

Membrane fluctuations in erythrocytes are linked to MgATP-dependent dynamic assembly of the membrane skeleton

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ABSTRACT The observation of low-frequency fluctuations of the cell membrane in erythrocytes and in several nucleated cells suggests that this phenomenon may be a general property of the living cell. A study of these fluctuations in human erythrocytes and its ghosts has now been carried out using a novel optical method based on point dark field microscopy. We have demonstrated that the reestablishment of membrane fluctuations in erythrocyte ghosts is dependent on MgATP but does not necessarily require the restoration of the biconcave shape. The results imply that the dominant component of membrane fluctuations are metabolically dependent and suggest the existence of a dynamic mechano-chemical coupling within the membrane skeleton network induced by MgATP.

INTRODUCTION

Low-frequency fluctuations (0.2–30 Hz) of the cell membrane have been previously observed in erythrocytes (1–5). The more recent finding of similar fluctuations in several nucleated cells (6) suggests that this phenomenon may be a general property of the living cell. These membrane fluctuations may affect several processes such as cell-cell and cell-substrate adhesion (7), endocytosis, stretch activated ion fluxes (8–11), or hydrolysis of phospholipids by phospholipases (12). So far it has been assumed that the sole source of these fluctuations in erythrocytes originates from thermally excited undulations of the cell membrane (1–5). This study suggests that the dominant component of cell membrane fluctuations depends on a mechanochemical dynamic assembly of the membrane skeleton induced by the presence of MgATP.

MATERIALS AND METHODS

Measurement of cell membrane fluctuations

The measurement of local mechanical fluctuations of the cell membrane was carried out on human red blood cells (RBC) and ghosts, by a novel optical method based on point dark field microscopy (6). Using cells, attached to a cover glass, we illuminate a very small area (0.25 μm^2) at the cell edge and record cell membrane displacements (6) by monitoring the time-dependent changes of light reflection and scattering. The fluctuation of the light intensity depends on the changes of the membrane area 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 ($\delta F/F$) from the cell surface and the amplitude of the cell edge displacement was achieved by moving the cover glass, with attached glutaraldehyde fixed cells, by a calibrated vibrator. Linearity of $\delta F/F$ with displacement was observed over distances as long as 300–400 nm (corresponding to $\delta F/F$ of 20–25%). The sensitivity of the experimental setup was $\sim 1\%$ (15–20 nm). Human RBC were attached in the experimental chamber to a poly-L-lysine (65.5 kD) treated cover glass in a PBS solution containing 1 mg/ml BSA. Registration of the maximal peak-to-peak fluctuations of the scattered light from the edge of a cell, ghost, or a skeleton shell in relation to the constant intensity of the scattered light was performed during 1–2 min in each case. 4–10 cells or ghosts were analyzed on each of the cover glasses. In some cases we registered fluctuations on the same ghosts in the absence and presence of MgATP. In most cases we choose cells and ghosts of similar shape and size. Usually we analyzed 3–10 independent preparations of cells and ghosts. All measurements were carried out at 25–28° C.

The fluctuation amplitudes are expressed as mean \pm SD. The significance of the difference between two samples was determined by a Student's *t*-test.

The local character of membrane displacements emerges when comparing fluctuation amplitudes from relatively large (1–2 μm^2) and small (0.25 μm^2) areas. The fluctuation amplitudes obtained from the small area were always greater than those obtained from the larger one, reflecting the confinement of membrane fluctuations to microdomains, without displacement of the whole cell.

Preparation of hypotonic, low pH, and saponin ghosts

Hypotonic ghosts

Human erythrocytes, obtained immediately before experiment from a healthy donor, were washed twice by PBS solution (130 mM NaCl, 10 mM glucose, 11 mM phosphate buffer pH 7.4, and 1 mg/ml BSA). RBC were attached in the experimental chamber to a poly-L-lysine treated cover glass in a PBS solution containing 1 mg/ml BSA. The volume ratio of cells/solution in the chamber was 1:3,000. Hypoosmotic ghosts were obtained in the chamber from the attached RBC after perfusion by a hypoosmotic solution of 1:30 diluted PBS pH 7.4. After hemolysis

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the ghosts were washed twice with 1:30 diluted PBS solution and then with PBS.

Low pH ghosts

Human RBC were attached in the experimental chamber to a poly-L-lysine treated cover glass in a PBS solution containing 1 mg/ml BSA. Hemolysis of the RBC was carried by a low pH hemolyzing solution (4 mM MgSO₄, 2.5 mM KCl, 1 mg/ml Dextran-FITC [4 kD], 0.07 mM acetic acid, and 10% hemolyzate, pH 3.9) at 4° C. The 10% (vol/vol) hemolyzate was obtained from the same blood by diluting RBC 1:10 in the hemolyzing solution and pelleting of the ghosts by centrifugation. After incubation with the hemolyzing solution for 2–5 min the solution in the chamber was replaced by incubation solution (150 mM KCl, 10 mM MgSO₄, 1 mg/ml Dextran-FITC, Tris buffer pH 7.8 and 1–2% hemolyzate). 2 mM MgATP was added to the hemolyzing and incubation solutions. Resealing of the open ghosts was carried by incubating the ghosts for 30 min at 26–28° C. Dextran-FITC was used as a marker for the complete resealing of the ghosts. After resealing, the solution in the chamber was replaced by PBS.

