductance regulator [175], are components of signal transduction pathways. These molecules are responsible for the deformation-induced ATP release from RBCs that, in turn, regulates vascular resistance [152].

Mechanical stress causes plasma membrane disruption in cells (Fig. 5). Physiological shear stress increases erythrocyte membrane permeability to monovalent cations [176] and Ca^{2+} [177] (Fig. 5). Mechanical disruption of the membrane and activation of mechano-sensitive ion channels are major routes for shear stress-induced ion fluxes. Cell swelling, used as an analog of shear stress, opens mechano-sensitive Ca^{2+} -permeable cation channels in human RBCs [90], whereas during shrinkage, Ca^{2+} influx occurs via non-selective cation channels that are inhibited by swelling and increase in intracellular Cl^- [178]. Furthermore, stretch-activated inwardly rectifying anion channels are present in human red cells [179].

In the cytosol, Ca^{2+} activates at least such regulatory proteins as calmodulin, Ca^{2+} /calmodulin-dependent protein kinase (Fig 5), phospholipase C and the phosphoinositide turnover, and protein kinase C. Ca^{2+} is tightly implicated in the regulation of membrane structural and mechanical properties via its binding to calmodulin [180, 181, 182] (Fig. 5). The main targets of calmodulin are the Ca^{2+} pump, adducin, protein 4.1 and spectrin [1, 124].

Age-dependent elevation of cytosolic Ca^{2+} followed by activation of the Gardos channel, scramblase and calpain (Fig 5) was proposed as a mechanism responsible for the triggering of programmed cell death and removal

of dying erythrocytes from circulation [183]. Erythrocyte ageing is accompanied by an increase in the activity of mechano-sensitive Ca^{2+} -permeable cation channels [90] and by decrease in the activity of the Ca^{2+} pump [184].

Cell shrinkage, membrane blebbing and breakdown of phosphatidylserine (PS) asymmetry, typical features of apoptosis in nucleated cells [2] (Fig. 5), are also triggered in red cells by increase in intracellular Ca^{2+} when induced by the Ca^{2+} ionophore ionomycin [185-187]. The mechanism of erythrocyte apoptosis appears to be conservative in evolution [188]. Ca^{2+} influx through cation channels appears to be regulated by Bcl-XL, suppressor of apoptosis, and Bak, promoter of apoptosis, both expressed in mature human red cells [189]. Human plasma contains survival factors that increase erythrocyte viability in culture [189]. One such factor could be erythropoietin that inhibits Ca^{2+} -permeable cation channels [6]. Interestingly, high concentrations of urea and NaCl to which erythrocytes are exposed in kidney medulla exert an antiapoptotic effect on erythrocytes [190].

Recently, Ca^{2+} entry through non-selective cation channels which are activated by osmotic shock, oxidative stress, energy depletion and Cl^- removal was proposed as a major signal for induction of apoptosis in erythrocytes [191]. However, additional mechanisms triggering apoptosis of red cells appear to exist since removal of extracellular Ca^{2+} only partially blunted apoptosis after osmotic shock [191]. Enhanced influx of Ca^{2+} activates the scramblase that transfers PS to the outer leaflet of the membrane [192] (Fig 5). Exposure of PS on the cell surface allows phagocytic cells to recognize and eliminate dying erythrocytes from circulation [193]. Mechanical stress-induced Ca^{2+} entry also activates Gardos channels [194] (Fig. 5) thus leading to cell shrinkage and facilitating engulfment of erythrocytes by phagocytes. Furthermore, asymmetry of phospholipids in the cell membrane improves the membrane mechanical stability [195], whereas increase in cytosolic Ca^{2+} induces vesiculation of the erythrocyte membrane [196].

Another target of Ca^{2+} is calpain, a neutral Ca^{2+} -dependent protease, that is translocated to the membrane and activated by autolysis in the presence of Ca^{2+} [197]. Cytoskeletal proteins [198], protein kinase C [199] and protein tyrosine phosphatase 1B [200] undergo proteolysis by calpain. In contrast, the age-related decline of the Ca^{2+} pump activity is not due to proteolysis by calpain [201].

Nevertheless, sudden entry of Ca^{2+} into the cytosol upon mechanical injury of the membrane or by opening Ca^{2+} -permeable channels not only stimulates proapoptotic mechanisms but also is suggested to initiate local repair

Fig. 4. Scheme illustrating involvement of NO in RBC signalling. OxyHb binds NO produced in the lungs and then transfers and delivers it to peripheral tissues [153]. NO can also be obtained from external sources (e.g. nitrovasodilators). In addition, red cells appear to express nitric oxide synthase (NOS) [156, 158], which may be activated by shear stress (dashed arrows), like in endothelial cells [170, 211]. RBCs also possess soluble guanylyl cyclase (sGC) [159] and protein kinase G (PKG) [140, 159]. The main targets of NO are the Na/H exchange [159], K-Cl cotransport [140, 163] and RBC mechanical properties [158, 165-167].

Fig. 5. Scheme illustrating shear stress-induced Ca^{2+} -dependent mechanisms in RBCs. Shear stress induces Ca^{2+} entry via mechanosensitive Ca^{2+} -permeable non-selective cation channels [90] and via plasma membrane disruption. Effects of Ca^{2+} on red cell mechanical properties are mediated by direct binding of Ca^{2+} to cytoskeletal proteins (e.g. spectrin [126]), via calmodulin [180-182] and via Ca^{2+} -dependent enzymes (e.g. calpain [198]). Activation of scramblase and Gardos channels may trigger apoptosis in red cells [191]. Also, elevation in intracellular Ca^{2+} may lead to local repair of the cytoskeleton through formation of actomyosin II complex (italics, dashed arrows) [202].

of the cytoskeleton through formation of actomyosin II complexes [202] (Fig. 5). Since erythrocyte spectrin has

binding sites for Ca^{2+} and Mg^{2+} (Fig. 3B), it was suggested that alterations of the mechanical properties of spectrin

Fig. 6. Overall scheme showing links between Hb-cytoskeletal protein interactions, cellular metabolites and shear stress-induced changes, and the rheological properties of RBCs.

upon divalent cation binding may be important for the maintenance of erythrocyte integrity under shear stress [126] (Fig. 5). Reversible energy-independent contractions of red cell ghosts occur in response to an increase in the concentration of monovalent and divalent cations [203]. Ca^{2+} and Mg-ATP induce reversible shape changes of triton-treated erythrocyte ghosts [204], Ca^{2+} being responsible for the contraction of such ghosts. The role of divalent cations on Hb-membrane binding [32, 118] and its effect on membrane rigidification and human RBC filterability [118] has been described above).

