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Kinetics of erythrocyte swelling and membrane hole formation in hypotonic media

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Abstract

Red blood cell (RBC) swelling and membrane hole formation in hypotonic external media were studied by measuring the time-dependent capacitance, C, and the conductance, G, in the beginning of the β-dispersion range. At high and moderate osmolarities of the external solution the capacitance reaches a steady-state whereas at low osmolarities it reveals a biphasic kinetics. Examination of RBC suspensions exposed to different concentrations of $HgCl_2$ demonstrates that water transport through mercury-sensitive water channel controls RBC swelling. Unlike the capacitance, an increase in the conductance to a stationary level is observed after a certain delay. A comparison of $G(t)$ curves recorded for the suspensions of the intact cells and those treated with cytochalasin B or glutaraldehyde demonstrates the significant effect of the membrane viscoelasticity on the pore formation. It is shown that the stretched membrane of completely swollen RBC retains its integrity for a certain time, termed as the membrane lifetime, t_{memb}. Therefore, the resistivity of RBCs to a certain osmotic shock may be quantified by the distribution function of RBC(t_{memb}).

Keywords: Red blood cell; Lysis; Hypotonic medium; Capacitance/conductance

1. Introduction

Assessments of the osmotic fragility, defined as the sensitivity of RBCs to the osmotic shock, are widely used to elucidate mechanisms of ionic and molecular transport across the plasma membrane and for diagnosis of certain hematological diseases [1]. Experimentally, the conventional osmotic fragility test consists of measuring the intensity of light transmitted through the suspension of RBCs in hypotonic external media. It is assumed that at $\lambda = 540$ nm, where measurements are usually performed, only Hb contributes to the light absorption. In accordance with experimentally confirmed theory [2] suggested for turbid media with large light absorbing particles [3,4], the expression for the time-dependent intensity of light, $I(t)$, transmitted through dilute RBC suspensions, where the multiple light scattering may be ignored, is written as follows:

$$I(t)/I_o = \exp\{-\varepsilon_{Hb} \bullet \{Hb_{ext}](t) \bullet l\} +$$

Abbreviations: RBC, red blood cell; EDL, electrode double layer; IR, infrared; PBS, phosphate-buffered saline; GA, glutaraldehyde; LIS, low ionic strength solution

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$$\exp\{-l\bullet\mathrm{Hct}\bullet[\varepsilon_{\mathrm{Hb}}\bullet[\mathrm{Hb}_{\mathrm{cyt}}](t) + K[\langle r_{RBC}(t)\rangle/\lambda\,,$$

$$n_{\mathrm{cyt}}(t)/n_{\mathrm{ext}}(t)]\bullet\pi\langle r_{\mathrm{RBC}}(t)\rangle^2]\bullet\mathrm{Hct}\} \quad (1)$$

where $\varepsilon_{\mathrm{Hb}}$, l, K, $\langle r_{\mathrm{RBC}}\rangle$, n and Hct are the extinction coefficient of Hb, the optical length, the scattering coefficient, the mean effective radius of RBCs, the refractive index and the volume fraction of the cells termed hematocrit for blood and RBC suspensions, respectively; subscripts ext and cyt stand for the external medium and cytosol.

The first exponential term in the right-hand side of this expression is due to the light absorption by the external Hb; the first and the second addend in braces of the second exponential term represent the attenuation of the incident light by the absorption of the intracellular Hb and by RBC scattering, respectively. Obviously this equation which includes two unknown variable parameters (i.e., $\langle r_{\mathrm{RBC}}\rangle$ and $\mathrm{Hb}_{\mathrm{ext}}$)[1] has no solution. However, since $I(t)$ is usually measured at the maximum of the Hb absorption band, it is believed that the light attenuation is predominantly caused by the Hb absorption. Indeed, if the scattering term in Eq. 1 is omitted, this equation may be used to extract the extra- (or intra-)cellular Hb concentration from the transmittance. Unfortunately, this assumption which is usually taken for granted, is at least disputable. In contrast to the Raleigh scattering, the intensity of light scattered by particles larger than the wavelength of the incident light depends weakly on the wavelength in the visible region [5]. This means that except for suspensions of erythrocyte ghosts in which $n_{\mathrm{cyt}} \approx n_{\mathrm{ext}}$, the scattering term cannot be omitted even if measurements are performed at the maximum of the Hb absorption band. The experimental data [6] provide direct evidence for this conclusion.

To circumvent the problem associated with the need to consider both light scattering and absorption, it was recently suggested to monitor RBC lysis by measuring the light intensity in the IR region ($\lambda = 808$ nm) [7] where the light absorption by Hb is negligible and therefore lysis-induced changes in the transmittance are due only to light scattering. In order to distinguish between changes in RBC refractive index cased by the swelling and Hb leakage, it was tentatively assumed that the rate of swelling and the time which is required to form hemolytic holes are the same for all RBCs in a population. These assumptions, however, do not seem realistic. Thus, it may be concluded that the optical approach fails to distinguish between different stages of RBC transformations in hypo(hyper)tonic media.