Saponin ghosts

Human RBC were treated by a similar procedure to the one described for the preparation of low pH ghosts to the stage where RBC were attached to the cover glass. Hemolysis of the RBC was carried by perfusing the chamber with 50 µl of 0.005–0.01% saponin in PBS for 1 min and then washing out the saponin by 100 µl of PBS.

Heat treatment of RBC

Human RBC were attached to the cover glass of the experimental chamber. Heat treatment was carried by incubating the RBC in the chamber at 50° C for 5 min.

RESULTS AND DISCUSSION

In an attempt to verify the hypothesis that membrane fluctuations are driven only by thermal energy we studied the effect of MgATP, the ubiquitous metabolic source of energy, on membrane oscillations in three types of RBC ghosts. These were prepared by hypoosmotic, low pH, or saponin treatments. The measurement of cell membrane oscillations in human RBC shows a 10–20% relative change of the scattered light intensity (corresponding to a displacement of 170–340 nm [6]). Conversion of RBC, attached to a cover glass, to open spherical ghosts after a hypoosmotic treatment, leads to the abolition of fluctuations ($\leq 1\%$). Similarly, conversion of RBC to open spherical ghosts, after low pH treatment, leads to a decrease of membrane fluctuations from $18.2 \pm 5.0\%$ ($n = 24$) down to $2.4 \pm 1.7\%$ ($n = 8$, $p < 0.01$ related to the RBC) (Fig. 1). Upon resealing these ghosts in an incubation solution devoid of MgATP, the spherical closed ghosts change their shape into echinocyte-like ones, which possess low levels of fluctuation ($4.9 \pm 3.6\%$, $n = 17$). However, resealing the ghosts in the presence of 2 mM MgATP results in

temperature and time-dependent shape transformation from echinocyte-like to flat ghosts and subsequently to biconcave ones. In parallel to these shape changes we observe an increase of the amplitude of fluctuation in the flat ghosts to $10.7 \pm 6.6\%$ ($n = 14$, $P < 0.01$ related to the echinocyte-like ghosts) and the restoration of the fluctuation to $22.9 \pm 8.8\%$ ($n = 50$, $P < 0.01$ related to flat ghosts) in the biconcave ghosts (Fig. 1). The high values of standard deviation, obtained in the various measurements seem to be independent on the size (n) of the studied sample, suggesting the existence of an inherent population heterogeneity of cells and ghosts. The above results imply an apparent coupling between incorporation of MgATP, ghost shape transformation (from a spherical to a biconcave ghost) and the restoration of the cell membrane fluctuations. MgATP-dependent shape transformations have been previously observed in RBC ghosts (13–15).

To distinguish between the effect of ghost shape transformation and the direct effect of MgATP on cell membrane fluctuations we examined fluctuations in nonresealable ghosts obtained by saponin treatment. Saponin treatment of RBC converts them into open, flat, or spherical ghosts (with pores bigger than 10 nm), leading to a decrease of fluctuation from $11.7 \pm 3.8\%$ ($n = 66$) to $4.7 \pm 2.0\%$ ($n = 27$, $P < 0.01$) (Fig. 2). On perfusing the saponin ghosts with PBS containing 2 mM MgATP, we observed the restoration of fluctuation back to $11.8 \pm 4.0\%$ ($n = 67$, $P < 0.01$ related to the saponin ghosts in the absence of MgATP) without an accompanying change in the ghosts' shape. Consequent washing out the MgATP from the ghosts led to a reduction of fluctuation to $5.8 \pm 1.7\%$ ($n = 33$, $P < 0.01$ related to saponin ghosts in the presence of MgATP). MgATP exerted its effects in the concentration range of 0.5–2 mM. Substitution of MgATP by MgGTP (2 mM) was not able to restore fluctuation in saponin ghosts. Furthermore, perfusion of the ghosts with a solution containing 2 mM MgAMP-PNP, a nonhydrolyzable analogue of ATP, did not lead to any increase in cell membrane fluctuations.

