Oxygenation-deoxygenation cycle of erythrocytes modulates submicron cell membrane fluctuations

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ABSTRACT Low frequency submicron fluctuations of the cell membrane were recently shown to be characteristic for different cell types, nevertheless their physiological role is yet unknown. Point dark-field microscopy based recordings of these local displacements of cell membrane in human erythrocytes, subjected to cyclic oxygenation and deoxygenation, reveals a reversible decrease of displacement amplitudes from 290 ± 49 to 160 ± 32 nm, respectively. A higher rate of RBC adhesion to a glass substratum is observed upon deoxygenation, probably due to a low level of fluctuation amplitudes. The variation in the amplitude of these displacements were reconstituted in open RBC ghosts by perfusing them with composite solutions of 2,3 diphosphoglycerate, Mg^{+2}, and MgATP, which mimic the intracellular metabolite concentrations in oxygenated and deoxygenated erythrocytes. The mere change in intracellular Mg^{+2} during oxygenation-deoxygenation cycle is sufficient to explain these findings. The results imply that the magnitude of fluctuations amplitude is directly connected with cell deformability. This study suggests that the physiological cycle of oxygenation-deoxygenation provides a dynamic control of the bending deformability and adhesiveness characteristics of the RBC via a Mg^{+2}-dependent reversible assembly of membrane-skeleton proteins. The existing coupling between oxygenation-deoxygenation of the RBC and its mechanical properties is expected to play a key role in blood microcirculation and may constitute an example of a general situation for other circulating blood cells, where the metabolic control of cytoskeleton dynamics may modulate their dynamic mechanical properties.

INTRODUCTION

Microdisplacements of the cell membrane, possessing amplitudes of 20–250 nm in the frequency range of 0.2–20 Hz, were recently observed in red blood cells (RBC) (1, 4) and in several nucleated cells (1, 2). The detection of these cell membrane fluctuations (CMF) in various types of cells suggests that this phenomenon may be a general property of the living cell. CMF involve local ($\leq 0.25\ \mu^2$) bending deformability of the cell membrane. The primary mode of RBC deformation in narrow blood capillaries is a folding or bending about the longitudinal axis of the capillary (3). In view of the large amplitude of CMF and their occurrence in regions of high positive curvature of the cell membrane, it is expected that membrane fluctuations are driven not only by thermal energy. Indeed, we have recently shown that a major underlying mechanism of these cell membrane movements in RBC is a MgATP dependent, mechanochemical dynamic assembly of membrane-skeleton proteins (4). In an attempt to examine the physiological significance of this phenomenon in erythrocytes, we have now investigated the effect of oxygenation-deoxygenation cycle on cell membrane displacements of RBC.

MATERIALS AND METHODS

Preparation of RBC and RBC saponin ghosts

Human erythrocytes, obtained immediately before experiment from a healthy donor and washed twice by PBS solution (130 mM NaCl, 10 mM glucose, 5.5 mM phosphate buffer, pH 7.4, and 1 mg/ml BSA), were introduced into the experimental chamber at a low density so that the volume ratio of cells:solution in the chamber was 1:3,000.

Deoxygenated red blood cells were obtained by bubbling N_2 through a suspension of red blood cells in a closed box for 5–25 min and then placing them in a chamber that was resealed in the closed box. The reoxygenation was carried out by opening the chamber to air and perfusing it by PBS. The state of deoxygenation of the erythrocytes in the closed chamber was monitored by measuring absorption spectral changes in the 450–650 nm range in a separate control chamber, prepared in the same box in parallel to the experimental chamber. The state of deoxygenation was maintained in these chambers for 1–1.5 h.

Preparation of saponin ghosts was carried out by a similar procedure to the stage where RBC were attached to the cover glass, in the experimental chamber containing a solution of 1 mg/ml BSA in PBS. This was followed by hemolysis of the RBC by perfusing the chamber with 50 μl of 0.0075% saponin in PBS solution for 1 min and then washing out the saponin by 100 μl of "KCl solution" (130 mM KCl, 50 mM K-Hepes, 10 mM glucose, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml BSA, pH 7.8).