Interpretation of experimental results obtained by spectrophoto(nephelo)metry faces also another problem. In the general case, the time-dependent intensity of light transmitted through RBC suspension is affected by the following processes: (a) the swelling of RBCs, (b) the hole formation, (c) the intracellular Hb diffusion to the membrane hole(s), (d) the passage of Hb through the hemolytic hole(s) and (e) the Hb diffusion in the extracellular medium. The light intensity changes until Hb reaches the steady-state homogeneous distribution in the external medium. The time required to reach the steady-state Hb distribution is therefore:

$$t^{-1} = t_{\mathrm{swel}}^{-1} + t_{\mathrm{hole}}^{-1} + t_{\mathrm{inter}}^{-1} + t_{\mathrm{pas}}^{-1} + t_{\mathrm{ext}}^{-1} \quad (2)$$

where subscripts swel, hole and pas denote RBC swelling, the hole formation and the Hb transit through the hole, respectively.

The kinetics of the transmittance reflect either the swelling or the Hb leakage or a superposition of these processes provided that t_{swel}, t_{pas}, $t_{\mathrm{hole}} \gg t_{\mathrm{inter}}$, t_{ext}. Since the osmotic fragility of RBCs is determined in quiescent suspensions with $\mathrm{Hct} \approx 2\times10^{-3}$–$4\times10^{-3}$ v/v [1], the mean intermembrane separation, l, in these dilute suspensions varies from 2.0×10^{-3} to 2.5×10^{-3} cm. The value of t_{ext} is calculated applying the Einstein relation: $t_{\mathrm{ext}} = 2\times^2/D_{\mathrm{Hb}}$, where $x = l/2$ and D_{Hb} is the diffusion coefficient of Hb. Inserting in this relation the calculated values of l and a realistic value of D_{Hb} (10^{-8} cm^2/s) yields $t_{\mathrm{ext}} \approx 13$–$20$ min, whereas the experimental observations show that "lysis is virtually complete at the end of 30 min" [1]. The following question arises: 'Does a time-dependent intensity of light transmitted through

[1] If $[\mathrm{Hb}]_{\mathrm{ext}}$ (or $[\mathrm{Hb}_{\mathrm{cyt}}]$) are known and assuming that the external medium and cytosol may be treated as aqueous Hb solutions, values of n_{ext} and n_{cyt} may be calculated with the use of the empirical Gladstone–Dale formula which relates the refractive index of a molecular solution to its chemical composition.

quiescent, dilute RBC suspension reflect solely the Hb diffusion in the continuous medium?'

The main conclusion from this analysis is that optical monitoring can hardly provide reliable information about RBC lysis. It thus becomes clear that other methodological approaches are required to investigate the osmotic fragility of RBCs.

Since dielectric spectra depend on RBC morphology and the conductivities of cytosol, RBC membrane and the external medium, it seemed quite reasonable to check whether dielectroscopy or its simplified versions may provide more adequate information about RBC lysis. The main advantage of this approach is that it is capable of differentiating between RBC swelling in hypotonic media and the lysis-induced hole formation. Furthermore, unlike optical methods, the problems associated with the diffusion of the intracellular content are no longer important.

If measurements are performed for the parallel circuit mode, the conductance and the capacitance of RBC suspensions relate to the components of the equivalent electrical network of the suspension (Fig. 1) as follows:

$$G = \{R_{cyt} + R_{memb}/[1 + \omega C_{memb} R_{memb})^2]\}^{-1} + R_{ext}^{-1} \quad (3)$$

$$C = [1 + (\omega C_{memb} R_{memb})^2]/\omega^2 C_{memb} R_{memb}^2 \quad (4)$$

where ω is the circular frequency.

Investigations of the RBC lysis at a relatively high

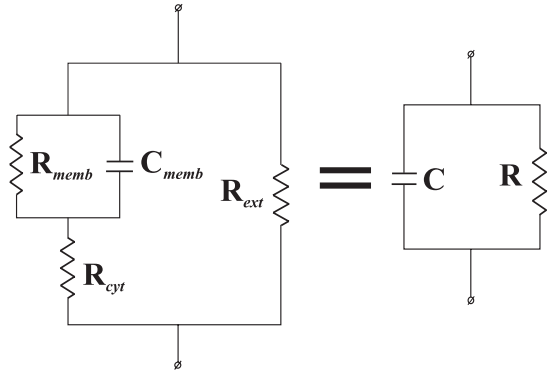

Fig. 1. The equivalent electrical circuit of RBC suspension; subscripts memb, cyt and ext stand for the membrane, cytosol and the external medium.

osmolality of the hypotonic solution revealed the formation of a single hole in the membrane [8]. If G and C of dilute RBC suspension (Hct $\leq$ 0.04–0.05 v/v) are measured in the beginning of the β-dispersion range ($f \approx$ 0.1–0.2 MHz), regardless of whether or nor a single hemolytic hole with a radius of 100–1000 Å [9] is formed, $(\omega C_{memb} R_{memb})^2 \gg 1$ and $\{R_{cyt} + 1/[(\omega C_{memb})^2 R_{memb}]\}^{-1} \ll R_{ext}^{-1}$. Given these non-equalities, Eqs. 3 and 4 reduce to:

$$G \approx R_{ext}^{-1} \quad (5)$$

and

$$C \approx C_{memb} \quad (6)$$

The conductance of dilute cellular suspensions measured in the beginning of the Maxwell-Vagner polarization range approaches the DC one, which obeys the Bruggeman-type equation [10]:

$$G = G_o \bullet (1 - \mathrm{Hct})^m \quad (7)$$

where G_o and m are the conductance of the suspending medium and the shape-dependent parameter, respectively.

Although this equation predicts a shape dependence of the suspension conductivity, no measurable differences between conductivities of suspensions of spherocytic, echinocytic and randomly oriented discocytic RBCs were found [11,12]. Therefore, the m value for spheres ($m = 2/3$ [10]) was used for calculations of G_o. Provided that the ion concentration of the suspending medium is lower than that of cytosol, a time-dependent increase in G_o quantitatively reflects the ion leakage from lysed RBCs.

