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Review

Historical View and Some Unsolved Problems in Red Blood Cell Membrane Research

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Abstract

The article provides a comprehensive overview of biological membrane lipid composition and distribution and ion transport processes, focusing particularly on red blood cells (RBCs). It begins with a historical perspective, detailing the introduction of the terms 'cell' and 'membrane' in biological sciences, and the development of the fluid-mosaic model of membrane structure. Early findings on ion transport highlighted the non-equilibrium distribution of Na^+ and K^+ across cell membranes, leading to the discovery of the Na^+/K^+ pump. The article delves into the lipid composition of RBC membranes, emphasising the roles of various lipids, including cardiolipin, and the concept of lipid rafts. These rafts, enriched with sphingolipids and cholesterol, play crucial roles in cellular processes. Variations in RBC shapes are discussed, with biophysical theories explaining transformations and pathological conditions affecting RBC morphology, such as sickle cell anaemia. Na^+ and K^+ transporters in RBC membranes are explored, highlighting the almost ubiquitous presence of the Na^+/K^+ pump (absent in Carnivora RBCs) and various ion channels, including the Gárdos and Piezo1 channels. The article notes species-specific differences in ion transport mechanisms and the activation or suppression of transporters during RBC maturation. The mechanism of residual ion transport is examined, questioning whether a $Na^+(K^+)/H^+$ antiporter exists in the human RBC membrane. Residual ion fluxes are mediated by this antiporter, influenced by the fatty acid composition of the RBC membrane. The outlook section underscores the need for further research to fully understand the complexities of RBC membrane structure and function, suggesting that many questions remain unanswered despite significant advances.

Keywords: red blood cell; membrane lipid composition; lipid rafts; red blood cell shapes; red blood cell deformability; residual (leak) ion transport

1. Introduction

This publication addresses various open questions in red blood cell (RBC) membrane research. Its main goal is to assist young scientists in this field by providing insights into past research and considerations for future experiments and theoretical calculations. The content was presented at the 25th Meeting of the European Red Cell Society (ERCS) in April 2024 on Ameland, The Netherlands. The talk was dedicated to the memory of Prof. Dr. Joseph F. Hoffman (USA) and Prof. Dr. h.c. Herrmann Passow (Germany), who passed away on May 19, 2022, and November 21, 2023, respectively, both at the age of 98 years. These eminent scientists significantly contributed to our understanding of the RBC membrane's structure and function.

2. Milestones of our Understanding of the Structure of Biological Membranes and Ion Transport Processes Across Membranes

First of all, it is interesting to know who first used the words "cell" and "membrane". The term "cell" was introduced by the English researcher Robert Hooke in 1665 [1], when he described the cellular structure of cork observed through a microscope. Identifying the first use of the term "membrane" is more challenging. To the best of our knowledge, it was the Swiss botany professor Carl Wilhelm von Nägeli [2] who introduced the term "membrane" in 1855 while working on osmosis in plant cells. Before and even after that time, the term "plasmalemma" was commonly used to describe the boundary of a biological cell.

In 1899, Overton [3] described the cell membrane as a structure of unknown components with holes permeable to water. In 1925, Gorter and Grendel [4] were the first to propose that a lipid bilayer forms a biological membrane. Significant progress was made between 1935 and 1943, when Danielli *et al.* [5] and Davson *et al.* [6] proposed a lipid bilayer with proteins on both membrane surfaces. Robertson [7] expanded on this in 1981 by introducing sugar elements on one surface of the membrane.

Our current understanding of biological membranes is based on the "fluid-mosaic model" developed by Singer and Nicolson in 1972 [8]. This model posits that membrane lipids are in a fluid-crystalline state and distributed relatively homogeneously within the membrane, with proteins inserted like islands in a mosaic. However, at least three

extensions to the fluid-mosaic model should be considered: (i) Lipids in general also exist in crystalline states, with phase transition temperatures higher than the membrane's surrounding temperature, leading to both fluid-crystalline and crystalline domains (e.g., [9]). (ii) Lipids and proteins are distributed asymmetrically, not only laterally but also transversally [10,11]. (iii) In addition to forming a bilayer structure, lipids in biological membranes can also exist in non-bilayer structures [12].

Understanding the historical views and ideas about ion transport across biological membranes is also very interesting. The first significant findings were published by Abderhalden in 1897 [13]. Using chemical methods, he discovered that in human RBCs, the extracellular Na^+ concentration is higher than the intracellular Na^+ concentration, while the opposite is true for K^+ – with a higher K^+ concentration inside the cell than outside. It is worth noting that a non-equilibrium distribution of Na^+ and K^+ was observed even earlier. In 1894, Zaleski [14] attributed these findings to Carl Schmidt's work from the 1850s.

At the end of the 19th century and later, physiologists believed that the cell membrane must be impermeable to ions (e.g., Gürber [15,16]). However, in 1923, van Slyke *et al*. [17] described the high permeability of the RBC membrane to Cl^-. In 1936, Fenn and Cobb [18] discovered that muscle cell membranes are permeable to Na^+ and K^+. Using radioactive isotopes, Cohn and Cohn [19], Dean *et al*. [20], and Eisenmann *et al*. [21] demonstrated between 1939 and 1941 that the RBC membrane is permeable to Na^+ and K^+. These findings led to the hypothesis of the Na^+/K^+ pump, developed by Dean [22] and Krogh [23] between 1941 and 1946. In 1957, Skou [24] identified the Na^+/K^+-ATPase in crab nerves as the enzymatic basis for the Na^+/K^+ pump, an achievement for which he received the Nobel Prize in Chemistry in 1997. Subsequent characterisation of ion transport via the pump was carried out by Glynn (e.g., [25]), Sachs (e.g., [26]), and Hoffman and Kregenow (e.g., [27]). In 1952, Hodgkin and Huxley [28] provided a quantitative description of the action potential through alterations in membrane permeability for monovalent cations, earning them the Nobel Prize in Physiology or Medicine in 1963. Initially, these permeability changes were thought to involve a carrier mechanism. However, in 1955, Hodgkin and Keynes [29] proposed the existence of membrane pores (channels) for ion movement.

From 1965 to 1978, there was significant debate about the existence of ion channels. Ultimately, Hille [30] and Armstrong [31] identified ion channels with two key features: (i) a selectivity filter and (ii) an opening mechanism (gate). In 1976, Neher and Sakmann [32] developed the "patch-clamp" technique to investigate single ion channels, a breakthrough that earned them the Nobel Prize in Physiology or Medicine in 1991. In the years following the invention of the patch-clamp technique, also RBCs were investigated extensively by this method [33,34].

3. Phospholipid Composition and Distribution of RBC Membranes in Different Species. The Role of Cardiolipin and Plasmalogens in Biological Membranes, Lipid Rafts, and Lipid Scramblase

When investigating the function of lipids in biological membranes, particularly in RBC membranes, only five or six classes are typically considered. These include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), and cholesterol. However, other lipids have been studied to a much lesser extent. These include lysolipids (e.g., lysoPC), glycolipids (cerebrosides and gangliosides), and especially cardiolipin. Cardiolipin, characterised by two phosphate groups and four fatty acids, is primarily located in the inner mitochondrial membrane. It is believed to be crucial for the optimal activity of several mitochondrial proteins and is also associated with various diseases, such as neuronal dysfunction [35,36]. Although the role of cardiolipin in the RBC membrane is not fully understood, it may be significant for the activity of certain membrane proteins through direct interaction (see section 6 on membrane protein-lipid interactions). Additionally, cardiolipin might play a role in maintaining the balance of reactive oxygen species.

Nelson [37] and Wessels and Veerkamp [38] described the phospholipid head group composition (PC, SM, PE, PS, and others) of RBC membranes from various species (rat, dog, horse, guinea pig, rabbit, cat, human, pig, cow, sheep). The RBC membrane lipid composition of RBCs of different mammalian species including PI has been summarised by Kotyk and Janácek [39]. It was found that the variability of PE and PS among these species is relatively low. In contrast, the PC and SM content vary significantly. For example, dog and rat RBC membranes have low SM (10.8% and 12.8%, respectively) and high PC content (46.9% and 47.6%, respectively), while cow and sheep RBC membranes have high SM content (46.2% and 51.0%, respectively) and no PC. When comparing the PI content in the RBC membrane across species, significant differences are observed. To current knowledge, RBCs from horses have the lowest PI content (0.3%), while RBCs from cats have the highest (7.4%) [39]. The head group composition of the lipids in the RBC membrane in mice does not differ significantly from that in rats [40]. However, the lipid head group composition of camel RBCs shows significant differences compared to human RBCs. Specifically, camel RBCs have a much lower PC content but a much higher PE, with PS and SM levels nearly identical to those in human RBCs [41]. Additionally, other studies have shown that compared to sheep and goats, camels have significantly higher PC, SM, and cholesterol levels in their RBC membrane (e.g., [42]). The cholesterol content of RBCs across various mammalian species generally does not differ significantly, maintaining a total phospholipid/cholesterol mo-

Table 1. Asymmetrical transversal distribution of the major lipids in the human and bovine RBC membrane [48].

Lipid	Human inner (%)	Human outer (%)	Bovine inner (%)	Bovine outer (%)
Phosphatidylcholine	14	44	-	-
Sphingomyelin	10	44	-	100
Phosphatidylethanolamine	48	12	67	-
Phosphatidylserine	28	-	33	-

lar ratio close to 1, except in camel RBCs, where the ratio is slightly higher [42,43]. Nelson investigated also the fatty acid composition of the phospholipids in the human RBC membrane, as reported in [44]. The main phospholipids (PC, PE, PS, SM, PI, lysoPC) contain various fatty acids. In brackets, the trivial names are provided: 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 20:0 (arachidic acid), 20:3 (eicosatrinoic acid), 20:4 (arachidonic acid), 22:0 (behenic acid), 22:1 (erucic acid), 22:4 (docosatetraenoic acid), 22:5 (docosapentaenoic acid), and 22:6 (docosahexaenoic acid). Other authors investigated the fatty acid composition of the membrane lipids of RBCs from different mammalian species (rats, rabbits, guinea pigs, sheep, cows, pigs, dogs, and cats) [39]. They also identified the presence of the fatty acids 12:0 (lauric acid, only in sheep, 14:0 (myristic acid, only in sheep and rats), and 18:3 (α-linolenic acid, only in sheep). The fatty acids 20:0 and 20:3 were only found in sheep and sheep and rats, respectively. Furthermore, the fatty acids 20:0, 22:1, and 22:4 could not be detected in the membrane lipids of RBCs of the different species [39]. However, it is possible that other fatty acids are present in the RBC membrane lipids, but likely in minor concentrations.