In order to mimic the main features of the metabolic composition of oxygenated and deoxygenated RBC in saponin ghosts we perfused them with two different solutions. The first solution consisted of a "KCl solution" in the presence of 1.2 mM MgATP, 6.1 mM DPG, 0.4 mM MgDPG, and 0.15 mM Mg^{+2} ("oxygenated solution"), which mimics the metabolite composition of the oxygenated RBC (9, 11). The second solution consisted of a "KCl solution" in the 1.2 mM MgATP, 0.75 mM DPG, 0.15 mM MgDPG and 0.6 mM Mg^{+2} ("deoxygenated solution") which mimics the metabolite composition of the deoxygenated RBC (9–11).

Measurement of cell membrane fluctuations and spectral analysis

Cell membrane fluctuations were measured by a novel optical method based on point dark field microscopy (1, 2, 4). Human RBC were introduced into an experimental chamber, consisting of two coverglasses separated by a distance of 0.2 mm. Incubation for 20–30 min in the chamber at 24–27°C allowed the cells to attach to the cover glass. A very small area (0.25 μm^2) at the cell edge was illuminated and the time-dependent intensity changes of the reflected and scattered light

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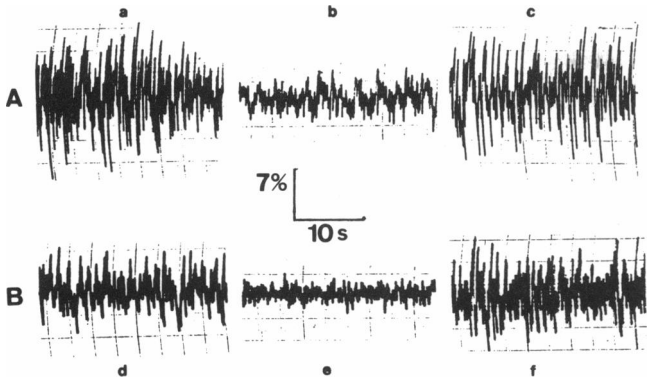

FIGURE 1 Changes of cell membrane fluctuations in RBC during oxygenation-deoxygenation cycle and in RBC saponin ghosts perfused with solutions that mimic metabolite changes during this cycle. Recordings of membrane fluctuations measured on: human red blood cells (*A*) in oxygenated (*a*), deoxygenated (*b*), and reoxygenated (*c*) states as well as in saponin ghosts (*B*) of red blood cells perfused with 2,3 diphosphoglycerate, Mg^{+2}, and MgATP solutions that mimic the oxygenated and deoxygenated states. (*d*) cell membrane fluctuations after perfusion of saponin ghosts with "oxygenated solution"; (*e*) cell membrane fluctuations following perfusion of saponin ghosts with "deoxygenated solution"; (*f*) cell membrane fluctuations of reperfused saponin ghosts with "oxygenated solution" after being exposed to a "deoxygenated solution".

were recorded. The fluctuation of the light intensity depends on the changes of the membrane area position moving in and out of the focused light spot near the cell edge. A linear dependence between the relative change in the scattered and reflected light ($\delta I/I$) from the cell surface and the amplitude of the cell edge displacement was achieved by moving the coverglass, with attached glutaraldehyde fixed cells, by a calibrated vibrator (1). Linearity of $\delta I/I$ with displacement was observed over distances as long as 340 nm. A relative change in light scattering of 1% corresponds to a cell membrane displacement of ~17 nm (1). The sensitivity of the experimental set-up was ~1% (17 nm). All measurements were carried out at 24–27°C. Fast Fourier transformation (FFT) of light scattering fluctuations due to cell membrane displacements, in the frequency range 0.5–18.0 Hz, was followed by squaring the displacement amplitude at each frequency (at 0.02 Hz resolution). After averaging the squared amplitude over 0.2 Hz intervals, the spectra were normalized, in terms of squared amplitude, at 0.5 Hz.