The swelling of RBCs exposed to a hypoosmotic shock increases the effective radius of the cells. The theory of the Maxwell–Vagner polarization [13,14] and the experimental results [15] point out that the mean effective radius of the dispersed particles in RBC suspensions relates to the capacitance measured in the beginning of the β-dispersion, as follows:

$$C \propto (9 C_{RBC}/4\varepsilon_o) \bullet \langle r_{RBC} \rangle \bullet \mathrm{Hct}/(1 + 0.5 \bullet \mathrm{Hct})^2 \quad (8)$$

where C_{RBC} and ε_o are the specific membrane capacitance and the permittivity of free space.

However, changes in the capacitance are expected to be observed not only due to RBC swelling. It is quite possible that RBCs with many large hemolytic holes are formed in the suspension with a low osmo-

lality of the hypotonic medium. Depending on the number of hemolytic holes and their radii, the membrane capacitance must be completely, or partially, shorted out by a low membrane resistance thereby reducing the contribution of the capacitive resistance ($1/\omega C_{memb}$) to the impedance of the equivalent electrical scheme (Fig. 1). Experimentally, the tendency of blood impedance to approach purely resistive behavior will be manifested by a decrease in the capacitance. Thus, it is expected that at high and moderate osmolarities of the hypotonic solution the capacitance will increase to a stationary level, whereas at low osmolarities it will reveal a biphasic kinetics. In the latter case, the ascending and descending branches of a $C(t)$ curve will reflect RBC swelling and the formation of strongly porated RBCs, respectively.

Thus, it is anticipated that measuring the capacitance and conductance at a frequency far below the mid-point of the β-dispersion range will allow a differentiation between morphological transformations of RBCs and the formation of hemolytic hole(s) in the membrane. In order to clarify whether this prediction is correct, this study was undertaken.

2. Materials and methods

2.1. Blood samples and RBC treatment

Blood units, within 7 days after donation, were supplied by the blood bank of the Soroka University Medical Center (Beer-Sheva, Israel). The lysis of both intact and treated RBCs was investigated. To clarify whether water diffusion across the lipid bilayer or through water channels plays the main role in RBC swelling, the cells were incubated with $HgCl_2$ – a classical water channel blocker (e.g., [16]). In these experiments, the following protocol was used: (1) plasma was removed by aspiration after 10 min centrifugation at $300 \times g$; (2) $HgCl_2$ was dissolved in plasma; (3) RBC pellet and plasma containing different concentrations of $HgCl_2$ were mixed. Measurements were performed after incubation with $HgCl_2$ for approx. 1 h.

The effect of the membrane viscoelasticity on the kinetics of RBC lysis was studied using cells treated with cytochalasin B (Sigma Chemical, USA) or glutaraldehyde, GA (Sigma). The former reagent increases RBC deformability whereas the latter one decreases it. To treat the cells with cytochalasin B, 1 ml of saline containing 0.5 mg cytochalasin B was added to 9 ml of whole blood. To prepare the suspension of the treated cells with the same Hct as in whole blood, after incubation with cytochalasin B at room temperature for 3 h, 1 ml of the external medium was removed by aspiration after 10 min centrifugation.

Cells with the enhanced membrane rigidity were prepared as follows: plasma was removed as described above, the RBC pellet was washed once with phosphate-buffered saline (PBS) and finally, PBS containing different concentrations of GA was added to the pellet. RBC suspensions were examined after incubation with GA for approx. 30 min.

2.2. External media

RBC lysis was studied in the glycerol (0.3 M; Sigma) containing external media. Unlike the standard glycerol lysis test [17], the isosmotic low ionic strength solution (30.8 mM NaCl, 1.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4 and 240 mM glycine; pH 6.8) was used instead of saline or PBS. This low ionic strength solution, LIS, is applied in clinical hematology [1]. The osmolality of the hypotonic media was varied by changes in the ratio of LIS volume to that of distilled water. The application of LIS was motivated by the following reasons. (a) The scale of lysis-induced changes in the conductance and consequently the accuracy of its measurements increases as the difference between the conductivities of the external medium and cytosol increases. (b) The impedance of the measuring chamber filled with RBC suspension is the sum of those of electrodes and RBC suspension. Although the absolute value of the electrode impedance correlates negatively with the conductivity of the external solution, its contribution to the total impedance reduces with a decrease in the conductivity of the external medium. (c) The frequency range at which the dispersion of the electrode double layer, EDL, is observed, depends on the product of $I \bullet D_{ion}$, where I and D_{ion} are the ionic strength and the ion diffusion coefficient. In suspensions with low conducting media, the dispersion of EDL is shifted towards lower frequencies which should be below the

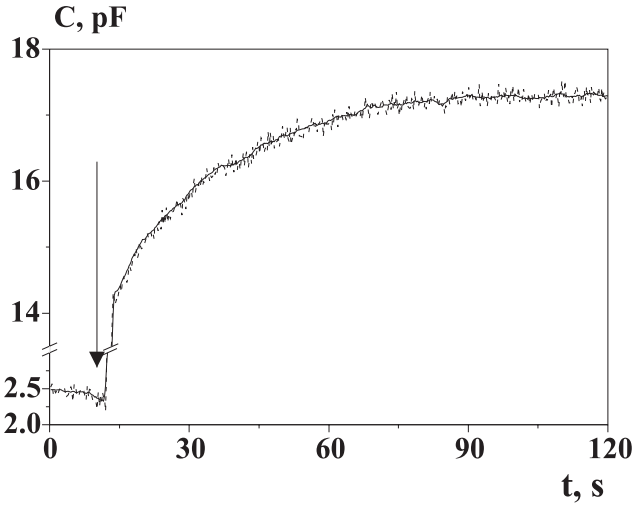

Fig. 2. Comparison between recorded (dashed line) and smoothed (solid line) capacitance signals. The vertical arrow indicates the instant when blood was added to the hypotonic solution.