Thus the MgATP-driven restoration of fluctuation in the saponin ghosts is independent of shape transformation. Furthermore, washing of saponin ghosts with a low ionic strength (1:300 diluted PBS) solution (known to induce irreversible dissociation of cytoskeletal elements from the membrane), followed by addition of 2 mM MgATP, does not lead to the resumption of membrane fluctuations. Treatment of the saponin ghosts by Triton X-100 (4% volume per volume of saponin ghosts) yields a membrane skeleton by dissolution of the overlying membrane (16–17). Repeating this procedure, in the presence of 2 mM MgATP, leads to a time-dependent

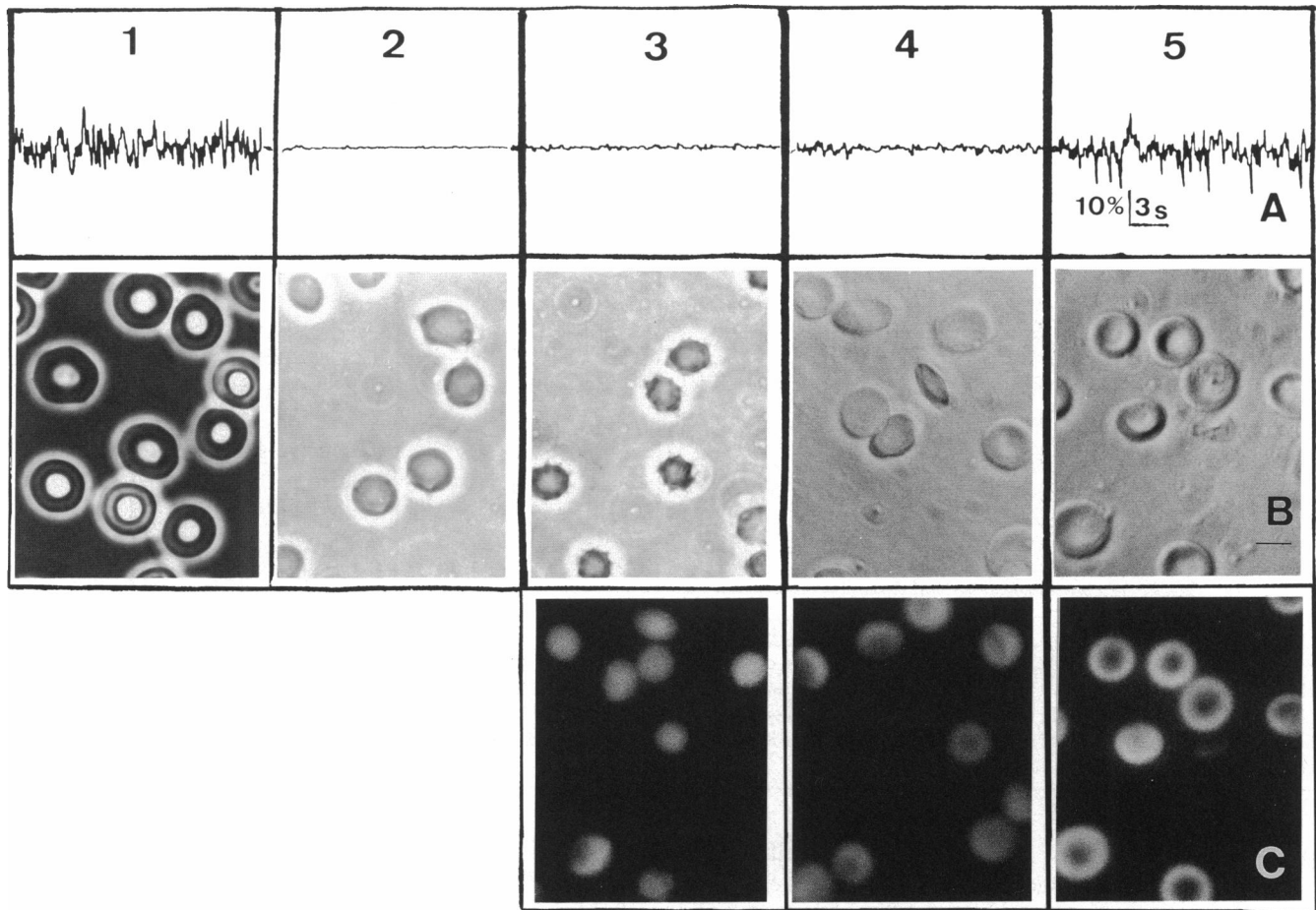


FIGURE 1 Reconstitution of cell membrane fluctuations in resealed ghosts, prepared at low pH in the presence of MgATP, coincides with membrane smoothing and transformation into flat and biconcave shape. (Row A) Fluctuations of the relative change ($\delta F/F$ %) in the intensity of reflected and scattered light, where δF is the peak-to-peak change in the intensity of light and F is the constant intensity of the reflected and scattered light. The fluctuation changes shown from left to right: (1) RBC; (2) open ghost in the hemolyzing solution containing 2 mM MgATP; (3) closed ghost in PBS after resealing in incubation solution containing 2 mM MgATP; (4) flat ghost; (5) biconcave ghost. (Row B) Phase contrast microscopy of (1) RBC and (2) to (5) ghosts. (Row C) Fluorescence of Dextran-FITC trapped in the resealed ghosts. Scale bar: 5 μm .