It seems important to highlight the spatial aspects of calcium signalling. The concept of physiologically meaningful signalling via highly localized and transient increases in Ca^{2+} has been developed in the last few years [205, 206]. Indeed, the diffusion of Ca^{2+} in the cytosol is very limited and an increase in Ca^{2+} in this compartment can therefore be kept localized to some extent. The calculated effective ranges for the distance from the source (membrane Ca -channels, Ca^{2+} stores, etc.) are $0.1\ \mu\text{m}$ for free Ca^{2+} before it is buffered and $5\ \mu\text{m}$ for buffered Ca^{2+} [207]. As a consequence, individual portions of the membrane can be regulated in a manner largely autonomous compared to the rest of the cell. It is tempting to speculate that both the local reversible ATP-dependent

and -independent Ca^{2+} -induced changes in membrane mechanical properties may facilitate passage of erythrocytes through the microcirculation.

It is noteworthy that both Ca^{2+} -mediated pathways and NO-induced events are triggered almost simultaneously in the microcirculation where increased Ca^{2+} influx can impair deformability of the erythrocyte under mechanical stress [208]. In contrast, NO is known to decrease the intracellular Ca^{2+} concentration [209] and it might be speculated that NO-dependent mechanisms are used by RBCs to counterbalance the deleterious action of Ca^{2+} . Indeed, NO and Ca^{2+} have opposite effects on the fluidity of RBC membranes [165]. Deformability of whole erythrocytes can be decreased by Ca^{2+} [208] and increased by NO [158, 166]. NO is involved in the regulation of the shear stress response in endothelial cells [170] and leukocytes [210]. Moreover, in endothelial cells, the anti-apoptotic effect of shear stress is partly mediated by NO [211]. In human erythrocytes, Ca^{2+} increases tyrosine phosphorylation of band 3 [212] because of the Ca^{2+} -induced dissociation of protein tyrosine phosphatase from band 3 [213]. In fibroblasts, actin, Ca^{2+} and tyrosine phosphorylation act co-ordinately to produce a mechanoprotective response to stretching [214].

Based on these interactions, it is tempting to propose that in erythrocytes, cellular signals involving NO- and Ca²⁺-dependent mechanisms and tyrosine phosphorylation act co-ordinately to develop a cytoprotective response to mechanical stress.

Conclusions

Independently of the number of O₂ sensors in erythrocytes, a range of O₂-dependent interactions between Hb, cytoplasmic factors and cytoskeletal proteins appear to be of physiological significance (Fig 6). So far, preferential binding of deoxygenated Hb to the cytoplasmic domain of band 3 (cdB3) has been considered as a main mechanism linking the oxygenation-deoxygenation cycle with diverse O₂-dependent membrane functions (Fig. 6). The data reviewed here seem to indicate that multiple modes of interaction

between Hb and membrane proteins exist and that these interactions are dependent on the oxygenation-deoxygenation state of the cell (Fig. 6). A cardinal question is whether the activity of individual transporters is separately regulated through association with Hb, or whether they are collectively controlled through association of Hb at one key site, such as cdB3 (Fig. 6). Due to the absence of band 3 in lamprey RBCs and of DPG binding to Hb in sheep erythrocytes, these cells could be valuable tools to answer this question.

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References

- Minetti G, Low PS: Erythrocyte signal transduction pathways and their possible functions. *Curr Opin Hematol* 1997;4:116-121.
- Gulbins E, Jekle A, Ferlinz K, Grassme H, Lang F: Physiology of apoptosis. *Am J Physiol Renal Physiol* 2000;279:F605-F615.
- Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D: Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 1998;78:247-306.
- Pedersen SF, Hoffmann EK, Mills JW: The cytoskeleton and cell volume regulation. *Comp Biochem Physiol A Mol Integr Physiol* 2001;130:385-399.
- Maeno E, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci U S A* 2004;97:9487-9492.
- Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B, Risler T, Wieder T, Lang F: Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 2003;14:2750-2757.
- Rivera A, Jarolim P, Brugnara C: Modulation of Gardos channel activity by cytokines in sickle erythrocytes. *Blood* 2002;99:357-603.
- Buemi M, Marino D, Floccari F, Ruello A, Nostro L, Aloisi C, Marino MT, Di Pasquale G, Corica F, Frisina N: Effect of interleukin 8 and ICAM-1 on calcium-dependent outflow of K⁺ in erythrocytes from subjects with essential hypertension. *Curr Med Res Opin* 2004;20:19-24.
- Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- Gibson JS, Cossins AR, Ellory JC: Oxygen-sensitive membrane transporters in vertebrate red cells. *J Exp Biol* 2000;203:1395-1407.
- Tuvia S, Levin S, Korenstein R: Oxygenation-deoxygenation cycle of erythrocytes modulates submicron cell membrane fluctuations. *Biophys J* 1992;63:599-602.
- Tuvia S, Moses A, Gulayev N, Levin S, Korenstein R: Beta-adrenergic agonists regulate cell membrane fluctuations of human erythrocytes. *J Physiol* 1999;516:781-792.
- Barbul A, Zipser Y, Nachles A, Korenstein R: Deoxygenation and elevation of intracellular magnesium induce tyrosine phosphorylation of band 3 in human erythrocytes. *FEBS Lett* 1999;455:87-91.
- Low PS, Rathinavelu P, Harrison ML: Regulation of glycolysis via reversible enzyme binding to the membrane protein, band 3. *J Biol Chem* 1993;268:14627-14631.
- Weber RE: Adaptations for oxygen transport: Lessons from fish hemoglobins. In: Di Prisco G, Giardina B, Weber RE, editors. *Hemoglobin Function in Vertebrates, Molecular Adaptation in Extreme and Temperate Environments*. Milano: Springer-Verlag Italia, 2000:23-37.
- Wenger RH: Mammalian oxygen sensing, signalling and gene regulation. *J Exp Biol* 2000;203:1253-1263.
- Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M, Wenger RH: Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. *Biochem Biophys Res Com* 2001;288:757-764.
- Motais R, Garcia Romeu F, Borgese F: The control of Na⁺/H⁺ exchange by molecular oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer. *J Gen Physiol* 1987;90:197-207.
- Berenbrink M, Volkel X, Heisler N, Nikinmaa M: O₂ dependent K⁺ fluxes in trout red cells: the nature of O₂ sensing revealed by the affinity, cooperativity and pH dependence of the transport. *Journal of Physiology-London* 2000;526:69-80.
- Bogdanova AY, Nikinmaa M: Reactive oxygen species regulate oxygen-sensitive potassium flux in rainbow trout erythrocytes. *J Gen Physiol* 2001;117:181-190.
- Burmester T, Weich B, Reinhardt S, Hankeln T: A vertebrate globin expressed in the brain. *Nature* 2000;407:520-523.
- Burmester T, Ebner B, Weich B, Hankeln T: Cytooglobin: A novel globin type ubiquitously expressed in vertebrate tissues. *Mol Biol Evol* 2002;19:416-421.
- Trent JT, Hargrove MS: A ubiquitously expressed human hexacoordinate hemoglobin. *J Biol Chem* 2002;277:19538-19545.