β-dispersion range. (d) Examinations of suspensions with a low Hct implies small swelling-induced changes in the capacitance. Therefore, the capacitance must be measured with the highest possible sensitivity and accuracy. The expression for the phase angle, θ, of the suspension is written as follows: $tg\theta = \omega \cdot \varepsilon_0 \cdot \varepsilon/\sigma$, where ε and σ are the permittivity and conductivity of the suspension. This equation indicates that low conducting external media should be used to improve the sensitivity of capacitance measurements. Nevertheless, even using these media, because of low Hcts, the noise-to-signal ratio was relatively high. Therefore, capacitance signals were smoothed by FFT filtering (the number of points for smoothing was 5). The comparison of the original and smoothed signals shown in Fig. 2 points out that this data processing does not change the shape of $C(t)$ curves.

2.3. Measurements of the conductance and capacitance

The measurements were carried out for the parallel circuit mode using a HP 4285A LCR meter equipped with a HP 16047A test fixture (Yokogawa Hewlett-Packard, Tokyo, Japan). A low voltage test signal (10 mV) was employed in order to reduce the current density; the time required for each measurement was 200 ± 15 ms. Raw experimental data were transferred via a GPIB interface to a computer and then the measured capacitance was corrected for the effects of the series inductance (5.2×10^{-8} H) and the stray capacitance (1.5 pF).

The conductance and capacitance of RBC suspensions at a frequency of 0.2 MHz were measured in a plexiglas cylindrical chamber (1.8 cm diameter) fitted with two opposing nonpolarizable Ag/AgCl electrodes (0.8 cm diameter; INV Co., CA, USA) embedded in the chamber's wall at equal distances from the bottom of the chamber and the top level of the suspension (~ 2 cm).

To eliminate the effect of RBC sedimentation on the measured signals, hypotonic solutions and the suspension obtained after the addition of blood to these solutions were mixed with a magnetic stirrer (model F-13, Field Electric, Haifa, Israel). No measurable effect of stirring on the level of electromagnetic noises was observed.

Although electrode polarization at $f = 0.2$ MHz is no longer a serious problem even for polarizable electrodes [14], a priori, one could not completely rule out that transient signals are affected by shear-induced changes in the electrode adsorption of plasma surface-active molecules, an increase in the apparent diffusion coefficient caused by rotation of the cells in the shear flow [18] and alterations in the Warburg impedance of the electrodes. To determine to what extent shear-induced changes in the electrode impedance affect time dependencies of the capacitance and the conductance, $G(t)$ and $C(t)$ curves measured for stirred and unstirred conditions were compared. The similarity of the signals recorded during a rather short time (~ 150 s) (data not shown) clearly indicates that shear-induced changes in the electrode impedance have no measurable effect on $G(t)$ and $C(t)$.

Addition of blood to the low conducting hypotonic solution and RBC lysis increase the ion concentration in the external medium thus causing a decrease in the thickness of the electrode Gouy layer and an increase in its conductivity. Therefore, the transient signals might be also affected by these changes in EDL. To clarify whether the time-scale of this effect is comparable with that of lysis-induced changes in G and C, the measurements were performed before and after addition of the same volumes of blood and native plasma to the same volume