It should be mentioned that also new data on the fatty acid composition of RBC membrane lipids based on modern or sensitivity-improved measuring techniques (e.g., gas chromatography) are available. In most cases, authors are investigating the effect of diets on the fatty acid composition (examples for RBCs of cats and rats see [45,46]), or the effect of diseases on the fatty acid composition (example for RBCs of dogs, see [47]). Analysing the transversal distribution of lipids in the RBC membrane reveals interesting differences between species. Table 1 (Ref. [48]) shows the asymmetrical transversal distribution of SM, PC, PE, PS in human and bovine RBC membranes. Notably, it is intriguing that the outer leaflet of the bovine RBC membrane is composed entirely of sphingomyelin.

The diverse lipid compositions of RBC membranes of various species suggest their significance in RBC flexibility (see section 5). Additionally, the phase transition temperatures of these lipids, determined by both their head groups and fatty acid content, vary relative to body temperature, indicating not all lipids maintain a fluid-crystalline state. It is important to note that the fluidity of the lipid bilayer and consecutively of the cell membrane is determined by its lipid composition. However, these findings suggest the formation of lipid microdomains (lipid rafts) within biological membranes since a random distribution of lipids in both fluid-crystalline and crystalline states appears energetically unfavourable. Lipid rafts, characterised by their detergent-insolubility, are enriched with sphingolipids and cholesterol. Sphingolipids, such as SM and glycosphingolipids, exist in a crystalline phase with saturated fatty acids, forming distinct phases within the plasma membrane of eukaryotic cells. Rafts also harbour glycosylphosphatidylinositol (GPI)-anchored proteins, with raft diameters typically ranging from 10 nm to 200 nm and containing up to 50 GPI-anchored proteins. Larger sub-micrometric domains with diameters between 300 nm and 500 nm have also been reported. The existence of lipid rafts was first suggested by Simons and Ikonen in 1997 [49], and the concept has since been further developed by researchers such as Sharma *et al*. [50], Carquin *et al*. [51,52], and Conrad *et al*. [53]. Currently, at least 150 different human GPI-anchored proteins have been identified [54]. Examples of GPI-anchored proteins in the human RBC membrane include protectin (CD59) and Complement Decay Accelerating Factor (CD55) [54]. Rafts represent dynamic structures, with lipids and proteins residing within them for periods ranging from seconds to minutes. Identifying rafts in living cells remains a contentious subject. Future research should prioritise understanding their physiological functions and properties, including interactions with the cytoskeleton and protein receptors, crucial for signal transduction. Notably, Minetti's group [55,56] has significantly contributed to our comprehension of rafts in RBC membranes. An important finding regarding the RBC membrane was described by Conrad *et al*. [53]. Using fluorescence and confocal microscopy, three distinct lipid domains were identified: (i) cholesterol-enriched domains associated with high curvature areas of the RBC, (ii) ganglioside GM1/PC/cholesterol-enriched domains present in low curvature areas, and (iii) SM/PC/cholesterol-enriched domains also present in low curvature areas. Cholesterol- and SM-enriched domains in the RBC membrane have also been reported by other studies [51,52]. Additionally, the molecular organisation within these different domains has been described in more detail [57].

Another intriguing aspect is the presence of plasmalogens in the RBC membrane. Plasmalogens are a subclass of glycerophospholipids characterised by a vinylether bond at the sn-1 position and polyunsaturated fatty acid at the sn-2 position. While their role in biomembranes is still un-

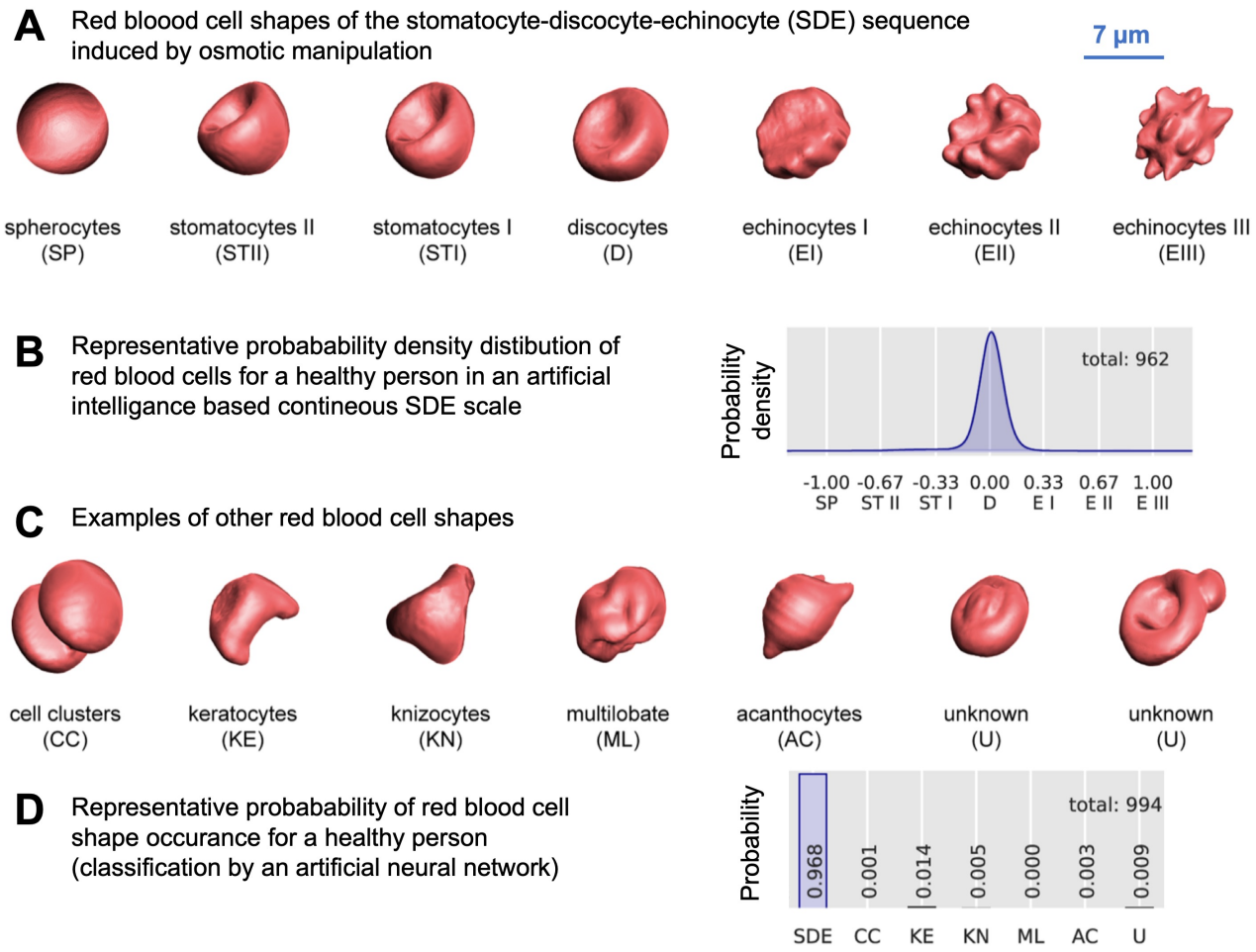

Fig. 1. Overview of red blood cell (RBC) shapes. (A) represents 3D-rendered RBC from confocal microscopy recordings in the stomatocyte-discocyte-echinocyte (SDE) sequence. These cell shapes were induced by osmotic manipulation. Recently, the SDE sequence was proposed to be matched on an analogue scale from -1 to +1 [65]. (B) shows the distribution of the occurrence of the RBCs of the SDE sequence in a blood sample from a healthy person. (C) shows 3D images of examples of other cell shapes not covered by the SDE sequence. In blood samples of healthy donors such shapes occur very rarely. (D) indicates this probability compared to the RBCs of the SDE sequence. Reproduced with permission from Simionato *et al*, PLoS Computational Biology; published by PLoS, 2021 [65].

der discussion, it is believed that plasmalogens contribute to the physical and chemical properties of the membrane and act as antioxidants, protecting unsaturated fatty acids and lipoproteins from oxidative stress [58,59]. Plasmalogens have been identified in neuronal, immune, and cardiovascular cells [59] and are also present in the RBC membrane [60,61]. Therefore, in future, it seems of importance to investigate their role in the RBC membranes. In addition, the biophysical properties of plasmalogens and their implications for certain diseases, such as Alzheimer's disease, should be considered. For more details, we refer to the reviews by Honsho and Fujiki [62,63].

Finally, very recent research showed significant lipid remodelling during reticulocyte maturation and the RBC ageing process beyond [64]. Lipid analysis showed that cholesterol and SM increase, while PC and PS decrease as reticulocytes mature into RBCs. Specific phospholipid subclasses change during the ageing of RBCs, with some approaching the composition of plasma lipoproteins. Furthermore, VPS13A, a lipid transport protein, is present in reticulocytes and decreases with RBC maturation, potentially playing a role in lipid exchange. The findings challenge the traditional view that RBC membrane maturation is solely linked to membrane skeleton assembly, suggesting a more complex process involving lipid remodelling [64]. The concrete molecular regulation of lipid remodelling during RBC aging is among the unsolved problems in RBC membrane research.