increase in fluctuation amplitude, before the occurrence of irreversible contraction of the skeletal network (17). Along the same lines, short heat treatment of the intact RBC, under conditions known to induce dissociation and denaturation of spectrin, leads to a threefold decrease in amplitude of fluctuations from $14.8 \pm 6.4\%$ ($n = 10$) to $5.0 \pm 4.9\%$ ($n = 37, P < 0.01$). Under these conditions, no change of cell shape is observed.

Our results indicate that the MgATP-dependent cell membrane fluctuations occur in biconcave and flat low pH ghosts, in saponin ghosts and in skeleton shells. However, in situations where local mechanical stresses exist (e.g., in spheric and echinocyte-like low pH ghosts) the amplitude of fluctuation is strongly diminished. This can be attributed to the nonlinear mechanical character-

istics of the cell membrane and the spectrin-actin network, revealed by the increase of the elastic moduli at large stresses (24). The results obtained imply the direct involvement of the membrane skeleton in the fluctuation process. Such fluctuations may reflect a MgATP-dependent, temporal association of the membrane proteins in the network junctions. These fluctuations occur in the semi-expanded state of the membrane skeleton, where expansion and contraction forces are balanced (16–18).

The present study suggests that cell membrane fluctuations are associated with a MgATP-dependent mechano-chemical dynamic assembly of the skeletal proteins. It would be of importance to investigate the existence of a similar mechano-chemical dynamic cou-

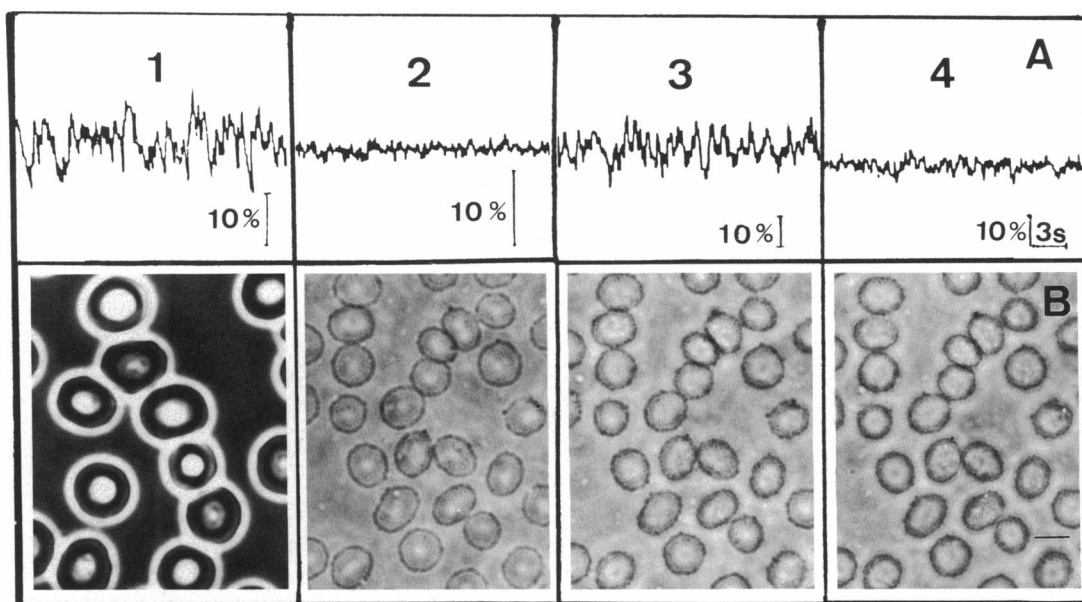


FIGURE 2 Reconstitution of cell membrane fluctuations in open, nonresealable, saponin-treated ghosts in presence of MgATP is independent of ghost shape changes. (Row A) The fluctuation changes shown from left to right: (1) RBC; (2) ghost in PBS after saponin (0.005%) treatment; (3) ghost in PBS + 2 mM MgATP; (4) ghost in PBS after washing out of MgATP. (Row B) Phase contrast microscopy of (1) RBC and (2) to (4) ghosts. Scale bar: 5 μm . A decrease of up to 15% in the ghosts' size is seen upon the MgATP addition. After washing out of MgATP the ghosts' size is not changed.

pling in nucleated cells by optical (6) and cell-poking (19) methods. The foregoing observations share a common feature with the dynamic instability of microtubule growth (20–22) and form an experimental data base for a broader area dealing with the role of oscillating dynamics in cellular functions (23).

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