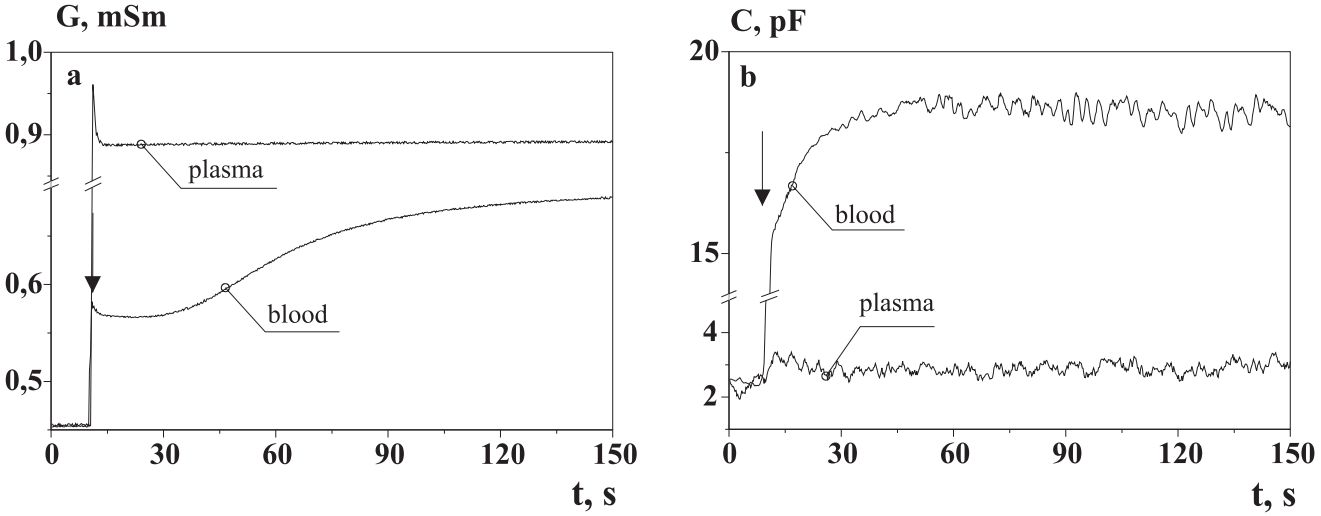

Fig. 3. Time courses of the conductance (a) and the capacitance (b) recorded before and after 1 ml of blood or plasma were added to 12 ml of the hypotonic solution. The water/LIS ratio in the hypotonic solution is 2.2. The instant when blood or plasma were added is indicated by vertical arrows.

of the hypotonic solution. The results of these experiments, shown in Fig. 3, indicate that long-term changes in both G and C are observed only for RBC suspensions. Therefore one may conclude that these changes are associated with processes in the bulk of RBC suspensions.

Another potential source of artifacts might be due to changes in the spatial Hct distribution from strongly nonhomogeneous (instantaneously after blood was added to the hypotonic solution) to homogeneous. To determine the time required to reach the uniform RBC distribution, G and C were recorded before and after 1 ml of the suspension of GA fixed RBCs in PBS ([GA] = 1.0%; Hct = 0.58 v/v) was added to 12 ml of isotonic LIS or PBS. It was found that stationary levels of G and C are reached within 2–3 s (data not shown). Thus, changes in the spatial RBC distribution affect the measured signals only during this initial time interval.

To compare data obtained for suspensions with different Hcts and with different external media, time courses of G and C shown in the figures represent the difference between values of the signals at a certain time and at $t = 2$–3 s.

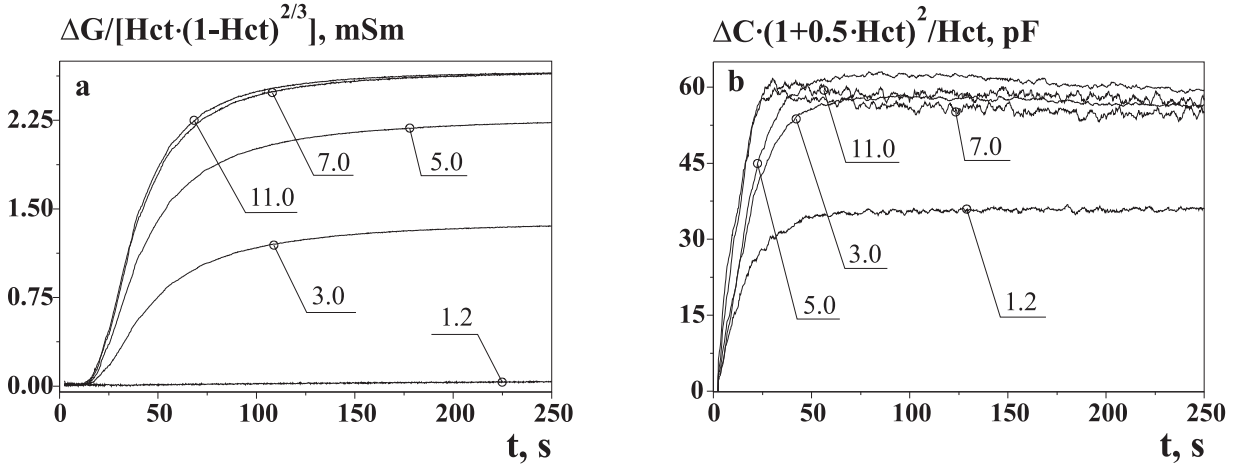

Fig. 4. The effect of the hypotonic solution/blood ratio (shown in figures) on the time-dependent changes in the conductance of the external medium (a) and on the rate of RBC swelling (b). The ratio of water to LIS in the external medium is 2.25.

Addition of blood to the hypotonic solution and ion leakage from lysed RBCs increase the osmolality of the external medium. In this case, experimental results may depend not only on intrinsic RBC properties and the initial osmolality of the hypotonic solution, but may also be affected by the hypotonic solution/blood ratio. Comparison of time-dependent conductances of the external medium normalized to Hct $\{\Delta G/[\mathrm{Hct}\bullet(1-\mathrm{Hct})^{2/3}] = \Delta G_o/\mathrm{Hct}\}$ and of kinetics of RBC swelling $[\Delta C\bullet(1+\mathrm{Hct})^2/\mathrm{Hct} \propto \Delta r_{RBC}]$ shown in Fig. 4 indicate that this effect must be considered at ratios of the hypotonic solution/blood smaller than 7. Therefore, to avoid this problem, apart from the suspensions incubated with $HgCl_2$, experiments were performed at the hypotonic solution/blood ratio of 12.

3. Results

3.1. The effect of the osmolality

Time courses of ΔG and ΔC recorded after 1 ml of whole blood (Hct = 0.48 v/v) was added to 12 ml of the hypotonic solutions with different osmolarities, are shown in Fig. 5. As predicted, biphasic kinetics of the capacitance are observed for suspensions with low osmolarities of the external medium (Fig. 5b). The osmolality of these solutions has a small influence on the ascending branch of the kinetic curves, whereas quite measurable effect of the osmolality on the descending branch is observed: the kinetics of decrease in the capacitance slows down and their stationary levels increase as the osmolality increases. A further increase in the osmolality abolishes the descending branch of $C(t)$ curves and slows down the initial kinetics of the capacitance (Fig. 5c).