4. Variations in RBC Shapes

Under physiological conditions, human RBCs typically have a biconcave shape and are referred to as discocytes. When subjected to volume changes, such as alterations in the osmolarity of the surrounding medium as

performed for Fig. 1A (Ref. [65]), the RBCs undergo a series of shape transformations in the sequence stomatocyte-discocyte-echinocytes (SDE). The normal distribution of these cell shapes under physiological conditions is illustrated in Fig. 1B. Experimentally, other methods can also induce similar RBC shape changes, as documented in the literature (e.g., [66]). Various biophysical theories have been proposed to explain the mechanisms behind these shape transformations. Additionally, unusual or pathological RBC shapes (for selected examples see Fig. 1C) can occur under physiological conditions, though at a very low frequency, as depicted in Fig. 1D.

Furthermore, pathological RBC shapes are characteristic of specific diseases and have even given their names to some of them [67], such as drepanocytes (sickle cells), which are associated with sickle cell disease. This condition is explained by the replacement of normal haemoglobin (mainly HbA) with abnormal haemoglobin (HbS), which alters the interaction between haemoglobin molecules and the inner membrane surface of RBCs. Keratocytes and schistocytes often appear after cardiac or vascular surgery, while dacrocytes are associated with thalassemia, leukaemia, toxicity, and haemolytic anaemias. Codocytes are indicative of hypochromic anaemias. Acanthocytes are linked to a group of rare hereditary neurodegenerative disorders known as neuroacanthocytosis syndromes [68]. Elliptocytes, which are prominent in various anaemias like hereditary elliptocytosis, are also of significant interest.

Camel RBCs exclusively adopt an ellipsoid shape, termed ovalocytes. Bessis' atlas comprehensively covers these shapes [69]. Notably, human elliptocytes lack the increased resistance to osmotic haemolysis observed in camel cells. Camels can lose significant body weight (30–40%) and rapidly consume vast amounts of water (up to 200 litres), leading to considerable water uptake of the RBCs resulting in their volume expansion. Camel RBCs can increase their volume to a much larger extent compared with human RBCs [70]. Possible explanations of such differences are still a matter of debate.

5. Deformability of RBCs in Different Mammalian Species

The deformability is one of the most, if not the most important property of the RBC. It is crucial for their ability to pass through narrow capillaries without rupturing, which is essential for maintaining efficient blood flow [71]. This flexibility varies significantly among different mammalian species, reflecting adaptations to their unique environmental challenges and physiological requirements. RBC deformability is influenced by several factors, including the structural integrity of the cell membrane — such as the lipid composition (see section 3, above) and the membrane proteins (their abundance, interaction and activity), the organization of the cytoskeleton, and the viscosity of the cytoplasm. However, the exact relation and contributions of all mentioned parameters is among the unsolved problems in RBC membrane research [72]. Additionally, there is a biomolecular signaling component, namely the interplay between mechano-sensitive ion channels, such as Piezo1 and the Gárdos channel (see section 6, below). The activation of the mechano-sensitive channel allows Ca^{2+} to enter the RBC, which activates the Gárdos channel resulting primarily in the loss of K^+, followed by Cl^- and water, finally leading to a volume decrease [73]. Nevertheless, the biconcave shape of RBCs in most mammals (see section 4, above) is a key feature that enhances their flexibility, allowing them to undergo significant deformation while traversing capillaries [74]. Structural properties of the RBC membrane are subject to variation across species, depending on their environmental and physiological needs. Camels have evolved RBCs with unique characteristics [70] to survive in the extreme conditions of the desert (see sections 3 and 4, above). For comparative studies of RBC deformability see, e.g., Amin and Sirs [75], Nemeth *et al.* [76], and Plasenzotti *et al.* [77]. There is a wide range of methods to investigate the deformability of RBCs [78]. It includes micropipette aspiration [79], atomic force spectroscopy [80], optical tweezers [81], ektacytometry [82], and microfluidic assays [83], just to name a few.

6. Cation Transporters in the RBC Membrane, Including the Activation or Suppression of these Transporters during RBC Maturation

A wide array of ion transporters, particularly for Na^+ and K^+, are now recognized in the RBC membrane. Nearly all cells possess a Na^+/K^+ pump crucial for establishing the Na^+ and K^+ gradient across the cell membrane. Interestingly, mature RBCs of dogs and cats lack this pump, relying on alternative mechanisms for maintaining Na^+ and K^+ gradients (details see next paragraph (iii)) [84–86]. Besides the pump, the RBC membrane harbours various carriers, including the Na^+-K^+-$2Cl^-$ symporter (NKCC), K^+-Cl^- symporter (KCC), Na^+-dependent amino acid transporters, $Na^+(Mn^+)/Mg^{2+}$ antiporter, Na^+/Li^+ antiporter, Na^+/H^+ antiporter (NHE1), band 3 (anion transporter), which can act as $NaCO_3^-/Cl^-$ antiporter, and $Na^+(K^+)/H^+$ antiporter (e.g., [87]). Further details on the $Na^+(K^+)/H^+$ antiporter are provided below (see section 7). The presence of ion channels adds complexity to the picture. Initially, only the Ca^{2+}-activated K^+ channel (Gárdos channel) was known [88], which years later was identified as KCNN4 [89]. This was followed by the description of the non-selective, voltage-dependent cation (NSVDC) channel in human RBCs [90–92]. Fig. 2 (Ref. [88,89,93–104]) illustrates the current state of knowledge. The Gárdos channel and the Piezo1 channel have both been confirmed with molecular evidence [105]. It is highly probable that Piezo1 accounts for most of the measurements

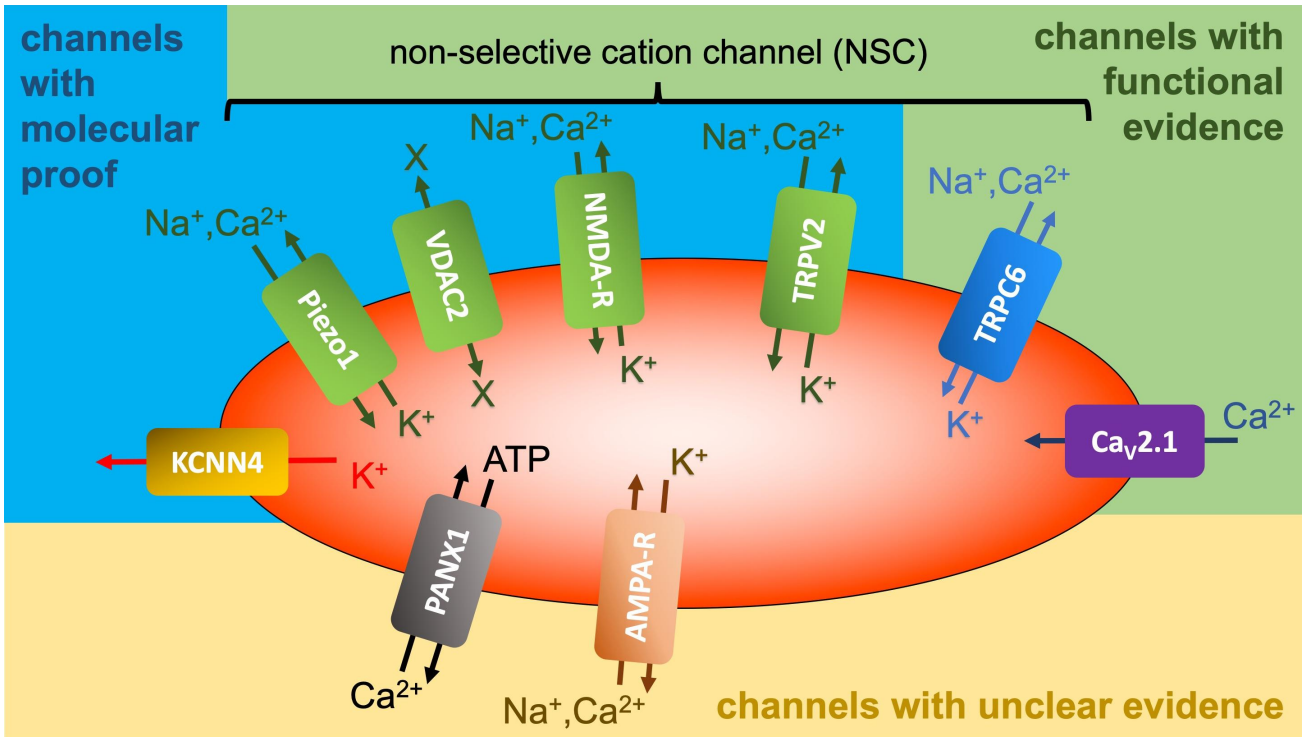

Fig. 2. Overview of ion channels reported to be present in RBCs. Gárdos Channel (KCNN4) [88,89]; piezo1 [93,94]; voltage-dependent anion channel type 2 (VDAC2) [95]; n-methyl-d-aspartate receptor (NMDA-R) [96]; transient receptor potential channel of vanilloid type 2 (TRPV2) [97,98]; transient receptor potential channel of canonical type 6 (TRPC6) [99,100]; $Ca_V2.1$, voltage-dependent Ca^{2+} Channel 2.1 [101,102]; pannexin 1 (PANX1) [103]; α-amino-3-hydroxy-5-methylisoxacol-4-propion acid receptor (AMPA-R) [104]; X: high permeability for small ions (Na^+, K^+, Cl^-) but also glutamate, ATP, Ca^{2+}, acetylcholine, dopamine, tris etc. Drawn by Keynote (Apple Inc., Cupertino, CA, USA).

previously attributed to the NSVDC channel [93,94,106]. However, there are additionally non-selective cation channels in the RBC membrane, such as VDAC2 – Voltage-Dependent Anion Channel type 2 [95], NMDA-R – N-Methyl-D-Aspartate Receptor [96], TRPV2 - Transient Receptor Potential Channel of Vanilloid type 2 [97,98], which may also account for specific previous NSVDC channel recordings. These channels have been unequivocally confirmed on the molecular level. However, there are also channels where standard molecular techniques, such as Western blots or proteomic mass spectrometry, fail or yield inconsistent results. This can be attributed to the detection limits of the methods combined with a low copy number of the channels per cell [105]. Despite these challenges, the effects of the channel openings can still be observed by applying specific agonists or antagonists or by a specific activation of a signalling cascade and measuring an expected consecutive cellular response. We refer to these as channels described mainly based on functional evidence. Channels in this category include TRPC6 – Transient Receptor Potential Channel of Canonical type 6 [99,100] and $Ca_V2.1$ – Voltage-Dependent Calcium Channel 2.1 [101,102]. In contrast, there are isolated, episodic reports of ion channels in RBCs [103,104] that have not been confirmed by other research groups; we categorize these as channels with unclear evidence. Further investigations are required to determine the actual repertoire of ion channels in the RBC membrane as well as their genesis and physiological functions [107].