- 24 Roesner A, Fuchs C, Hankeln T, Burmester T: A globin gene of ancient evolutionary origin in lower vertebrates. Evidence for two distinct globin families in animals. *Mol Biol Evol* 2004;In press.
- 25 Sun YJ, Jin KL, Mao XO, Zhu YH, Greenberg DA: Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci USA* 2001;98:15306-15311.
- 26 Fordel E, Geuens E, Dewilde S, Rottiers P, Carmeliet P, Grooten J, Moens L: Cytoglobin expression is upregulated in all tissues upon hypoxia: an *in vitro* and *in vivo* study by quantitative real-time PCR. *Biochem Biophys Res Com* 2004;319:342-348.
- 27 Sun Y, Jin K, Peel A, Mao XO, Xie L, Greenberg DA: Neuroglobin protects the brain from experimental stroke *in vivo*. *PNAS* 2003;100:3497-3500.
- 28 Kriegl JM, Bhattacharyya AJ, Nienhaus K, Deng P, Minkow O, Nienhaus GU: Ligand binding and protein dynamics in neuroglobin. *PNAS* 2002;99:7992-7997.
- 29 Arnone A, Perutz MF: Structure of inositol hexaphosphate- human deoxyhaemoglobin complex. *Nature* 1974;249:34-36.
- 30 Ellory JC, Gibson JS, Stewart GW: Pathophysiology of abnormal cell volume in human red cells. *Contrib Nephrol* 1998;123:220-239.
- 31 Walder JA, Chatterjee R, Steck TL, Low PS, Musso GF, Kaiser ET, Rogers PH, Arnone A: The interaction of hemoglobin with the cytoplasmic domain of Band 3 of the human erythrocyte membrane. *J Biol Chem* 1984;259:10238-10246.
- 32 Ducis I, Kandrach A, Racker E: Stimulation of ^{32}P i transport into human erythrocyte ghosts and reconstituted vesicles by Mg^{2+} and hemoglobin. *J Biol Chem* 1988;263:8544-8550.
- 33 Jensen FB, Brahm J: Kinetics of chloride transport across fish red blood cell membranes. *J Exp Biol* 1995;198:2237-2244.
- 34 Jensen FB, Wang T, Jones DR, Brahm J: Carbon dioxide transport in alligator blood and its erythrocyte permeability to anions and water. *Am J Physiol* 1998;274:R661-71.
- 35 Galtieri A, Tellone E, Romano L, Misiti F, Bellocco E, Ficarra S, Russo A, Di Rosa D, Castagnola M, Giardina B, Messana I: Band-3 protein function in human erythrocytes: effect of oxygenation-deoxygenation. *Biochim Biophys Acta-Biomembranes* 2002;1564:214-218.
- 36 Drew C, Ball V, Robinson H, Clive Ellory J, Gibson JS: Oxygen sensitivity of red cell membrane transporters revisited. *Bioelectrochemistry* 2004;62:153-158.
- 37 Murphy JR: Erythrocyte metabolism .2. Glucose metabolism and pathways. *J Lab & Clin Med* 1960;55:286-302.
- 38 Rapoport I, Berger H, Rapoport SM, Elsner R, Gerber G: Response of glycolysis of human erythrocytes to transition from oxygenated to deoxygenated state at constant intracellular pH. *Biochim Biophys Acta* 1976;428:193-204.
- 39 Messana I, Orlando M, Cassiano L, Pennacchietti L, Zuppi C, Castagnola M, Giardina B: Human erythrocyte metabolism is modulated by the O_2 -linked transition of hemoglobin. *FEBS Lett* 1996;390:25-28.
- 40 Hamasaki N, Asakura T, Minakami S: Effect of oxygen tension on glycolysis in human erythrocytes. *J Biochem Tokyo* 1970;68:157-161.
- 41 Jagger JE, Bateman RM, Ellsworth ML, Ellis CG: Role of erythrocyte in regulating local O_2 delivery mediated by hemoglobin oxygenation. *Am J Physiol Heart Circ Physiol* 2001;280:H2833-H2839.
- 42 Weber RE, Voelter W, Fago A, Echner H, Campanella E, Low PS: Modulation of red cell glycolysis: interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins. *Am J Physiol Regul Integr Comp Physiol* 2004;287:R454-R464.
- 43 Rauenbuehler PB, Cordes KA, Salhany JM: Identification of the hemoglobin binding sites on the inner surface of the erythrocyte membrane. *Biochim Biophys Acta* 1982;692:361-370.