Adhesion kinetics

RBC suspension in PBS was placed in a camera consisting of two coverglasses separated by a distance of 100 μm and the cells were allowed to sediment. At different time intervals the camera was turned upside-down and analyzed after additional 40 min. The number of cells that remained adhered to the upper glass as well as the cells that sedimented to the lower glass, at the different incubation times, were counted. Thus, the percentage of adhered cells was calculated for each time point. For the initial time points the percentage of adhered cells was corrected by multiplying it by the sedimentation factor due to incomplete sedimentation at the initial time points.

RESULTS AND DISCUSSION

Measurements of cell membrane fluctuations (CMF) were carried out on single human RBC and their corresponding saponin ghosts by our recently developed point dark-field microscopy (1, 2, 4). Oxygenated RBC reveal (Fig. 1 *a*) CMF with amplitude of displacements of 290 ± 49 nm (mean ± SD) (n = 131). Deoxygenation results in an decrease of the fluctuation amplitude up to 160 ± 32 nm (n = 92) (Fig. 1 *b*). When deoxygenated cells (revealing a small amplitude of displacement) were reoxygenated, their amplitude of fluctuation was restored to the level of 268 ± 63 nm (n = 37) (Fig. 1 *c*). Thus, a cyclic change of oxygenation-deoxygenation is accompanied by reversible amplitude changes of cell membrane fluctuations. Under deoxygenation-reoxygenation conditions, no shape change of the RBC was detected by phase contrast microscopy, ruling out the possibility that changes in membrane shape may be responsible for the observed change in fluctuations.

The normalized power spectra of membrane displacement fluctuations of the oxygenated and deoxygenated RBC are shown in Fig. 2. The power spectra of oxygenated RBC can be fitted to a function of the type: $y = a/f^b$ (curve), where f is frequency, a and b are constants (a = 0.485 ± 0.004 [mean ± SD] and b = 1.40 ± 0.01). The normalized power spectra of cell membrane displace-

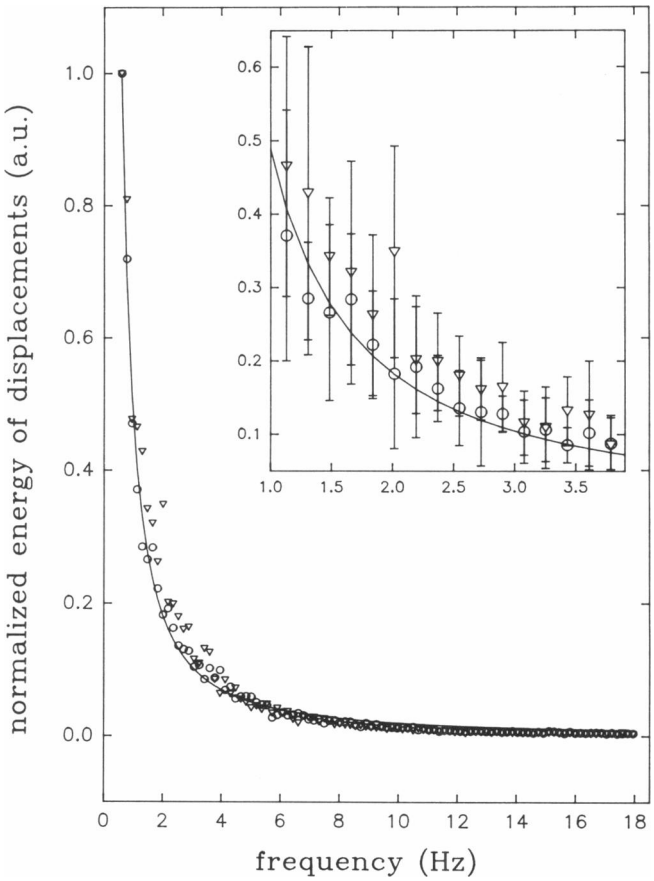

FIGURE 2 Normalized power spectra of cell membrane displacements in oxygenated (*open circle*) and deoxygenated (*open triangle*) red blood cells. Averaging of the normalized power spectra of oxygenated (n = 6) and deoxygenated (n = 7) RBC yields the final power spectra shown in this figure. The spectra were normalized in terms of squared amplitude at 0.5 Hz. (*Inset*) Normalized expanded power spectra in the frequency range of 1.0–4.0 Hz, where the bars represent the SD of each data point.