Another intriguing aspect is the variation in ion transport pathways among RBCs of different species and their modulation during RBC maturation. For instance, (i) KCC is present in young but silent in mature human RBCs [108]. However, in mature RBCs it can be activated by different manoeuvres [109]. KCC is present in low K^+-type (LK) sheep RBCs but absent in high K^+-type (HK) sheep RBCs [110,111]. The K^+ content in sheep RBCs is under genetic control, resulting in either low K^+-containing (LK) or high K^+-containing (HK, similar to RBCs of most mammalian species). For more details, we refer to [109,112]. No differences in the lipid composition between these two RBC types have been observed [113]. (ii) Voltage-activated cation transport occurs in HK but not in LK sheep RBCs [114]. On the contrary, the low ionic strength (LIS) induced residual (leak) cation transport is present in LK but not HK sheep RBCs ([115], for LIS effect see section 7). (iii) Despite the absence of the Na^+/K^+ pump in mature dog and cat RBCs, these cells possess a Ca^{2+}/Na^+ antiporter [116,117] absent

in RBCs of other mammals, enabling Na^+ gradient generation based on the Ca^{2+} gradient realised by the Ca^{2+} pump (which is present in all mammalian cells) [118]. Assuming the existence of NKCC in dog and cat RBCs, a K^+ gradient is ultimately established. (iv) Notably, Ouabain, a Na^+/K^+ pump inhibitor, requires much higher concentrations for rat and mouse RBCs compared to human RBCs [119,120]. (v) Approximately 10% of Japanese cows lack band 3 protein crucial for gas exchange in their RBC membrane, raising questions about their survival mechanism [121].

It has long been known that the activity of integral membrane proteins, including ion transporters, can be influenced by the lipid environment within the membrane. It is now broadly accepted that the function of membrane proteins can be affected by the head group or fatty acid (or both) of the surrounding lipids. In addition to such specific effects, which are not fully understood, also non-specific effects, influenced by the physical properties of membrane lipids, e.g., the structure and the fluidity of the membrane, play a role in the regulation of membrane proteins [122–124]. In general, the investigation of lipid-protein interaction is complicated since three different possibilities have to be taken into consideration, the role of (i) bulk lipids, (ii) boundary or annular lipids, representing the first shell of the membrane protein coat, and (iii) specifically bound lipids at the membrane protein surface. One example of such effects is the Na^+/K^+ pump affected by the lipid environment via both general (physical) and specific (chemical) interactions [125,126]. In the case of the RBC membrane, specific effects of membrane lipids on transport proteins have been demonstrated. These findings stem from a study using different mammalian species, by reconstitution of purified proteins in lipid bilayer structures, e.g., liposomes, or by altering the lipid composition of RBCs through the use of a phospholipid exchange protein (PLEP) or using right-site-out membrane vesicles [127]. Furthermore, the activity of certain transport proteins has been shown to depend on the molar phospholipid/cholesterol ratio, which has been modified by incubating RBCs with lipid vesicles containing varying amounts of cholesterol (for details see [128]). Another aspect not yet fully explored is how the movement of membrane lipids, particularly their transmembrane movement, affects the activity of integral membrane proteins. As this issue lies beyond the scope of this paper, interested readers are referred to a variety of reviews (e.g., [129]). However, it is worth mentioning that SM does not translocate from the outer to the inner membrane leaflet. Consequently, sheep and cow RBCs, which exclusively contain SM in the outer membrane layer, do not require scramblases, as reported by Nguyen *et al.* [130].

While numerous ion transporters exist in the RBC membrane, our understanding of pathways for trace metal ions such as zinc, copper, cobalt, nickel, chromium, manganese, iron, and cadmium remains limited [131]. Identifying and characterizing specific transporters for these ions is imperative for future research. Additionally, elucidating the dynamics, including transporter movement during ion translocation, is essential alongside determining the 3D structure of membrane transport proteins post-crystallization.

7. The Mechanism of Residual (Leak) Ion Transport: Is there a $Na^+(K^+)/H^+$ Antiporter in the Human RBC Membrane? How does Ion Transport Depend on the Fatty Acid Composition of the RBC Membrane?

The question remains whether electrodiffusion of a particular ion can occur in the human RBC membrane when all specific pathways for this ion (pumps, carriers, channels) are inhibited. It has long been assumed that the remaining residual (leak) ion flow is attributable to simple electrodiffusion [132–136]. In several publications, we have demonstrated that the observed residual fluxes of Na^+ and K^+ are mediated by a $Na^+(K^+)/H^+$ antiporter (e.g., [87,137,138]). This notion stems from studies [87,137,138] of residual K^+ efflux in low ionic strength (LIS) solutions. It has been established for some time that the residual K^+ efflux, specifically the (ouabain + bumetanide + ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA))-insensitive K^+ efflux, significantly increases when RBCs are transferred from physiological solutions to LIS media [139]. Ouabain, bumetanide, and EGTA are used to inhibit the K^+ transport mediated by the Na^+/K^+ pump, the Na^+-K^+-$2Cl^-$-symporter, and the Gárdos (Ca^{2+}-activated K^+) channel, respectively.

Experimental data illustrating this phenomenon are presented in Fig. 3. Theoretically, an increase in K^+ efflux can be anticipated based on electrodiffusion mechanisms. This expectation arises from a shift of transmembrane potential, from approximately –12 mV in physiological solutions to roughly +50 mV in LIS solutions due to reduced NaCl concentration. However, the observed flux-increase surpassed predictions based on the Goldman flux equation:

$$J_j = P_j z_j^2 \frac{V_m F^2}{RT} \times \frac{c_{ji} - c_{j0}\, e^{-\frac{z_j V_m F}{RT}}}{1 - e^{-\frac{z_j V_m F}{RT}}}$$

with J_j – outward flux of the ion j, P_j – membrane permeability for ion j, V_m – transmembrane potential, c_{ji} – intracellular concentration of ion j, c_{jo} – extracellular concentration of ion j, and z_j – valence of ion j. F, R, and T – are the Faraday constant, gas constant, and absolute temperature, respectively. Thus, the Goldman flux equation (equation above) describes the electrodiffusion of an ion in dependence on the driving force, which includes the ion concentrations of both sides of the membrane and the transmembrane potential [140]. For an illustration of the effect, see Fig. 3.

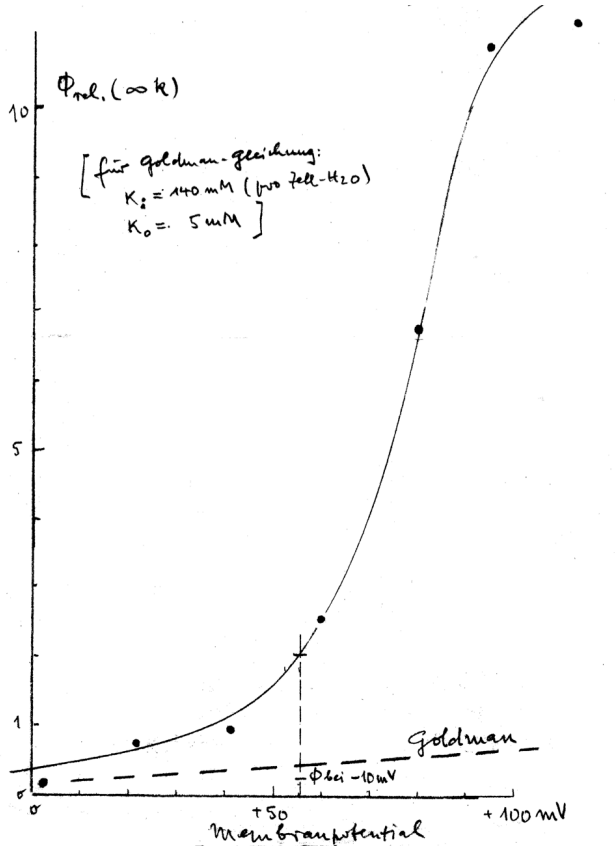

Fig. 3. K^+ efflux of human RBCs depending on the membrane potential, i.e., on the extracellular NaCl concentration of isotonic solution (the NaCl reduction was compensated by sucrose). The figure (hand drawing) represents one of the oldest data of K^+ efflux in low ionic strength (LIS) solutions, probably obtained from experiments carried out before the 1960s and is a personal gift of H.J. Schatzmann (former Director of the Pharmacological Institute of the University Bern (Switzerland) to Ingolf Bernhardt.

Surprisingly, the K^+ efflux in bovine and HK sheep RBCs remained largely unchanged in LIS solutions compared to physiological ones [141–143]. Thus, we conducted comprehensive measurements of all four residual fluxes for K^+ and Na^+ — efflux and influx for both — in human RBCs. Surprisingly, all four fluxes exhibited significant increases in solutions with decreasing ionic strength [137]. This phenomenon defies explanation solely through electrodiffusion, as it would necessitate two fluxes increasing and two decreasing under uniform changes of the driving force for all four. Various mechanisms were considered, with several possibilities eliminated based on arguments presented as early as 2003 [87]. The most plausible explanation for the observed effect was the involvement of a $Na^+(K^+)/H^+$ antiporter [87,137,138]. A K^+/H^+ exchange has been also described in trout RBCs [144].