Unlike the capacitance, an increase in the conductance to stationary values is observed after a certain delay. The time delay before the conductance starts to change becomes longer and the steady-state level of the conductance decreases as the osmolality increases (Fig. 5a). Measurements performed during 20 min for the hypotonic solution with the water/LIS ratio of 0.5 did not reveal measurable changes in the conductance (data not shown).

The findings shown in Fig. 5 may qualitatively be explained as follows. Depending on the osmolality of the suspending medium, the increase in the capacitance reflects either RBC swelling alone or a superposition of this process and the formation of strongly porated RBCs. The formation of membrane hole(s) causes an ion flux into the external medium which is manifested by an increase in the conductance. Although it is postulated (but, to our knowledge, never was experimentally verified) that lysis occurs immediately after the cells increase their volume by ~70% [1], the results obtained at the highest osmolality of the external solutions (Fig. 5a,c; water/LIS = 0.8) indicate that even a significant extent of swelling does not lead to hole formation. Opposite to what is often thought, findings showing an increase in the conductance while the capacitance already reached the steady state (Fig. 5a,b), suggest that the membranes of swollen, spherical RBCs may remain unbroken for a certain time. If so, one may assume that the delay time is the sum of t_{swel} and the lower limit of the lifetime of the stretched

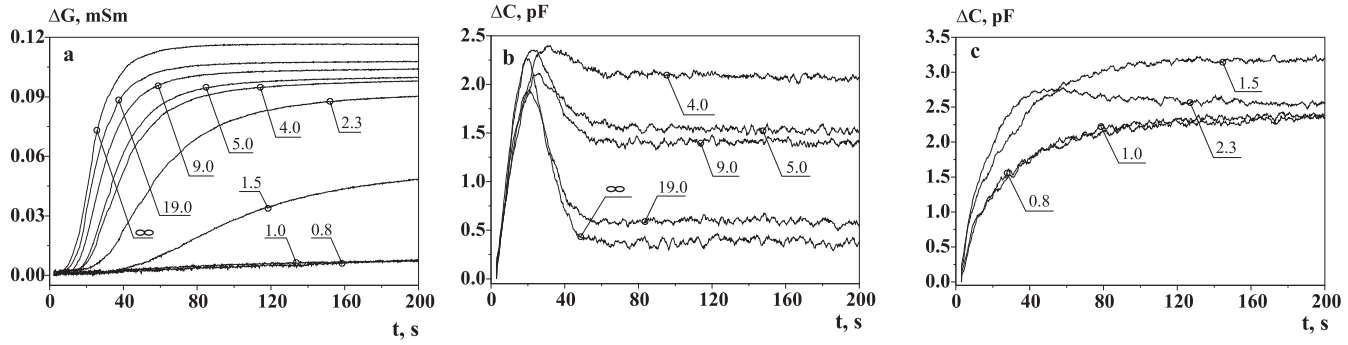

Fig. 5. Time-dependent changes in the conductance (a) and capacitance (b,c) recorded after 1 ml of whole blood (Hct = 0.48 v/v) was added to 12 ml of hypotonic media. The ratios of water to LIS in the hypotonic media are shown in each panel.

membranes under influence of a given osmotic pressure.

3.2. The lysis of RBCs incubated with the water channel blocker

In order to clarify whether water diffusion across the lipid bilayer or water transport through the mercury-sensitive aquaporin water-channel proteins controls RBC swelling, suspensions of RBCs treated with different concentrations of $HgCl_2$ were examined. In these experiments the volume ratio of the hypotonic medium to RBC suspension was increased to 5.5 to improve the sensitivity of the capacitance measurements. The results of these measurements (Fig. 6a) point out that the rate of swelling and the steady-state size of RBCs correlate negatively with the concentration of this water channel inhibitor. The steady-state volume of RBCs treated with 3.5 mM $HgCl_2$ is smaller than that of nontreated cells by a factor of $\sim$1.4, thus indicating inhibition of a significant number of the water channel. Note, however, that these data alone do not allow quantitative characterization of the inhibitory $HgCl_2$ effect. The osmotic water permeability is usually characterized by the hydraulic conductivity, L_p [19]:

$$L_p = 2.8 \bullet r_{RBC} / [6 \bullet t_{1/2} \bullet (M + \pi)] \quad (9)$$

where $t_{1/2}$, M and π are the half-time for water flow equilibration, the volumetric elastic (or tangent modulus for plasma membranes which are non-linear elastic materials) and the intracellular osmotic pressure.

By assuming that the membrane viscoelasticity is not affected by $HgCl_2$, this equation may be used to extract L_p values from the capacitance data alone. However, the significant effect of $HgCl_2$ on the hole formation (Fig. 6b) which may presumably be accounted for by changes in RBC viscoelastic properties, suggests that the capacitance data alone may not be sufficient for a quantitative characterization of the inhibitory $HgCl_2$ effect.