- 44 Lebbar I, Stetzkowski-Marden F, Mauffret O, Cassoly R: Interactions of actin and tubulin with human deoxyhemoglobin. Their possible occurrence within erythrocytes. *Eur J Biochem* 1987;170:273-277.
- 45 Tuvia S, Levin S, Bitler A, Korenstein R: Mechanical fluctuations of the membrane-skeleton are dependent on F-actin ATPase in human erythrocytes. *J Cell Biol* 1998;141:1551-1561.
- 46 Cossins AR, Weaver YR, Lykkeboe G, Nielsen OB: Role of protein phosphorylation in control of K flux pathways of trout red blood cells. *Am J Physiol* 1994;267(36):C1641-C1650.
- 47 Sørensen B, Weber RE: Effects of oxygenation and the stress hormones adrenaline and cortisol on the viscosity of blood from trout, *Oncorhynchus mykiss*. *J Exp Biol* 1995;198:953-959.
- 48 Jensen FB, Jakobsen MH, Weber RE: Interaction between haemoglobin and synthetic peptides of the N- terminal cytoplasmic fragment of trout band 3 (AE1) protein. *J Exp Biol* 1998;201:2685-2690.
- 49 Campbell EH, Gibson JS: Oxygen-dependent K^+ fluxes in sheep red cells. *J Physiol (London)* 1998;506:679-688.
- 50 Chaimanee P, Yuthavong Y: Binding of haemoglobin to spectrin of human erythrocytes. *FEBS Lett* 1977;78:119-123.
- 51 Devogel M, Leonis J, Vincentelli J: The alteration of the functional properties of human haemoglobin by spectrin. *Experientia* 1977;33:1429-1450.
- 52 Liu SC, Palek J: Hemoglobin enhances the self-association of spectrin heterodimers in human erythrocytes. *J Biol Chem* 1984;259:11556-11562.
- 53 Vincentelli J, Fraboni A, Paul C, Schnek AG: The influence of experimental conditions on the spectrin-hemoglobin interaction. *Biol Chem Hoppe Seyler* 1989;370:63-66.
- 54 Cassoly R: Evidence against the binding of native hemoglobin to spectrin of human erythrocytes. *FEBS Lett* 1978;85:357-360.
- 55 Shaklai N, Frayman B, Fortier N, Snyder M: Crosslinking of isolated cytoskeletal proteins with hemoglobin: a possible damage inflicted to the red cell membrane. *Biochim Biophys Acta* 1987;915:406-414.
- 56 Datta P, Chakrabarty SB, Chakrabarty A, Chakrabarti A: Interaction of erythroid spectrin with hemoglobin variants: implications in beta-thalassemia. *Blood Cells Molecules and Diseases* 2003;30:248-253.
- 57 Marttila ONT, Nikinmaa M: Binding of b-adrenergic antagonists 3H-DHA and 3H-CGP 12177 to intact rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) red blood cells. *Gen Comp Endocrinol* 1988;70:429-435.
- 58 Chang SH, Low PS: Regulation of the glycophorin C-protein 4.1 membrane-to-skeleton bridge and evaluation of its contribution to erythrocyte membrane stability. *J Biol Chem* 2001;276:22223-22230.
- 59 Bunn HF, Ransil BJ, Chao A: The interaction between erythrocyte organic phosphates, magnesium ion, and hemoglobin. *J Biol Chem* 1971;246:5273-5279.
- 60 Janmey PA: The cytoskeleton and cell signaling: Component localization and mechanical coupling. *Physiol Rev* 1998;78:763-781.
- 61 Papakonstanti EA, Vardaki EA, Stournaras C: Actin cytoskeleton: a signaling sensor in cell volume regulation. *Cell Physiol Biochem* 2000;10:257-264.
- 62 Weaver YR, Cossins AR: Protein tyrosine phosphorylation and the regulation of KCl cotransport in trout erythrocytes. *Pflügers Arch* 1996;432:727-734.
- 63 Lauf PK, Theg BE: Chloride dependent K^+ flux induced by N-ethylmaleimide in genetically low K^+ sheep and goat erythrocytes. *Biochem Biophys Res Com* 1980;92:1422-1428.
- 64 Lauf PK: K^+Cl^- cotransport - sulfhydryls, divalent-cations, and the mechanism of volume activation in a red-cell. *J Membr Biol* 1985;88:1-13.