Despite accumulating evidence supporting this hypothesis, we unfortunately failed to demonstrate the presence of such a transporter in the RBC membrane at the molecular level. To date, 13 isoforms of Na^+/H^+ antiporters have been identified in biological membranes, with isoforms 1–9 relatively well-characterized [145]. While NHE1–NHE5 are typically found in cell membranes, NHE6–NHE9 have been primarily detected in organelle membranes, leading to the assumption that NHE6–NHE9 are absent from cell membranes [146]. However, recent evidence has suggested the presence of NHE9 in the plasma membrane of inner ear hair cell bundles [147].

Furthermore, NHE7 and NHE9 not only exchange Na^+ but also K^+ for H^+. However, attempts to detect the presence of NHE7 in the human RBC membrane using mass spectrometry and fluorescent antibodies were unsuccessful. Our focus on NHE7 stemmed from limited knowledge about NHE9 at the time of our investigations. Future research should explore the hypothesis of NHE9's presence in the human RBC membrane. Recent support for the presence of a $Na^+(K^+)/H^+$ antiporter in the human RBC membrane comes from various other findings.

As already mentioned, unlike human RBCs, HK sheep and bovine RBCs did not exhibit increased residual K^+ efflux in LIS solutions, despite experiencing similar changes of the transmembrane potential. Our research demonstrated that residual K^+ efflux of RBCs of different species correlates with lipid composition of the RBC membrane, particularly the content of arachidonic acid. Furthermore, we observed an increased residual K^+ flux in LIS solutions of new-born calf RBCs, which diminished over time (blood taken between one day and six weeks after birth of the calves), as their arachidonic acid content decreased (correlation coefficient between flux in LIS solution and arachidonic acid content of the RBC membrane of the calves: 0.951) [141,143]. A recent study has shown that NHE9 activity is modulated by phosphatidylinositol-4,5-bisphosphate (PIP_2), with potential implications for its interaction with arachidonic acid-enriched membranes [148]. This provides a plausible explanation for our earlier findings. In summary, residual transport of Na^+ and K^+ across the RBC membrane appears to be specific and mediated by a cation/proton antiporter in humans. Future research should aim to elucidate how monovalent cations traverse the RBC membrane when all known specific pathways (pumps, channels, carriers) are inhibited.

8. Conclusion

Although we have a substantial understanding of the structure and function of the RBC membrane, many questions remain unanswered. Notably, the RBC was almost always the first cell type studied when new methods for investigating biological cells were introduced. The only exception was the patch-clamp technique since RBCs are "designed" to pass through small capillaries, which made it difficult to create pipette geometry where RBCs could be "patched" and not just "sucked in the pipette" [149]. How-

ever, today, patch-clamp studies of channels in the RBC membrane are routine (e.g. [150]) and can even be performed using automated patch-clamp devices (e.g. [151]).

In this review we focussed on original findings, some dating back to the 1960s or 1970s, as we recognised that these data are often forgotten or unknown to younger scientists. Occasionally these measurements are repeated many years after the original investigations, yet the earlier work is often ignored and not cited in subsequent publications. Building on earlier investigations and employing modern techniques, it will be possible to address unsolved questions regarding RBC membranes. Some of these questions have been discussed in this paper.

Abbreviations

EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; ERCS, European Red Cell Society; GPI, glycosylphosphatidylinositol; Hb, haemoglobin; HK, high potassium; KCC, K^+-Cl^- symporter; LIS, low ionic strength; LK, low potassium; NHE, Na^+/H^+ antiporter; NKCC, Na^+-K^+-$2Cl^-$ symporter; NSVDC, non-selective, voltage-dependent cation; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PIP_2, phosphatidylinositol-4,5-bisphosphate; RBC, red blood cell; SM, sphingomyelin.

Author Contributions

IB and LK made substantial contributions to the conception and design of the work. Both authors wrote the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Hooke R. Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses. With observations and inquiries thereupon. The Royal Society: London. 1665.

[2] Nägeli C. In Pflanzenphysiologische Untersuchungen. Heft 1. Friedrich Schulthess: Zürich. 1855. (In German)

[3] Overton E. Über die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermutlichen Ursachen und ihre Bedeutung für die Physiologie. Vierteljahresschrift der Naturforschenden Gesellschaft in Zürich. 1899; 44: 88–114. (In German)

[4] Gorter E, Grendel F. On bimolecular layers of lipoids on the chromocytes of the blood. The Journal of Experimental Medicine. 1925; 41: 439–443. https://doi.org/10.1084/jem.41.4.439

[5] Danielli JF, Davson H. A contribution to the theory of permeability of thin films. Journal of Cellular and Comparative Physiology. 1935; 5: 495–508. https://doi.org/10.1002/jcp.1030050409

[6] Dawson H, Danielli JF. In The permeability of natural membranes. University Press: Cambridge. 1943.

[7] Robertson JD. Membrane structure. Journal of Cellular Biology. 1981; 91: 189–204.

[8] Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science (New York, N.Y.). 1972; 175: 720–731. https://doi.org/10.1126/science.175.4023.720

[9] Silvius JR. In Thermotropic phase transition of pure lipids in model membranes and their modifications by membrane proteins. John Wiley and Sons Inc.: New York. 1982.

[10] Bretscher MS. Asymmetrical lipid bilayer structure for biological membranes. Nature: New Biology. 1972; 236: 11–12. https://doi.org/10.1038/newbio236011a0

[11] Bretscher MS. Membrane structure: some general principles. Science (New York, N.Y.). 1973; 181: 622–629. https://doi.org/10.1126/science.181.4100.622

[12] Cullis PR, de Kruijff B. Lipid polymorphism and the functional roles of lipids in biological membranes. Biochimica et Biophysica Acta. 1979; 559: 399–420. https://doi.org/10.1016/0304-4157(79)90012-1

[13] Abderhalden E. Zur quantitativen Analyse des Blutes. Zeitschrift für Physiologische Chemie. 1897; 23: 521–531. (In German) https://doi.org/10.1515/bchm2.1897.23.6.521

[14] Zaleski SS. Carl Schmidt. Berichte der Deutschen Chemischen Gesellschaft. 1894; 27: 963–978. (In German)

[15] Gürber A. Die Salze des Blutes. Jahresbericht über die Fortschritte der Tierchemie oder der physiologischen und pathologischen Chemie. 1895; 24: 172–175. (In German)

[16] Gürber A. Salze des Blutes. II. Teil. Salze der Blutkörper. Habilitationsschrift, Königlich-Bayerische Julius-Maximilians-Universität: Würzburg. 1904. (In German)

[17] van Slyke DD, Wu H, McLean FC. Studies of gas and electrolyte equilibria in the blood. V. Factors controlling the electrolyte and water distribution in the blood. Journal of Biological Chemistry. 1923; 56: 765–849. https://doi.org/10.1016/S0021-9258(18)85558-2

[18] Fenn WO, Cobb DM. Electrolyte changes in muscle during activity. American Journal of Physiology. 1936; 115: 345–356. https://doi.org/10.1152/ajplegacy.1936.115.2.345

[19] Cohn WE, Cohn ET. Permeability of red corpuscles of the dog to sodium ion. Proceedings of the Society for Experimental Biology and Medicine. 1939; 41: 445–449. https://doi.org/10.3181/00379727-41-10705

[20] Dean RB, Noonan TR, Haege L, Fenn WO. Permeability of erythrocytes to radioactive potassium. The Journal of General Physiology. 1941; 24: 353–365. https://doi.org/10.1085/jgp.24.3.353

[21] Eisenmann AJ, Ott L, Smith PK, Winkler AW. A study of the permeability of human erythrocytes to potassium, sodium and inorganic phosphate by the use of radioactive isotopes. Journal of Biological Chemistry. 1940; 135: 165–173. https://doi.org/10.1016/S0021-9258(18)73173-6

[22] Dean RB. Theories of electrolyte equilibrium in muscle. Biological Symposium. 1941; 3: 331–348.

[23] Krogh A. The active and passive exchanges of inorganic ions through the surfaces of living cells and through living mem-