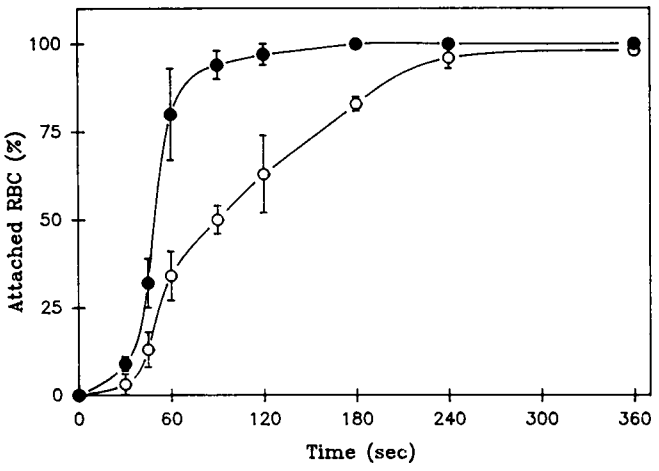

FIGURE 3 Different adherence kinetics of red blood cells in oxygenated (*open circle*) and deoxygenated (*closed circle*) states to a cover glass.

ments in oxygenated (*open circle*) and deoxygenated (*open triangle*) RBC are not statistically different except at 2.0 and 3.4 Hz (see *inset*), where the normalized squared displacements are, respectively, 2-fold ($P = 0.03$ by a Student's t test) and 1.5-fold ($P = 0.04$) higher for the deoxygenated state. A similar power spectrum of oxygenated RBCs was obtained previously (5) for thermal fluctuations of RBC thickness at the center of the RBC, where the membrane curvature is low. However, our investigation of membrane displacements at the edge of the RBC, where the membrane curvature is high (≈ 1 μm^{-1}), shows that these submicron membrane displacements are MgATP driven (4).

We suggest that the occurrence of metabolically driven CMF on the side surface of the RBC increases the bending deformability of the RBC and enables it to fold more efficiently upon entrance into blood capillaries. The higher fluctuation amplitudes of the oxygenated RBC, in comparison with the deoxygenated ones, is expected to be accompanied by a higher deformability of the oxygenated erythrocytes. Indeed, we have recently established a positive correlation between the maximal amplitude of membrane displacement and filterability of RBCs through a 5-μm pore filter (14).

An additional consequence of the change in the amplitudes of CMF, during the oxygenation-deoxygenation cycle, is its effect on RBC adhesion to a substrate. Stationary adhesion measurements of RBC to a glass substratum were previously reported (6). Examination of stationary adhesion to a coverglass shows a complete adhesion for both the oxygenated and deoxygenated RBC. However, the adhesion kinetics of RBC to a cover glass substratum shows a marked difference between the oxygenated and deoxygenated states (Fig. 3). In deoxygenated RBC 50% of the cell population is attached to the cover-glass within 47 s, whereas 50% of the oxygenated cells are attached only within 90 s. The change of adhesion kinetics may be related to the variation of membrane fluctuation amplitudes through the possible modulation of the repulsive forces, between the cell membrane and substratum (7, 8). Thus, oxygenation of RBC, which is accompanied by slower adhesion kinetics of erythrocytes, is expected to reduce the attachment of the RBC to the surface of the vessel's wall, thereby increasing the efficiency of the RBC to pass through blood capillaries.

In intact RBC the effect of oxygenation-deoxygenation on CMF may be attributed to a variation in the intracellular concentration of metabolites during the oxygenation-deoxygenation cycle. The oxygenation state of hemoglobin is a major effector of the intracellular levels of 2,3 diphosphoglycerate (DPG) and Mg^{+2} (9–11), which may be responsible for the modulation of the membrane fluctuations. To evaluate this possibility, we have examined the effects of 2,3 DPG and Mg^{+2} on the displacement amplitudes in RBC ghosts. We have chosen the characteristic concentrations of 2,3 DPG and Mg^{+2} for the oxygenated and the deoxygenated states of the RBC (9–11).