- 65 Zipser Y, Piade A, Kosower NS: Erythrocyte thiol status regulates band 3 phosphotyrosine level via oxidation/reduction of band 3-associated phosphotyrosine phosphatase. *FEBS Lett* 1997;406:126-130.
- 66 Bordin L, Brunati AM, Donella-Deana A, Baggio B, Toninello A, Clari G: Band 3 is an anchor protein and a target for SHP-2 tyrosine phosphatase in human erythrocytes. *Blood* 2002;100:276-282.
- 67 Gibson JS, Godart H, Ellory JC, Staines H, Honess NA, Cossins AR: Modulation of K⁺-Cl⁻ cotransport in equine red blood cells. *Exp Physiol* 1994;79:997-1009.
- 68 Honess NA, Gibson JS, Cossins AR: The effects of oxygenation upon the Cl dependent K flux pathway in equine red cells. *Pflügers Arch-Eur J Physiol* 1996;432:270-277.
- 69 Tyuma I, kamigawara Y, Imai K: pH dependence of the shape of the hemoglobin-oxygen equilibrium curve. *Biochim Biophys Acta* 1973;310:317-320.
- 70 Weber RE, Jensen FB, Cox RP: Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changueux models. Effects of nucleoside triphosphates and pH on oxygenation of tench hemoglobin. *J Comp Physiol* 1987;157B:145-152.
- 71 Merciris P, Hardy-Dessources MD, Sauvage M, Giraud F: Involvement of deoxygenation-induced increase in tyrosine kinase activity in sickle cell dehydration. *Pflügers Arch-Eur J Physiol* 1998;436:315-322.
- 72 Merciris P, Hardy-Dessources MD, Giraud F: Deoxygenation of sickle cells stimulates Syk tyrosine kinase and inhibits a membrane tyrosine phosphatase. *Blood* 2001;98:3121-3127.
- 73 Fathallah H, Coezy E, Deneef RS, Hardy-dessources MD, Giraud F: Inhibition of deoxygenation-induced membrane-protein dephosphorylation and cell dehydration by phorbol esters and okadaic acid in sickle cells. *Blood* 1995;86:1999-2007.
- 74 Salama A, Nikinmaa M: Effect of oxygen tension on catecholamine-induced formation of cAMP and on swelling of carp red blood cells. *Am J Physiol* 1990;259:C723-C726.
- 75 Borgese F, Sardet C, Cappadoro M, Pouyssegur J, Motaïs R: Cloning and expression of a cAMP-activated Na⁺/H⁺ exchanger - Evidence that the cytoplasmic domain mediates hormonal regulation. *Proc Natl Acad Sci USA* 1992;89:6765-6769.
- 76 Prat AG, Cunningham CC, Jackson GR, Jr., Borkan SC, Wang Y, Ausiello DA, Cantiello HF: Actin filament organization is required for proper cAMP-dependent activation of CFTR. *Am J Physiol* 1999;277:C1160-C1169.
- 77 Szaszi K, Kurashima K, Kaibuchi K, Grinstein S, Orlowski J: Role of the cytoskeleton in mediating cAMP-dependent protein kinase inhibition of the epithelial Na⁺/H⁺ exchanger NHE3. *J Biol Chem* 2001;276:40761-40768.
- 78 Putney LK, Denker SP, Barber DL: The changing face of the Na⁺/H⁺ exchanger, NHE1: structure, regulation, and cellular actions. *Annu Rev Pharmacol Toxicol* 2002;42:527-552.
- 79 Nikinmaa M, Cech JJ, Jr., Ryhanen EL, Salama A: Red cell function of carp (*Cyprinus carpio*) in acute hypoxia. *Exp Biol* 1987;47:53-58.
- 80 Jensen FB: Nitrite and red cell function in carp: control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. *J Exp Biol* 1990;152:149-166.
- 81 Nielsen OB, Lykkeboe G, Cossins AR: Oxygenation-activated K-fluxes in trout red-blood-cells. *Am J Physiol* 1992;263:C1057-C1064.
- 82 Jensen FB: Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O₂ and CO₂ transport. *Acta Physiol Scand* 2004;182:215-227.
- 83 Muto S, Ebata S, Okada K, Saito T, Asano Y: Glucocorticoid modulates Na⁺/H⁺ exchange activity in vascular smooth muscle cells by nongenomic and genomic mechanisms. *Kidney Int* 2000;57:2319-2333.
- 84 Ullrich V, Bachschmid M: Superoxide as a messenger of endothelial function. *Biochem Biophys Res Comm* 2000;278:1-8.
- 85 Stuhlmeier KM, Kao JJ, Wallbrandt P, Lindberg M, Hammarstrom B, Broell H, Paigen B: Antioxidant protein 2 prevents methemoglobin formation in erythrocyte hemolysates. *Eur J Biochem* 2003;270:334-341.
- 86 Nikinmaa M, Bogdanova A, Lecklin T: Oxygen dependency of the adrenergic Na⁺/H exchange in rainbow trout erythrocytes is diminished by a hydroxyl radical scavenger. *Acta Physiol Scand* 2003;178:149-154.
- 87 Gibson JS, Muzyamba MC: Modulation of Gardos channel activity by oxidants and oxygen tension: effects of 1-chloro-2,4-dinitrobenzene and phenazine methosulphate. *Bioelectrochemistry* 2004;62:147-152.
- 88 Guillemette G, Favreau I, Lamontagne S, Boulay G: 2,3-Diphosphoglycerate is a nonselective inhibitor of inositol 1,4,5-trisphosphate action and metabolism. *Eur J Pharmacol* 1990;188:251-260.
- 89 McConnel FM, Goldstein L: Intracellular signals and volume regulatory response in skate erythrocytes. *Am J Physiol* 1988;255:R982-R987.
- 90 Romero PJ, Romero EA: Effect of cell ageing on Ca²⁺ influx into human red cells. *Cell Calcium* 1999;26:131-137.
- 91 Ferrell CM, Lauf PK, Wilson BA, Adragna NC: Lithium and protein kinase C modulators regulate swelling-activated K-Cl cotransport and reveal a complete phosphatidylinositol cycle in low K sheep erythrocytes. *J Membr Biol* 2000;177:81-93.
- 92 Piacentini MP, Piatti E, Bucchini A, Fazi A, Accorsi A: Modification of inositol 1,4,5-trisphosphate concentration of human erythrocytes under „in vivo“ physiological conditions. *Biochem Mol Biol Int* 1996;38:1265-1269.
- 93 Davis FB, Davis PJ, Blas SD, Gombas DZ: Inositol phosphates modulate human red blood cell Ca²⁺-adenosine triphosphatase activity in vitro by a guanine nucleotide regulatory protein. *Metabolism* 1995;44:865-868.
- 94 Shiffer KA, Rood L, Emerson RK, Kuypers FA: Effects of phosphatidylinositol diphosphate on phospholipid asymmetry in the human erythrocyte membrane. *Biochemistry* 1998;37:3449-3458.
- 95 Bogdanova A, Ogunshola OO, Bauer C, Nikinmaa M, Gassmann M: Molecular mechanisms of oxygen-induced regulation of Na⁺/K⁺ pump. *Adv Exp Med Biol* 2004;536:231-238.
- 96 Hoffmann EK, Pedersen SF: Sensors and signal transduction in the activation of cell volume regulatory ion transport systems. *Contrib Nephrol* 1988;123:58-78.
- 97 Mongin AA, Orlov SN: Mechanisms of cell volume regulation and possible nature of the cell volume sensor. *Pathophysiology* 2001;8:77-88.
- 98 Jensen FB: Regulatory volume decrease in carp red blood cells: mechanisms and oxygenation-dependency of volume-activated potassium and amino acid transport. *J Exp Biol* 1995;198:155-165.
- 99 Orlov SN, Kolosova IA, Cragoe EJ, Gurlo TG, Mongin AA, Aksentsev SL, Konev SV: Kinetics and peculiarities of thermal inactivation of volume-induced Na⁺/H⁺ exchange, Na⁺,K⁺,2Cl⁻ cotransport and K⁺,Cl⁻ cotransport in rat erythrocytes. *Biochim Biophys Acta* 1993;1151:186-192.
- 100 Estevez AY, Bond T, Strange K: Regulation of ICl_{swell} in neuroblastoma cells by G protein signaling pathways. *Am J Physiol Cell Physiol* 2001;281:C89-C98.
- 101 Lominadze D, Dean WL: Involvement of fibrinogen specific binding in erythrocyte aggregation. *FEBS Lett* 2002;517:41-44.