branes generally. Proceedings of the Royal Society of London. Series B, Biological Sciences. 1946; 133: 140–200. https://doi.org/10.1098/rspb.1946.0008
[24] Skou JC. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochimica et Biophysica Acta. 1957; 23: 394–401. https://doi.org/10.1016/0006-3002(57)90343-8
[25] Glynn IM. Sodium and potassium movements in human red cells. The Journal of Physiology. 1956; 134: 278–310. https://doi.org/10.1113/jphysiol.1956.sp005643
[26] Sachs RJ. Na+/K+ pump. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 111–137). Springer: Berlin. 2003.
[27] Hoffman JF, Kregenow FM. The characterization of new energy dependent cation transport processes in red blood cells. Annals of the New York Academy of Sciences. 1966; 137: 566–576. https://doi.org/10.1111/j.1749-6632.1966.tb50182.x
[28] Hodgkin AL, Huxley AF. A quantitative description of membrane current and its application to conduction and excitation in nerve. The Journal of Physiology. 1952; 117: 500–544. https://doi.org/10.1113/jphysiol.1952.sp004764
[29] Hodgkin AL, Keynes RD. The potassium permeability of a giant nerve fibre. The Journal of Physiology. 1955; 128: 61–88. https://doi.org/10.1113/jphysiol.1955.sp005291
[30] Hille B. Ionic channels in nerve membranes. Progress in Biophysics and Molecular Biology. 1970; 21: 1–32. https://doi.org/10.1016/0079-6107(70)90022-2
[31] Armstrong CM. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. The Journal of General Physiology. 1971; 58: 413–437. https://doi.org/10.1085/jgp.58.4.413
[32] Neher E, Sakmann B. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature. 1976; 260: 799–802. https://doi.org/10.1038/260799a0
[33] Hamill OP. Ca and volume sensitive K channels in frog red blood cells. Pflugers Archiv. 1982; 394: R30. https://doi.org/10.1007/BF02580695
[34] Grygorczyk R, Schwarz W, Passow H. Ca2+-activated K+ channels in human red cells. Comparison of single-channel currents with ion fluxes. Biophysical Journal. 1984; 45: 693–698. https://doi.org/10.1016/S0006-3495(84)84211-3
[35] Jiang Z, Shen T, Huynh H, Fang X, Han Z, Ouyang K. Cardiolipin Regulates Mitochondrial Ultrastructure and Function in Mammalian Cells. Genes. 2022; 13: 1889. https://doi.org/10.3390/genes13101889
[36] Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Role of Cardiolipin in Mitochondrial Function and Dynamics in Health and Disease: Molecular and Pharmacological Aspects. Cells. 2019; 8: 728. https://doi.org/10.3390/cells8070728
[37] Nelson GJ. Lipid composition of erythrocytes in various mammalian species. Biochimica et Biophysica Acta. 1967; 144: 221–232. https://doi.org/10.1016/0005-2760(67)90152-x
[38] Wessels JM, Veerkamp JH. Some aspects of the osmotic lysis of erythrocytes. 3. Comparison of glycerol permeability and lipid composition of red blood cell membranes from eight mammalian species. Biochimica et Biophysica Acta. 1973; 291: 190–196. https://doi.org/10.1016/0005-2736(73)90411-2
[39] Kotyk A, Janacek K. In Membrane transport. An interdisciplinary approach. Plenum Press: New York. 1977.
[40] Shevchenko OG, Shishkina LN. Comparative analysis of phospholipid composition in blood erythrocytes of various species of mouse-like rodents. Journal of Evolutionary Biochemistry and Physiology. 2011; 47: 179–186. https://doi.org/10.1134/S0022093011020071
[41] Mirgani T. Lipid composition of camel erythrocytes. Journal of Arid Enviuronments. 1992; 22: 401–405. https://doi.org/10.1016/S0140-1963(18)30583-4
[42] Al-Qarawi AA, Mousa HM. Lipid concentrations in erythrocyte membranes in normal, starved, dehydrated and rehydrated camels (Camelus dromedarius), and in normal sheep (Ovis aries) and goats (Capra hircus). Journal of Arid Environments. 2004; 59: 675–683. https://doi.org/10.1016/j.jaridenv.2004.02.004
[43] Nelson GJ. Composition of neutral lipids from erythrocytes of common mammals. Journal of Lipid Research. 1967; 8: 374–379.
[44] Nelson GJ. In Blood lipids and lipoproteins: Quantitation, composition and metabolism. Wiley-Interscience: New York. 1972.
[45] Trevizan L, de Mello Kessler A, Brenna JT, Lawrence P, Waldron MK, Bauer JE. Maintenance of arachidonic acid and evidence of Δ5 desaturation in cats fed γ-linolenic and linoleic acid enriched diets. Lipids. 2012; 47: 413–423. https://doi.org/10.1007/s11745-011-3651-0
[46] Abbott SK, Else PL, Atkins TA, Hulbert AJ. Fatty acid composition of membrane bilayers: importance of diet polyunsaturated fat balance. Biochimica et Biophysica Acta. 2012; 1818: 1309–1317. https://doi.org/10.1016/j.bbamem.2012.01.011
[47] Crisi PE, Luciani A, Di Tommaso M, Prasinou P, De Santis F, Chatgilialoglu C, *et al*. The Fatty Acid-Based Erythrocyte Membrane Lipidome in Dogs with Chronic Enteropathy. Animals: an Open Access Journal from MDPI. 2021; 11: 2604. https://doi.org/10.3390/ani11092604
[48] Dijck PW, Zoelen EJ, Seldenrijk R, Deenen LL, Gier J. Calorimetric behaviour of individual phospholipid classes from human and bovine erythrocyte membranes. Chemistry and Physics of Lipids. 1976; 17: 336–343. https://doi.org/10.1016/0009-3084(76)90078-5
[49] Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997; 387: 569–572. https://doi.org/10.1038/42408
[50] Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, *et al*. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. Cell. 2004; 116: 577–589. https://doi.org/10.1016/s0092-8674(04)00167-9
[51] Carquin M, Pollet H, Veiga-da-Cunha M, Cominelli A, Van Der Smissen P, N'kuli F, *et al*. Endogenous sphingomyelin segregates into submicrometric domains in the living erythrocyte membrane. Journal of Lipid Research. 2014; 55: 1331–1342. https://doi.org/10.1194/jlr.M048538
[52] Carquin M, Conrard L, Pollet H, Van Der Smissen P, Cominelli A, Veiga-da-Cunha M, *et al*. Cholesterol segregates into submicrometric domains at the living erythrocyte membrane: evidence and regulation. Cellular and Molecular Life Sciences: CMLS. 2015; 72: 4633–4651. https://doi.org/10.1007/s00018-015-1951-x
[53] Conrard L, Stommen A, Cloos AS, Steinkühler J, Dimova R, Pollet H, *et al*. Spatial Relationship and Functional Relevance of Three Lipid Domain Populations at the Erythrocyte Surface. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology. 2018; 51: 1544–1565. https://doi.org/10.1159/000495645
[54] Kinoshita T. Biosynthesis and biology of mammalian GPI-anchored proteins. Open Biology. 2020; 10: 190290. https://doi.org/10.1098/rsob.190290
[55] Ciana A, Achilli C, Balduini C, Minetti G. On the association of lipid rafts to the spectrin skeleton in human erythrocytes. Biochimica et Biophysica Acta. 2011; 1808: 183–190. https://doi.org/10.1016/j.bbamem.2010.08.019
[56] Ciana A, Achilli C, Minetti G. Membrane rafts of the human red blood cell. Molecular Membrane Biology. 2014; 31: 47–57. https://doi.org/10.3109/09687688.2014.896485
[57] Himbert S, Alsop RJ, Rose M, Hertz L, Dhaliwal A, Moran-Mirabal JM, *et al*. The Molecular Structure of Human Red Blood

Cell Membranes from Highly Oriented, Solid Supported Multi-Lamellar Membranes. Scientific Reports. 2017; 7: 39661. https://doi.org/10.1038/srep39661

[58] Almsherqi ZA. Potential Role of Plasmalogens in the Modulation of Biomembrane Morphology. Frontiers in Cell and Developmental Biology. 2021; 9: 673917. https://doi.org/10.3389/fcell.2021.673917

[59] Koivuniemi A. The biophysical properties of plasmalogens originating from their unique molecular architecture. FEBS Letters. 2017; 591: 2700–2713. https://doi.org/10.1002/1873-3468.12754

[60] Moser AB, Steinberg SJ, Watkins PA, Moser HW, Ramaswamy K, Siegmund KD, *et al*. Human and great ape red blood cells differ in plasmalogen levels and composition. Lipids in Health and Disease. 2011; 10: 101. https://doi.org/10.1186/1476-511X-10-101

[61] Acar N, Berdeaux O, Juaneda P, Grégoire S, Cabaret S, Joffre C, *et al*. Red blood cell plasmalogens and docosahexaenoic acid are independently reduced in primary open-angle glaucoma. Experimental Eye Research. 2009; 89: 840–853. https://doi.org/10.1016/j.exer.2009.07.008

[62] Honsho M, Fujiki Y. Asymmetric Distribution of Plasmalogens and Their Roles-A Mini Review. Membranes. 2023; 13: 764. https://doi.org/10.3390/membranes13090764

[63] Honsho M, Fujiki Y. Plasmalogen homeostasis - regulation of plasmalogen biosynthesis and its physiological consequence in mammals. FEBS Letters. 2017; 591: 2720–2729. https://doi.org/10.1002/1873-3468.12743

[64] Minetti G, Dorn I, Köfeler H, Perotti C, Kaestner L. Insights from lipidomics into the terminal maturation of circulating human reticulocytes. Cell Death Discovery. 2025. (in press) https://doi.org/10.1038/s41420-025-02318-x

[65] Simionato G, Hinkelmann K, Chachanidze R, Bianchi P, Fermo E, van Wijk R, *et al*. Red blood cell phenotyping from 3D confocal images using artificial neural networks. PLoS Computational Biology. 2021; 17: e1008934. https://doi.org/10.1371/journal.pcbi.1008934

[66] Deuticke B. Membrane lipids and proteins as a basis of red cell shape and its alterations. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 27–60). Springer: Berlin. 2003.

[67] Simionato G, van Wijk R, Quint S, Wagner C, Bianchi P, Kaestner L. Rare Anemias: Are Their Names Just Smoke and Mirrors? Frontiers in Physiology. 2021; 12: 690604. https://doi.org/10.3389/fphys.2021.690604

[68] Peikert K, Storch A, Hermann A, Landwehrmeyer GB, Walker RH, Simionato G, *et al*. Commentary: Acanthocytes identified in Huntington's disease. Frontiers in Neuroscience. 2022; 16: 1049676. https://doi.org/10.3389/fnins.2022.1049676

[69] Bessis M. Corpuscles. Atlas of red blood cell shapes. Springer: Berlin. 1974.