Nonresealable saponin ghosts perfused with a "KCl solution" reveal a basal fluctuation of 110 ± 32 nm ($n = 40$), 2.3-fold lower than the observed displacement level of 250 ± 76 nm ($n = 32$) in the corresponding intact RBC. However, the fluctuation amplitudes were reconstituted to a level of 185 ± 59 nm ($n = 82$) by perfusing the ghosts with a solution, whose composition of metabolites mimics the oxygenated state of RBC (Fig. 1 *d*). When the same ghosts were perfused again with a composite solution of metabolites which mimics the deoxygenated state of RBC (Fig. 1 *e*), the fluctuation amplitude decreased to a level of 116 ± 29 nm ($n = 54$), ($P < 0.01$). Furthermore, when the same ghosts were subjected to an additional set of perfusions with the "oxygenated" (Fig. 1 *f*) and "deoxygenated" composite solutions, the fluctuation level at first increased to 151 ± 36 nm ($n = 35$; $P < 0.01$) and then decreased to a level of 104 ± 25 nm ($n = 27$) ($P < 0.01$), correspondingly.

The results show that the observed changes in membrane fluctuations may be fully accounted for by change in concentration of 2,3 DPG and Mg^{+2} during the oxygenation-deoxygenation cycle of RBC. Moreover, they demonstrate that cyclic changes in metabolite concentrations leads to reversible changes in the amplitude of displacement of the cell membrane. The MgATP level does not change much between the oxygenated and the deoxygenated states (9). In an attempt to evaluate whether a single metabolite is dominantly influencing the fluctuation amplitude, we have therefore examined separately the effects of DPG and Mg^{+2} in RBC ghosts. DPG alone (6 mM in "KCl solution") had no effect on the amplitude of fluctuations as compared with the amplitudes in the "KCl solution" (Table 1). Mg^{+2} alone had a bimodal effect on displacement amplitudes: upon increasing Mg^{+2} concentration from 0.1 to 0.4 mM, the amplitude of fluctuation increased steeply to a maximal

TABLE 1 **The effect of Mg^{+2} and 2,3-DPG on displacement amplitudes (nm) in open, nonresealable, saponin RBC ghosts**

Treatment	Displacement amplitudes (nm)
	mean ± SD
RBC	214 ± 39 (n = 24)
KCl (after saponin)	87 ± 27 (n = 22)
6 mM DPG	92 ± 26 (n = 26)
0.1 mM Mg^{+2}	104 ± 27 (n = 45)
0.25 mM Mg^{+2}	179 ± 43 (n = 47)
0.3 mM Mg^{+2}	216 ± 39 (n = 31)
0.4 mM Mg^{+2}	188 ± 46 (n = 40)
0.6 mM Mg^{+2}	110 ± 34 (n = 41)
0.75 mM Mg^{+2}	104 ± 24 (n = 37)
1.0 mM Mg^{+2}	102 ± 17 (n = 38)

Between measurement ghosts were washed by "KCl solution". n is the total number of cells studied, where 6–8 cells were examined in each experiment. The experiments are given in a sequential order.

level of 188 ± 46 nm (n = 40) (Table 1), but higher concentrations of Mg^{+2} resulted in a decrease of the displacement amplitudes to the basal level of 104 ± 24 nm (n = 37). The highest level of fluctuation was observed at 0.3 mM Mg^{+2}. Hence, the variation of intracellular Mg^{+2} during the oxygenation-deoxygenation cycle is suggested to be a major factor affecting cell membrane displacements.