3.3. The effect of RBC membrane viscoelasticity on the hole formation

To clarify the effect of RBC membrane viscoelasticity on the hole formation, suspensions of RBCs treated with cytochalasin B and GA were studied. Representative results for cytochalasin-treated RBCs suspended in the hypotonic media with two different osmolarities are presented in Fig. 7. Regardless of the osmolality, the treatment with cytochalasin B decreases the time delay of the hole formation (Fig. 7a,c). The effect of altered viscoelasticity of RBC membrane at high osmolality (water/LIS = 1.5) is manifested by faster kinetics of the conductance and a higher level of its steady state

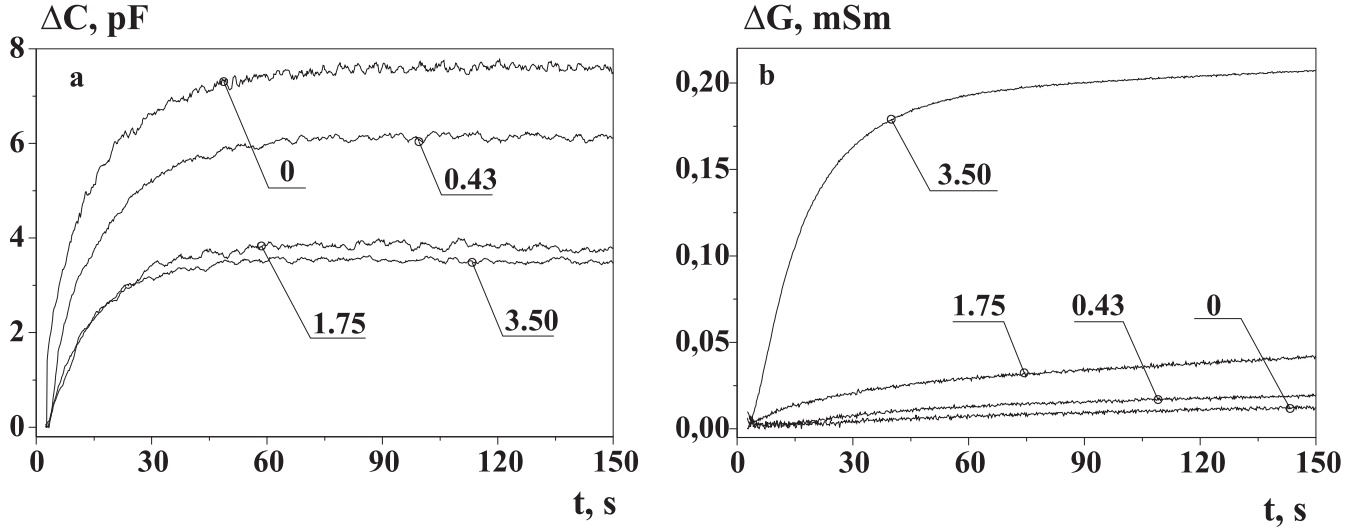

Fig. 6. The effect of $HgCl_2$ on the kinetics of the capacitance (a) and conductance (b). The concentrations of $HgCl_2$ (in mM) are shown in the figures. The water/LIS ratio in the hypotonic medium is 1.5. The transient signals were recorded after addition of 2.0 ml of RBC suspension (Hct = 0.65 v/v) to 11 ml of the hypotonic buffer.