- 102 Dahl Sv, Schliess F, Reissmann R, Gorg B, Weiergraber O, Kocalkova M, Dombrowski F, Haussinger D: Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver. *J Biol Chem* 2003;278:27088-27095.
- 103 Lepple-Wienhues A, Szabo I, Laun T, Kaba NK, Gulbins E, Lang F: The tyrosine kinase p56(lck) mediates activation of swelling-induced chloride channels in lymphocytes. *J Cell Biol* 1998;141:281-286.
- 104 Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ: Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci USA* 2003;100:13298-13302.
- 105 Colclasure GC, Parker JC: Cytosolic protein concentration is the primary volume signal for swelling-induced [K-Cl] cotransport in dog red cells. *J Gen Physiol* 1992;100:1-10.
- 106 Minton AP, Colclasure GC, Parker JC: Model for the role of macromolecular crowding in regulation of cellular-volume. *Proc Natl Acad Sci USA* 1992;89:10504-10506.
- 107 Vitoux D, Beuzard Y, Brugnara C: The effect of hemoglobin A and S on the volume- and pH-dependence of K-Cl cotransport in human erythrocytic ghosts. *J Membr Biol* 1999;167:233-240.
- 108 Sachs JR, Martin DW: The role of ATP in swelling-stimulated K-Cl cotransport in human red cell ghosts. Phosphorylation-dephosphorylation events are not in the signal transduction pathway. *J Gen Physiol* 1993;102:551-573.
- 109 Khan AI, Drew C, Ball SE, Ball V, Ellory JC, Gibson JS: Oxygen dependence of K⁺-Cl⁻ cotransport in human red cell ghosts and sickle cells. *Bioelectrochemistry* 2004;62:141-146.
- 110 Olivieri O, Vitoux D, Galacteros F, Bachir D, Blouquit Y, Beuzard Y, Brugnara C: Hemoglobin-variants and activity of the (K⁺Cl⁻) cotransport system in human erythrocytes. *Blood* 1992;79:793-797.
- 111 Nagel RL, Krishnamoorthy R, Fattoum S, Elion J, Genard N, Romero J, Fabry ME: The erythrocyte effects of haemoglobin O_{ARAB}. *Br J Haematol* 1999;107:516-521.
- 112 Dunham PB: Effects of urea on K-Cl cotransport in sheep red-blood-cells - Evidence for 2 signals of swelling. *Am J Physiol Cell Physiol* 1995;37:C1026-C1032.
- 113 Bard H, Fouron JC, Robillard JE, Cornet A, Soukini MA: Red-cell oxygen-affinity in fetal sheep - role of 2,3-DPG and adult hemoglobin. *J Appl Physiol* 1978;45:7-10.
- 114 Holland RA, Tibben EA, Hallam JF: Interaction between organic phosphates and sheep hemoglobins Interaction between organic phosphates and sheep hemoglobins. *Adv Exp Med Biol* 1990;277:191-197.
- 115 Chien S: Red cell deformability and its relevance to blood flow. *Annu Rev Physiol* 1987;49:177-192.
- 116 Cicha I, Suzuki Y, Tateishi N, Maeda N: Changes of RBC aggregation in oxygenation-deoxygenation: pH dependency and cell morphology. *Am J Physiol Heart Circ Physiol* 2003;284:H2335-H2342.
- 117 Speake PF, Gibson JS: Urea-stimulated K-Cl cotransport in equine red blood cells. *Pflugers Arch* 1997;434:104-112.
- 118 Friederichs E, Meiselman HJ: Effects of calcium permeabilization on RBC rheologic behavior. *Biorheology* 1994;31:207-215.
- 119 Bordin L, Clari G, Moro I, Dallavecchia F, Moret V: Functional-link between phosphorylation state of membrane-proteins and morphological-changes of human erythrocytes. *Biochem Biophys Res Comm* 1995;213:249-257.
- 120 Manno S, Takakuwa Y, Nagao K, Mohandas N: Modulation of erythrocyte-membrane mechanical function by beta-spectrin phosphorylation and dephosphorylation. *J Biol Chem* 1995;270:5659-5665.
- 121 Weber RE, Lykkeboe G: Respiratory adaptations in carp blood. Influences of hypoxia, red cell organic phosphates, divalent cations and CO₂ on hemoglobin-oxygen affinity. *J Comp Physiol* 1978;128B:127-137.
- 122 Murphy E: Mysteries of magnesium homeostasis. *Circ Res* 2000;86:245-248.
- 123 Mairbaur H, Hoffman JF: Internal magnesium, 2,3-diphosphoglycerate, and the regulation of the steady-state volume of human red blood cells by the Na/K/2Cl cotransport system. *J Gen Physiol* 1992;99:721-746.
- 124 Bennet V: The spectrin-actin junction of erythrocyte-membrane skeletons. *Biochim Biophys Acta* 1989;988:107-121.
- 125 Beaven GH, Parmar J, Nash GB, Bennett PM, Gratzer WB: Effect of magnesium ions on red cell membrane properties. *J Membr Biol* 1990;118:251-257.
- 126 Wallis CJ, Babitch JA, Wenegieme EF: Divalent cation binding to erythrocyte spectrin. *Biochemistry* 1993;32:5045-5050.
- 127 Kay FR: 2,3-Diphosphoglycerate, blood-oxygen dissociation and biology of mammals. *Comp Biochem Physiol A Physiol* 1977;57:309-316.
- 128 Bunn HF: Evolution of mammalian hemoglobin function. *Blood* 1981;58:189-197.
- 129 Mulquiney PJ, Kuchel PW: Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations: computer simulation and metabolic control analysis. *Biochem J* 1999;342 Pt 3:597-604.
- 130 Mulquiney PJ, Bubb WA, Kuchel PW: Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations in vivo kinetic characterization of 2,3-bisphosphoglycerate synthase/phosphatase using C-13 and P-31 NMR. *Biochem J* 1999;342:567-580.
- 131 Mulquiney PJ, Kuchel PW: Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations: equations and parameter refinement. *Biochem J* 1999;342:581-596.
- 132 Waugh RE: Effects of 2,3-diphosphoglycerate on the mechanical properties of erythrocyte membrane. *Blood* 1986;68:231-238.
- 133 Mentzer WC, Jr., Iarocci TA, Mohandas N, Lane PA, Smith B, Lazerson J, Hays T: Modulation of erythrocyte membrane mechanical stability by 2,3-diphosphoglycerate in the neonatal poikilocytosis/elliptocytosis syndrome. *J Clin Invest* 1987;79:943-949.
- 134 Chasis JA, Mohandas N: Erythrocyte membrane deformability and stability: two distinct membrane properties that are independently regulated by skeletal protein associations. *J Cell Biol* 1986;103:343-350.
- 135 Suzuki Y, Nakajima T, Shiga T, Maeda N: Influence of 2,3-diphosphoglycerate on the deformability of human erythrocytes. *Biochim Biophys Acta* 1990;1029:85-90.
- 136 Le Devehat C, Lemoine A, Cirette B, Ramet M: Pharmacological influences of pentoxifylline on red cell filterability and 2-3 diphosphoglycerate. *Scand J Clin Lab Invest Suppl* 1981;156:301-303.
- 137 Lee I, Kim JH, Levitt SH, Song CW: Increases in tumour response by pentoxifylline alone or in combination with nicotinamide. *Int J Radiat Oncol Biol Phys* 1992;22:425-429.
- 138 Yamamoto N, Yokota K, Yamashita A, Oda M: The effects of KBT-3022, a new anti-platelet agent, on hemorheological properties in guinea pigs. *Thromb Haemost* 1995;73:118-121.
- 139 Fernandes PR, Dewey MJ: Genetic control of erythrocyte volume regulation: effect of a single gene (rol) on cation metabolism. *Am J Physiol* 1994;267:C211-C219.
- 140 Adragna NC, White RE, Orlov SN, Lauf PK: K-Cl cotransport in vascular smooth muscle and erythrocytes: possible implication in vasodilation. *Am J Physiol Cell Physiol* 2000;278:C381-C390.