Mg^{+2} is known to be required for the association of tropomyosin with actin (12). Therefore, under deoxygenating conditions where free Mg^{+2} is high (0.5–0.75 mM) (10, 11), tropomyosin is expected to be associated with actin. However, in the fully oxygenated state of the RBC, where Mg^{+2} concentration is decreased, tropomyosin would not be able to bind actin (13). It may be expected that increased binding of tropomyosin to actin will lead to the increase of the mechanical stability of the membrane skeleton with a consequent decrease of cell-membrane fluctuations. Indeed, a decrease in fluctuation amplitudes is observed in saponin ghosts at Mg^{+2} concentrations higher or equal to 0.6 mM Mg^{+2}. This range of Mg^{+2} concentration coincides with concentration of Mg^{+2} at which tropomyosin binds to F-actin from muscle (13). It may be speculated that the two observed peaks in the normalized power spectrum of the deoxygenated RBC at 2.0 and 3.4 Hz (Fig. 2, *inset*) reflect two apparent characteristic relaxation times of $\approx$80 and $\approx$50 ms for the Mg^{+2}-dependent binding of tropomyosin to actin in the RBC. The stimulation of cell-membrane fluctuation by Mg^{+2} in the range of 0.25–0.3 mM (Table 1) probably reflects a different, yet unidentified, binding site for Mg^{+2}.

We suggest that the physiological process of oxygenation-deoxygenation provides a dynamic control of the bending deformability and adhesiveness characteristics of the RBC via a Mg^{+2}-dependent mechanism. The existing coupling between oxygenation-deoxygenation of the RBC and its mechanical properties is expected to play an important role in blood microcirculation. These results may constitute an example of a general situation for other circulating blood cells, where the metabolic control of cytoskeleton dynamics may modulate their attachment kinetics to endothelial cells as well as their deformability.

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REFERENCES

1. Krol, A. Ju., M. G. Grinfeldt, S. V. Levin, and A. D. Smilgavichus. 1990. Local mechanical oscillations of the cell surface within range 0.2–30 Hz. *Eur. Biophys. J.* 19:93–99.
2. Mittelman, L., S. Levin, and R. Korenstein. 1991. Fast cell membrane displacements in B lymphocytes: modulation by dihydrocytocalasin B and colchicine. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 293:207–210.
3. Chien, S. 1987. Red cell deformability and it's relevance to blood flow. *Annu. Rev. Physiol.* 49:177–192.
4. Levin, S., and R. Korenstein. 1991. Membrane fluctuations in erythrocytes are linked to MgATP dependent dynamic assembly of the membrane skeleton. *Biophys. J.* 60:733–737.
5. Brochard, F., and J. F. Lennon. 1975. Frequency spectrum of the flicker phenomena in erythrocytes. *J. de Physique (Paris).* 36:1035–1047.
6. Trommler, A., D. Gingell, and H. Wolf. 1985. Red blood cell experience electrostatic repulsion but make molecular adhesion with glass. *Biophys. J.* 58:835–841.
7. Helfrich, W. 1978. Steric interaction of fluid membranes in multilayer systems. *Z.f. Naturforschung.* 33A:303–315.
8. Evans, E. A., and V. A. Parsegian. 1986. Thermal-mechanical fluctuations enhance repulsion between bimolecular layers. *Proc. Natl. Acad. Sci. USA.* 83:7132–7136.
9. Petersen, A., S. R. Kristensen, K. P. Jacobsen, and M. Horder. 1990. ^{31}P-NMR measurements of ATP, ADP, 2,3-diphosphoglycerate and Mg^{2+} in human erythrocytes. *Biochim. Biophys. Acta.* 1035:165–174.
10. Flatman, P. W. 1980. The effect of buffer composition and deoxygenation on the concentration of ionized magnesium inside human red blood cells. *J. Physiol.* 300:19–30.
11. Flatman, P. W. 1988. The control of red cell magnesium. *Magnesium Res.* 1:5–11.
12. Fowler, V. M., and V. Bennett. 1984. Erythrocyte membrane tropomyosin: purification and properties. *J. Biol. Chem.* 259:5978–5989.
13. Bennett, V. 1990. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70:1029–1065.
14. Tuvia, S., S. Levin, and R. Korenstein. 1992. Correlation between local cell membrane displacements and filterability of human red blood cells. FEBS (Fed. Eur. Biochem. Soc.) Lett. In press.