[70] Pesen T, Haydaroglu M, Capar S, Parlatan U, Unlu MB. Comparison of the human's and camel's red blood cell deformability by optical tweezers and Raman spectroscopy. Biochemistry and Biophysics Reports. 2023; 35: 101490. https://doi.org/10.1016/j.bbrep.2023.101490

[71] Kuck L, Peart JN, Simmonds MJ. Active modulation of human erythrocyte mechanics. American Journal of Physiology. Cell Physiology. 2020; 319: C250–C257. https://doi.org/10.1152/ajpcell.00210.2020

[72] Lux SE, 4th. Anatomy of the red cell membrane skeleton: unanswered questions. Blood. 2016; 127: 187–199. https://doi.org/10.1182/blood-2014-12-512772

[73] Danielczok JG, Terriac E, Hertz L, Petkova-Kirova P, Lautenschläger F, Laschke MW, *et al*. Red Blood Cell Passage of Small Capillaries Is Associated with Transient Ca^{2+}-mediated Adaptations. Frontiers in Physiology. 2017; 8: 979. https://doi.org/10.3389/fphys.2017.00979

[74] Moreau A, Yaya F, Lu H, Surendranath A, Charrier A, Dehapiot B, *et al*. Physical mechanisms of red blood cell splenic filtration. Proceedings of the National Academy of Sciences of the United States of America. 2023; 120: e2300095120. https://doi.org/10.1073/pnas.2300095120

[75] Amin TM, Sirs JA. The blood rheology of man and various animal species. Quarterly Journal of Experimental Physiology (Cambridge, England). 1985; 70: 37–49. https://doi.org/10.1113/expphysiol.1985.sp002895

[76] Nemeth N, Kiss F, Klarik Z, Miko I. Comparative osmotic gradient ektacytometry data on inter-species differences of experimental animals. Clinical Hemorheology and Microcirculation. 2014; 57: 1–8. https://doi.org/10.3233/CH-2012-1620

[77] Plasenzotti R, Stoiber B, Posch M, Windberger U. Red blood cell deformability and aggregation behaviour in different animal species. Clinical Hemorheology and Microcirculation. 2004; 31: 105–111.

[78] Kim J, Lee H, Shin S. Advances in the measurement of red blood cell deformability: A brief review. Journal of Cellular Biotechnology. 2015; 1: 63–79.

[79] Evans EA, Hochmuth RM. Membrane viscoelasticity. Biophysical Journal. 1976; 16: 1–11. https://doi.org/10.1016/S0006-3495(76)85658-5

[80] Abay A, Simionato G, Chachanidze R, Bogdanova A, Hertz L, Bianchi P, *et al*. Glutaraldehyde - A Subtle Tool in the Investigation of Healthy and Pathologic Red Blood Cells. Frontiers in Physiology. 2019; 10: 514. https://doi.org/10.3389/fphys.2019.00514

[81] Ermolinskiy P, Lugovtsov A, Yaya F, Lee K, Kaestner L, Wagner C, *et al*. Effect of red blood cell aging in vivo on their aggregation properties in vitro: measurements with laser tweezers. Applied Sciences. 2020; 10: 7581. https://doi.org/10.3390/app10217581

[82] Hernández CA, Peikert K, Qiao M, Darras A, de Wilde JRA, Bos J, *et al*. Osmotic gradient ektacytometry - a novel diagnostic approach for neuroacanthocytosis syndromes. Frontiers in Neuroscience. 2024; 18: 1406969. https://doi.org/10.3389/fnins.2024.1406969

[83] Recktenwald SM, Lopes MGM, Peter S, Hof S, Simionato G, Peikert K, *et al*. Erysense, a Lab-on-a-Chip-Based Point-of-Care Device to Evaluate Red Blood Cell Flow Properties With Multiple Clinical Applications. Frontiers in Physiology. 2022; 13: 884690. https://doi.org/10.3389/fphys.2022.884690

[84] Sha'afi RI, Lieb WR. Cation movements in the high sodium erythrocyte of the cat. The Journal of General Physiology. 1967; 50: 1751–1764. https://doi.org/10.1085/jgp.50.6.1751

[85] Parker JC. Dog red blood cells. Adjustment of salt and water content in vitro. The Journal of General Physiology. 1973; 62: 147–156. https://doi.org/10.1085/jgp.62.2.147

[86] Gibson J. Comparative Physiology of red cell membrane transport. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 721–734). Springer: Berlin. 2003.

[87] Bernhardt I, Weiss E. Passive membrane permeability for ions and the membrane potential. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 83–109). Springer: Berlin. 2003.

[88] Gardos G. The function of calcium in the potassium permeability of human erythrocytes. Biochimica et Biophysica Acta. 1958; 30: 653–654. https://doi.org/10.1016/0006-3002(58)90124-0

[89] Hoffman JF, Joiner W, Nehrke K, Potapova O, Foye K, Wickrema A. The hSK4 (KCNN4) isoform is the Ca2+-activated K+ channel (Gardos channel) in human red blood cells. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100: 7366–7371. https://doi.org/10.1073/pn

as.1232342100
[90] Christophersen P, Bennekou P. Evidence for a voltage-gated, non-selective cation channel in the human red cell membrane. Biochimica et Biophysica Acta. 1991; 1065: 103–106. https://doi.org/10.1016/0005-2736(91)90017-3
[91] Kaestner L, Bollensdorff C, Bernhardt I. Non-selective voltage-activated cation channel in the human red blood cell membrane. Biochimica et Biophysica Acta. 1999; 1417: 9–15. https://doi.org/10.1016/s0005-2736(98)00240-5
[92] Kaestner L, Christophersen P, Bernhardt I, Bennekou P. The non-selective voltage-activated cation channel in the human red blood cell membrane: reconciliation between two conflicting reports and further characterisation. Bioelectrochemistry (Amsterdam, Netherlands). 2000; 52: 117–125. https://doi.org/10.1016/s0302-4598(00)00110-0
[93] Kaestner L, Egee S. Commentary: Voltage Gating of Mechanosensitive PIEZO Channels. Frontiers in Physiology. 2018; 9: 1565. https://doi.org/10.3389/fphys.2018.01565
[94] Zarychanski R, Schulz VP, Houston BL, Maksimova Y, Houston DS, Smith B, *et al*. Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis. Blood. 2012; 120: 1908–1915. https://doi.org/10.1182/blood-2012-04-422253
[95] Bouyer G, Cueff A, Egée S, Kmiecik J, Maksimova Y, Glogowska E, *et al*. Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are upregulated by Plasmodium falciparum. Blood. 2011; 118: 2305–2312. https://doi.org/10.1182/blood-2011-01-329300
[96] Makhro A, Hänggi P, Goede JS, Wang J, Brüggemann A, Gassmann M, *et al*. N-methyl-D-aspartate receptors in human erythroid precursor cells and in circulating red blood cells contribute to the intracellular calcium regulation. American Journal of Physiology. Cell Physiology. 2013; 305: C1123–C1138. https://doi.org/10.1152/ajpcell.00031.2013
[97] Belkacemi A, Trost CF, Tinschert R, Flormann D, Malihpour M, Wagner C, *et al*. The TRPV2 channel mediates Ca2+ influx and the Δ9-THC-dependent decrease in osmotic fragility in red blood cells. Haematologica. 2021; 106: 2246–2250. https://doi.org/10.3324/haematol.2020.274951
[98] Flormann D, Qiao M, Murciano N, Iacono G, Darras A, Hof S, *et al*. Transient receptor potential channel vanilloid type 2 in red cells of cannabis consumer. American Journal of Hematology. 2022; 97: E180–E183. https://doi.org/10.1002/ajh.26509
[99] Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, *et al*. TRPC6 contributes to the Ca(2+) leak of human erythrocytes. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology. 2008; 21: 183–192. https://doi.org/10.1159/000113760
[100] Wang J, Hertz L, Ruppenthal S, El Nemer W, Connes P, Goede JS, *et al*. Lysophosphatidic Acid-Activated Calcium Signaling Is Elevated in Red Cells from Sickle Cell Disease Patients. Cells. 2021; 10: 456. https://doi.org/10.3390/cells10020456
[101] Andrews DA, Yang L, Low PS. Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. Blood. 2002; 100: 3392–3399. https://doi.org/10.1182/blood.V100.9.3392
[102] Jansen J, Qiao M, Hertz L, Wang X, Fermo E, Zaninoni A, *et al*. Mechanistic ion channel interactions in red cells of patients with Gárdos channelopathy. Blood Advances. 2021; 5: 3303–3308. https://doi.org/10.1182/bloodadvances.2020003823
[103] Locovei S, Bao L, Dahl G. Pannexin 1 in erythrocytes: function without a gap. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103: 7655–7659. https://doi.org/10.1073/pnas.0601037103
[104] Föller M, Mahmud H, Gu S, Kucherenko Y, Gehring EM, Shumilina E, *et al*. Modulation of suicidal erythrocyte cation channels by an AMPA antagonist. Journal of Cellular and Molecular Medicine. 2009; 13: 3680–3686. https://doi.org/10.1111/j.1582-4934.2009.00745.x
[105] Petkova-Kirova P, Murciano N, Iacono G, Jansen J, Simionato G, Qiao M, *et al*. The Gárdos Channel and Piezo1 Revisited: Comparison between Reticulocytes and Mature Red Blood Cells. International Journal of Molecular Sciences. 2024; 25: 1416. https://doi.org/10.3390/ijms25031416
[106] Moroni M, Servin-Vences MR, Fleischer R, Sánchez-Carranza O, Lewin GR. Voltage gating of mechanosensitive PIEZO channels. Nature Communications. 2018; 9: 1096. https://doi.org/10.1038/s41467-018-03502-7
[107] von Lindern M, Egée S, Bianchi P, Kaestner L. The Function of Ion Channels and Membrane Potential in Red Blood Cells: Toward a Systematic Analysis of the Erythroid Channelome. Frontiers in Physiology. 2022; 13: 824478. https://doi.org/10.3389/fphys.2022.824478
[108] Hall AC, Ellory JC. Evidence for the presence of volume-sensitive KCl transport in 'young' human red cells. Biochimica et Biophysica Acta. 1986; 858: 317–320. https://doi.org/10.1016/0005-2736(86)90338-x
[109] Gibson JS, Ellory JC. K+-Cl- cotransport in vertebrate red cells. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 197–220), Springer: Berlin. 2003.
[110] Dunham PB, Ellory JC. Passive potassium transport in low potassium sheep red cells: dependence upon cell volume and chloride. The Journal of Physiology. 1981; 318: 511–530. https://doi.org/10.1113/jphysiol.1981.sp013881
[111] Lauf PK, Theg BE. A chloride dependent K+ flux induced by N-ethylmaleimide in genetically low K+ sheep and goat erythrocytes. Biochemical and Biophysical Research Communications. 1980; 92: 1422–1428. https://doi.org/10.1016/0006-291x(80)90445-3
[112] Gibson JS. Comparative physiology in red cell membrane transport. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 721–734). Springer: Berlin. 2003.
[113] Nelson GJ. Studies on the lipids of sheep red blood cells. I. Lipid composition in low and high potassium red cells. Lipids. 1967; 2: 64–71. https://doi.org/10.1007/BF02532003
[114] Halperin JA, Brugnara C, Van Ha T, Tosteson DC. Voltage-activated cation permeability in high-potassium but not low-potassium red blood cells. The American Journal of Physiology. 1990; 258: C1169–C1172. https://doi.org/10.1152/ajpcell.1990.258.6.C1169
[115] Erdmann A, Bernhardt I, Pittman SJ, Ellory JC. Low potassium-type but not high potassium-type sheep red blood cells show passive K+ transport induced by low ionic strength. Biochimica et Biophysica Acta. 1991; 1061: 85–88. https://doi.org/10.1016/0005-2736(91)90271-9
[116] Parker JC. Passive calcium movements in dog red blood cells: anion effects. The American Journal of Physiology. 1983; 244: C318–C323. https://doi.org/10.1152/ajpcell.1983.244.5.C318
[117] Parker JC. Urea alters set point volume for K-Cl cotransport, Na-H exchange, and Ca-Na exchange in dog red blood cells. The American Journal of Physiology. 1993; 265: C447–C452. https://doi.org/10.1152/ajpcell.1993.265.2.C447
[118] Romualdez A, Sha'afi RI, Lange Y, Solomon AK. Cation transport in dog red cells. The Journal of General Physiology. 1972; 60: 46–57. https://doi.org/10.1085/jgp.60.1.46
[119] Willis JS, Ellory JC. Ouabain sensitivity: Diversities and disparities. In Hoffman JF, Forbush III B (eds.) Current topics in membranes and transport (pp. 277–280). Academic Press: New York. 1983.
[120] Ihrig I, Schönheit C, Häussner W, Bernhardt I. Characterisation