- 141 Ortiz-Carranza O, Adragna NC, Lauf PK: Modulation of K-Cl cotransport in volume-clamped low-K sheep erythrocytes by pH, magnesium, and ATP. *Am J Physiol* 1996;271:C1049-C1058.
- 142 Haley BE, Hoffman JF: Interactions of a photo-affinity ATP analog with cation-stimulated adenosine triphosphatases of human red-cell membranes. *Proc Natl Acad Sci USA* 1974;71:3367-3371.
- 143 Shaklai N, Benitez L, Ranney HM: Binding of 2,3-diphosphoglycerate by spectrin and its effect on oxygen affinity of hemoglobin. *Am J Physiol* 1978;234:C36-C40.
- 144 Sheetz MP, Casaly J: 2,3-Diphosphoglycerate and ATP dissociate erythrocyte membrane skeletons. *J Biol Chem* 1980;255:9955-9960.
- 145 Moriyama R, Lombardo CR, Workman RF, Low PS: Regulation of linkages between the erythrocyte membrane and its skeleton by 2,3-diphosphoglycerate. *J Biol Chem* 1993;268:10990-10996.
- 146 Clari G, Ferrari S: Kinetic behaviour of human erythrocyte casein kinases. Effect of 2, 3- biphosphoglycerate and heparin. *Ital J Biochem* 1983;32:174-188.
- 147 Chao TS, Tao M: Effect of 2,3-diphosphoglycerate on the phosphorylation of protein 4.1 by protein kinase C. *Arch Biochem Biophys* 1991;285:221-226.
- 148 An XL, Takakuwa Y, Nunomura W, Manno S, Mohandas N: Modulation of band 3-ankyrin interaction by protein 4.1 - Functional implications in regulation of erythrocyte membrane mechanical properties. *J Biol Chem* 1996;271:33187-33191.
- 149 Sakashita K, Oonishi T, Ishioka N, Uyesaka N: Endothelin-1 improves the impaired filterability of red blood cells through the activation of protein kinase C. *Jpn J Physiol* 1999;49:113-120.
- 150 Oonishi T, Sakashita K: Ethanol improves decreased filterability of human red blood cells through modulation of intracellular signaling pathways. *Alcohol Clin Exp Res* 2000;24:352-356.
- 151 Ellsworth ML, Forrester T, Ellis CG, Dietrich HH: The erythrocyte as a regulator of vascular tone. *Am J Physiol Heart Circ Physiol* 1995;269:H2155-H2161.
- 152 Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ: ATP: the red blood cell link to NO and local control of the pulmonary circulation. *Am J Physiol Heart Circ Physiol* 1996;271:H2717-H2722.
- 153 Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA: Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 1997;276:2034-2037.
- 154 Pawloski JR, Hess DT, Stamler JS: Export by red blood cells of nitric oxide bioactivity. *Nature* 2001;409:622-626.
- 155 Bonaventura C, Taboy CH, Low PS, Stevens RD, Lafon C, Crumbliss AL: Heme redox properties of S-nitrosated hemoglobin A(0) and hemoglobin S - Implications for interactions of nitric oxide with normal and sickle red blood cells. *J Biol Chem* 2002;277:14557-14563.
- 156 Chen LY, Mehta JL: Evidence for the presence of L-arginine-nitric oxide pathway in human red blood cells: relevance in the effects of red blood cells on platelet function. *J Cardiovasc Pharmacol* 1998;32:57-61.
- 157 Kang ES, Ford K, Groluksky G, Wang YB, Chiang TM, Acchiardo SR: Normal circulating adult human red blood cells contain inactive NOS proteins. *J Lab & Clin Med* 2000;135:444-451.
- 158 Bor-Kucukatay M, Wenby RB, Meiselman HJ, Baskurt OK: Effects of nitric oxide on red blood cell deformability. *Am J Physiol Heart Circ Physiol* 2003;284:H1577-H1584.
- 159 Petrov V, Lijnen P: Regulation of human erythrocyte Na⁺/H⁺ exchange by soluble and particulate guanylate cyclase. *Am J Physiol* 1996;271:C1556-C1564.
- 160 Hassid A, Yao J, Huang SL: NO alters cell shape and motility in aortic smooth muscle cells via protein tyrosine phosphatase 1B activation. *Am J Physiol Heart Circ Physiol* 1999;277:H1014-H1026.
- 161 Brown C, Lin Y, Hassid A: Requirement of protein tyrosine phosphatase SHP2 for NO-stimulated vascular smooth muscle cell motility. *Am J Physiol Heart Circ Physiol* 2001;281:H1598-H1605.
- 162 Zipser Y, Kosower NS: Phosphotyrosine phosphatase associated with band 3 protein in the human erythrocyte membrane. *Biochem J* 1996;314:881-887.
- 163 Adragna NC, Lauf PK: Role of nitrite, a nitric oxide derivative, in K-Cl cotransport activation of low-potassium sheep red blood cells. *J Membr Biol* 1998;166:157-167.
- 164 Caramelo C, Riesco A, Outeirino J, Millas I, Blum G, Monzu B, Casado V, Sanchez L, Mosquera JR, Casado S, Farre AL: Effects of nitric oxide on red-blood-cells - Changes in erythrocyte resistance to hypotonic hemolysis and potassium efflux by experimental maneuvers that decrease nitric oxide. *Biochem Biophys Res Com* 1994;199:447-454.
- 165 Tsuda K, Kimura K, Nishio I, Masuyama Y: Nitric oxide improves membrane fluidity of erythrocytes in essential hypertension: An electron paramagnetic resonance investigation. *Biochem Biophys Res Comm* 2000;275:946-954.
- 166 Korb R, Gryglewski RJ: Nitric-oxide from polymorphonuclear leukocytes modulates red-blood-cell deformability *in vitro*. *Eur J Pharmacol* 1993;234:17-22.
- 167 Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG: Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am J Physiol Heart Circ Physiol* 2001;280:H2848-H2856.
- 168 Zamir N, Tuvia S, Riven-Kreitman R, Levin S, Korenstein R: Atrial natriuretic peptide: direct effects on human red blood cell dynamics. *Biochem Biophys Res Comm* 1992;188:1003-1009.
- 169 Ko KS, McCulloch CAG: Partners in protection: Interdependence of cytoskeleton and plasma membrane in adaptations to applied forces. *J Membr Biol* 2000;174:85-95.
- 170 Davies PF: Flow-mediated endothelial mechanotransduction. *Physiol Rev* 1995;75:519-560.
- 171 Shyy JY, Chien S: Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* 2002;91:769-775.
- 172 Gudi SRP, Clark CB, Frangos JA: Fluid flow rapidly activates G proteins in human endothelial cells - Involvement of G proteins in mechanochemical signal transduction. *Circ Res* 1996;79:834-839.
- 173 Olearczyk JJ, Stephenson AH, Lonigro AJ, Sprague RS: Heterotrimeric G protein G(i) is involved in a signal transduction pathway for ATP release from erythrocytes. *Am J Physiol Heart Circ Physiol* 2004;286:H940-H945.
- 174 Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ: Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release. *Am J Physiol Cell Physiol* 2001;281:C1158-C1164.
- 175 Sprague RS, Ellsworth ML, Stephenson AH, Kleinhenz ME, Lonigro AJ: Deformation-induced ATP release from red blood cells requires CFTR activity. *Am J Physiol Heart Circ Physiol* 1998;274:H1726-H1732.
- 176 Ney PA, Christopher MM, Heibel RP: Synergistic effects of oxidation and deformation on erythrocyte monovalent cation leak. *Blood* 1990;75:1192-1198.
- 177 Larsen FL, Katz S, Roufogalis BD, Brooks DE: Physiological shear stresses enhance the Ca²⁺ permeability of human erythrocytes. *Nature* 1981;294:667-668.
- 178 Huber SM, Gamper N, Lang F: Chloride conductance and volume-regulatory nonselective cation conductance in human red blood cell ghosts. *Pflügers Arch* 2001;441:551-558.
- 179 Egee S, Lapaix F, Decherf G, Staines HM, Ellory JC, Doerig C, Thomas SL: A stretch-activated anion channel is up-regulated by the malaria parasite *Plasmodium falciparum*. *J Physiol* 2002;542:795-801.