of the potassium influx in rat erythrocytes. General Physiology and Biophysics. 1992; 11: 377–388.
[121] Ban A, Sakai J, Koya M, Watanabe D, Abe S, Abe S, *et al*. Genetic control of red cell band 3 deficiency in Japanese black cattle. Journal of the Japan Veterinarary Medical Association. 1999; 52: 85–89.
[122] Sandermann H, Jr. Regulation of membrane enzymes by lipids. Biochimica et Biophysica Acta. 1978; 515: 209–237. https://doi.org/10.1016/0304-4157(78)90015-1
[123] Whitelegge J. Lipid Modulation of Membrane Protein Function. Cell Chemical Biology. 2018; 25: 803–804. https://doi.org/10.1016/j.chembiol.2018.07.003
[124] Fratti RA. Editorial: Effects of Membrane Lipids on Protein Function. Frontiers in Cell and Developmental Biology. 2021; 9: 675264. https://doi.org/10.3389/fcell.2021.675264
[125] Habeck M, Haviv H, Katz A, Kapri-Pardes E, Ayciriex S, Shevchenko A, *et al*. Stimulation, inhibition, or stabilization of Na,K-ATPase caused by specific lipid interactions at distinct sites. The Journal of Biological Chemistry. 2015; 290: 4829–4842. https://doi.org/10.1074/jbc.M114.611384
[126] Cornelius F, Habeck M, Kanai R, Toyoshima C, Karlish SJD. General and specific lipid-protein interactions in Na,K-ATPase. Biochimica et Biophysica Acta. 2015; 1848: 1729–1743. https://doi.org/10.1016/j.bbamem.2015.03.012
[127] Kuypers FA, Roelofsen B, Op den Kamp JA, Van Deenen LL. The membrane of intact human erythrocytes tolerates only limited changes in the fatty acid composition of its phosphatidylcholine. Biochimica et Biophysica Acta. 1984; 769: 337–347. https://doi.org/10.1016/0005-2736(84)90315-8
[128] Bernhardt I. Untersuchungen zur Regulation des Ouabain-insensitiven Membrantransports monovalenter Kationen an Erythrozyten [doctoral thesis]. Humboldt-University: Berlin. 1986. (In German)
[129] Pomorski T, Hrafnsdóttir S, Devaux PF, van Meer G. Lipid distribution and transport across cellular membranes. Seminars in Cell & Developmental Biology. 2001; 12: 139–148. https://doi.org/10.1006/scdb.2000.0231
[130] Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, *et al*. Regulation of phosphatidylserine exposure in red blood cells. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology. 2011; 28: 847–856. https://doi.org/10.1159/000335798
[131] Horn NM, Thomas AL, Oakley F. Trace metal transport. In Bernhardt I, Ellory JC (eds.) Red cell membrane transport in health and disease (pp. 435–450). Springer: Berlin. 2003.
[132] Davson H. Studies on the permeability of erythrocytes: The effect of reducing the salt content of the medium surrounding the cell. The Biochemical Journal. 1939; 33: 389–401. https://doi.org/10.1042/bj0330389
[133] Wilbrandt W. Die Ionenenpermeabilität der Erythrozyten in Nichtleiterlösungen. Pflügers Archiv. 1940; 242: 537–556. (In German) https://doi.org/10.1007/BF01751111
[134] Wilbrandt W, Schatzmann HJ. Changes in the passive cation permeability of erythrocytes in low electrolyte media. Ciba Foundation Study Group. 1960; 5: 34–52.
[135] LaCelle PL, Rothsteto A. The passive permeability of the red blood cell in cations. The Journal of General Physiology. 1966; 50: 171–188. https://doi.org/10.1085/jgp.50.1.171
[136] Donlon JA, Rothstein A. The cation permeability of erythrocytes in low ionic strength media of various tonicities. The Journal of Membrane Biology. 1969; 1: 37–52. https://doi.org/10.1007/BF01869773
[137] Richter S, Hamann J, Kummerow D, Bernhardt I. The monovalent cation “leak” transport in human erythrocytes: an electroneutral exchange process. Biophysical Journal. 1997; 73: 733–745. https://doi.org/10.1016/S0006-3495(97)78106-2
[138] Kummerow D, Hamann J, Browning JA, Wilkins R, Ellory JC, Bernhardt I. Variations of intracellular pH in human erythrocytes via K(+)(Na(+))/H(+) exchange under low ionic strength conditions. The Journal of Membrane Biology. 2000; 176: 207–216. https://doi.org/10.1007/s00232001089
[139] Bernhardt I, Hall AC, Ellory JC. Effects of low ionic strength media on passive human red cell monovalent cation transport. The Journal of Physiology. 1991; 434: 489–506. https://doi.org/10.1113/jphysiol.1991.sp018482
[140] Goldman DE. Potential, impedance, and rectification in membranes. The Journal of General Physiology. 1943; 27: 37–60. https://doi.org/10.1085/jgp.27.1.37
[141] Bernhardt I, Erdmann A, Glaser R, Reichmann G, Bleiber R. Influence of lipid composition on passive ion transport of erythrocytes. In Klein R, Schmitz B (eds.) Topics in lipid research (pp. 243–248). Royal Society of Chemistry: London. 1986.
[142] Erdmann A, Bernhardt I, Herrmann A, Glaser R. Species-dependent differences in the influence of ionic strength on potassium transport of erythrocytes. The role of membrane fluidity and Ca2+. General Physiology and Biophysics. 1990; 9: 577–588.
[143] Bernhardt I, Seidler G, Ihrig I, Erdmann A. Species-dependent differences in the effect of ionic strength on potassium transport of erythrocytes: the role of lipid composition. General Physiology and Biophysics. 1992; 11: 287–299.
[144] Fievet B, Guizouarn H, Pellissier B, Garcia-Romeu F, Motais R. Evidence for a K(+)-H+ exchange in trout red blood cells. The Journal of Physiology. 1993; 462: 597–607. https://doi.org/10.1113/jphysiol.1993.sp019571
[145] Winkelmann I. Structure, mechanisms, and regulation of sodium/proton exchangers [doctoral thesis]. Stockholm University: Sweden. 2021.
[146] Lawrence SP, Bright NA, Luzio JP, Bowers K. The sodium/proton exchanger NHE8 regulates late endosomal morphology and function. Molecular Biology of the Cell. 2010; 21: 3540–3551. https://doi.org/10.1091/mbc.E09-12-1053
[147] Hill JK, Brett CL, Chyou A, Kallay LM, Sakaguchi M, Rao R, *et al*. Vestibular hair bundles control pH with (Na+, K+)/H+ exchangers NHE6 and NHE9. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2006; 26: 9944–9955. https://doi.org/10.1523/JNEUROSCI.2990-06.2006
[148] Winklemann I, Matsuoka R, Meier PF, Shutin D, Zhang C, Orellana L, *et al*. Structure and elevator mechanism of the mammalian sodium/proton exchanger NHE9. The EMBO Journal. 2020; 39: e105908. https://doi.org/10.15252/embj.2020105908
[149] Kaestner L. Cation channels in erythrocytes - historical and future perspective. Open Biology Journal. 2011; 4: 27–34.
[150] Bernhardt L, Bogdanova A, Egee S. Calcium Channels and Calcium-Regulated Channels in Human Red Blood Cells. Advances in Experimental Medicine and Biology. 2020; 1131: 625–648. https://doi.org/10.1007/978-3-030-12457-1_25
[151] Rotordam MG, Fermo E, Becker N, Barcellini W, Brüggemann A, Fertig N, *et al*. A novel gain-of-function mutation of Piezo1 is functionally affirmed in red blood cells by high-throughput patch clamp. Haematologica. 2019; 104: e179–e183. https://doi.org/10.3324/haematol.2018.201160