- 180 Takakuwa Y, Mohandas N: Modulation of erythrocyte membrane material properties by Ca^{2+} and calmodulin. Implications for their role in regulation of skeletal protein interactions. *J Clin Invest* 1988;82:394-400.
- 181 Tanaka T, Kadowaki K, Lazarides E, Sobue K: Ca^{2+} -dependent regulation of the spectrin/actin interaction by calmodulin and protein 4.1. *J Biol Chem* 1991;266:1134-1140.
- 182 Nunomura W, Takakuwa Y, Tokimitsu R, Krauss SW, Kawashima M, Mohandas N: Regulation of CD44-protein 4.1 interaction by Ca^{2+} and calmodulin. Implications for modulation of CD44-ankyrin interaction. *J Biol Chem* 1997;272:30322-30328.
- 183 Romero PJ, Romero EA: The role of calcium metabolism in human red blood cell ageing: a proposal. *Blood Cells Mol Dis* 1999;25:9-19.
- 184 Romero PJ, Romero EA: Differences in Ca^{2+} pumping activity between subpopulations of human red cells. *Cell Calcium* 1997;21:353-358.
- 185 Daugas E, Cande C, Kroemer G: Erythrocytes: death of a mummy. *Cell Death Differ* 2001;8:1131-1133.
- 186 Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 2001;8:1197-1206.
- 187 Bratosin D, Estaquier J, Petit F, Arnould D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 2001;8:1143-1156.
- 188 Bratosin D, Estaquier J, Slomianny C, Tissier JP, Quatannens B, Bulai T, Mitrofan L, Marinescu A, Trandaburu I, Ameisen JC, Montreuil J: On the evolution of erythrocyte programmed cell death: apoptosis of *Rana esculenta* nucleated red blood cells involves cysteine proteinase activation and mitochondrion permeabilization. *Biochimie* 2004;86:183-192.
- 189 Walsh M, Lutz RJ, Cotter TG, O'Connor R: Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-X(L). *Blood* 2002;99:3439-3448.
- 190 Lang KS, Myssina S, Lang PA, Tanneur V, Kempe DS, Mack AF, Huber SM, Wieder T, Lang F, Duranton C: Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl^- . *Am J Physiol Renal Physiol* 2004;286:F1046-F1053.
- 191 Lang F, Lang KS, Wieder T, Myssina S, Birka C, Lang PA, Kaiser S, Kempe D, Duranton C, Huber SM: Cation channels, cell volume and the death of an erythrocyte. *Pflügers Arch-Eur J Physiol* 2003;447:121-125.
- 192 Zhou Q, Zhao J, Stout JG, Luhm RA, Wiedmer T, Sims PJ: Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem* 1997;272:18240-18244.
- 193 Connor J, Pak CC, Schroit AJ: Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. *J Biol Chem* 1994;269:2399-2404.
- 194 Johnson RM, Tang K: Induction of a Ca^{2+} -activated K^+ channel in human erythrocytes by mechanical stress. *Biochim Biophys Acta* 1992;1107:314-318.
- 195 Manno S, Takakuwa Y, Mohandas N: Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. *Proc Natl Acad Sci U S A* 2002;99:1943-1948.
- 196 Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R: Ca^{++} -dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. *Blood* 2002;99:2569-2577.
- 197 Inomata M, Hayashi M, Nakamura M, Saito Y, Kawashima S: Properties of erythrocyte membrane binding and autolytic activation of calcium-activated neutral protease. *J Biol Chem* 1989;264:18838-18843.
- 198 Boivin P, Galand C, Dhermy D: In vitro digestion of spectrin, protein 4.1 and ankyrin by erythrocyte calcium dependent neutral protease (calpain I). *Int J Biochem* 1990;22:1479-1489.
- 199 Al Z, Cohen CM: Phorbol 12-myristate 13-acetate-stimulated phosphorylation of erythrocyte membrane skeletal proteins is blocked by calpain inhibitors: possible role of protein kinase M. *Biochem J* 1993;296 (Pt 3):675-683.
- 200 Ciana A, Minetti G, Balduini C: Phosphotyrosine phosphatases acting on band 3 in human erythrocytes of different age: PTP1B processing during cell ageing. *Bioelectrochemistry* 2004;62:169-173.
- 201 Romero PJ, Salas V, Hernández C: Calcium pump phosphoenzyme from young and old human red cells. *Cell Biol Int* 2002;26:945-949.
- 202 Cibert C, Pruliere G, Lacombe C, Deprette C, Cassoly R: Calculation of a Gap restoration in the membrane skeleton of the red blood cell: possible role for myosin II in local repair. *Biophys J* 1999;76:1153-1165.
- 203 Johnson RM, Taylor G, Meyer DB: Shape and volume changes in erythrocyte ghosts and spectrin-actin networks. *J Cell Biol* 1980;86:371-376.
- 204 Jinbu Y, Sato S, Nakao M: Reversible shape change of triton-treated erythrocyte-ghosts induced by Ca^{2+} and Mg-ATP. *Nature* 1984;307:376-378.
- 205 Berridge MJ, Dupont G: Spatial and temporal signalling by calcium. *Curr Opin Cell Biol* 1994;6:267-274.
- 206 Marchant JS, Parker I: Functional interactions in Ca^{2+} signaling over different time and distance scales - Commentary. *J Gen Physiol* 2000;116:691-695.
- 207 Allbritton NL, Meyer T, Stryer L: Range of messenger action of calcium-ion and inositol 1,4,5-trisphosphate. *Science* 1992;258:1812-1815.
- 208 Oonishi T, Sakashita K, Uyesaka N: Regulation of red blood cell filterability by Ca^{2+} influx and cAMP-mediated signaling pathways. *Am J Physiol Cell Physiol* 1997;42:C1828-C1834.
- 209 Clementi E: Role of nitric oxide and its intracellular signalling pathways in the control of Ca^{2+} homeostasis. *Biochem Pharmacol* 1998;55:713-718.
- 210 Fukuda S, Yasu T, Predescu DN, Schmid-Schonbein GW: Mechanisms for regulation of fluid shear stress response in circulating leukocytes. *Circ Res* 2000;86:E13-E18.
- 211 Dimmeler S, Hermann C, Galle J, Zeiher AM: Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler Thromb Vasc Biol* 1999;19:656-664.
- 212 Minetti G, Piccinini G, Balduini C, Seppi C, Brovelli A: Tyrosine phosphorylation of band 3 protein in Ca^{2+} /A23187-treated human erythrocytes. *Biochem J* 1996;320:445-450.
- 213 Zipser Y, Piade A, Barbul A, Korenstein R, Kosower NS: Ca^{2+} promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. *Biochem J* 2002;368:137-144.
- 214 Glogauer M, Arora P, Yao G, Sokholov I, Ferrier J, McCulloch CAG: Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching. *J Cell Sci* 1997;110:11-21.