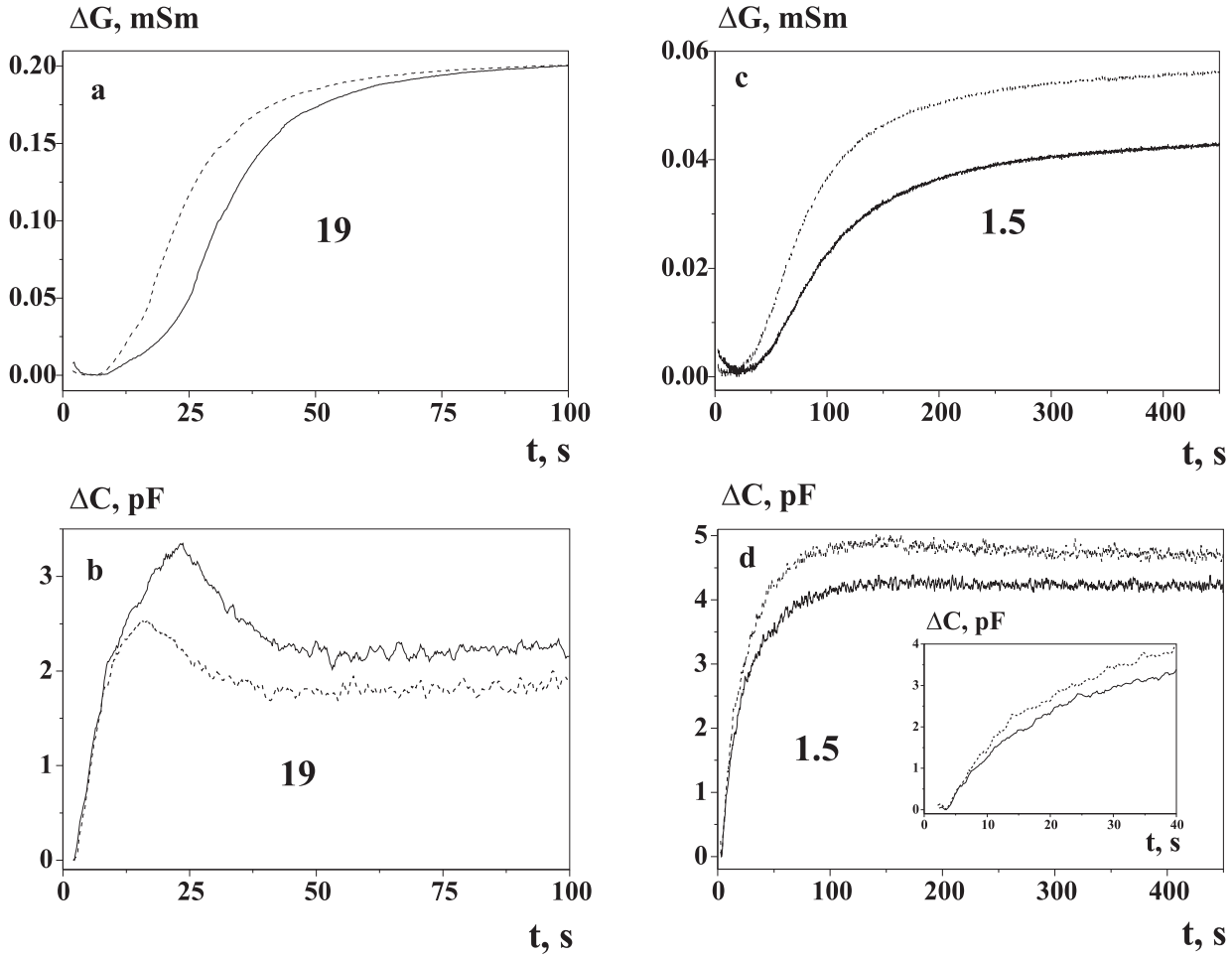

Fig. 7. Comparison between time courses of the capacitance (a,c) and conductance (b,d) recorded for suspension (Hct = 0.53 v/v) of intact (solid lines) and cytochalasin B-treated cells (dashed lines). The water to LIS ratio is shown in each panel. The inset in d shows the initial kinetics of the capacitance.

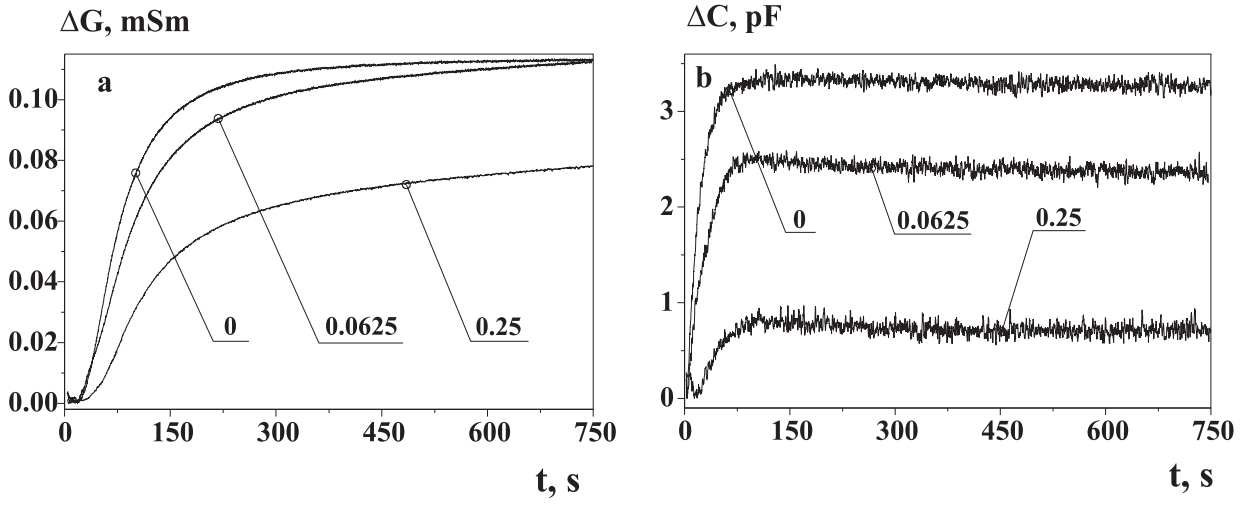

Fig. 8. The effect of RBC rigidity on the kinetics of the conductance (b) and capacitance (a). The GA concentrations (in %) are shown in each panel. The Hct value and the water/LIS ratio are 0.58 v/v and 2.3, respectively.

(Fig. 7c). For suspensions of cytochalasin B treated RBCs the maximum of the $C(t)$ curve at low osmolality (water/LIS = 19) reduces and its position is shifted towards shorter times (Fig. 7b), whereas at high osmolality, a higher steady-state level of the capacitance and faster its initial kinetics are observed (Fig. 7d). These results point out that the cells with more deformable membranes are characterized by a lower resistivity to the osmotic shock. If so, the opposite situation must be observed for RBCs with the enhanced membrane rigidity. In order to check this, suspensions of RBCs treated with GA were examined. As expected, the treatment with GA lowers the stationary levels of the conductance and the capacitance, slows down the rate of their increase and lengthens the time delay of the conductance (Fig. 8). The higher the GA concentration, the larger are these effects. Thus, the results shown in Figs. 7 and 8 clearly indicate that the membrane viscoelasticity is an important factor which affects the hole formation.

4. Discussion

RBCs suspended in hypotonic media undergo the following transformations: the swelling followed by the formation of hole(s) in the membrane. Although the results suggests that capacitance data may provide quantitative information about RBC swelling, it was decided, in order to keep the volume of this paper at a reasonable level, to focus this study on the hemolytic hole formation.

In general, the ion flux through aqueous holes depends not only on their diameter and the concentration gradient, but may also be affected by many other factors such as, for example, the transmembrane potential, concentration-dependent ion–ion interactions in a hole, the intensity of the local electrical field inside a hole, its position relative to the external electrical field and its intensity. Fortunately, given a large diameter of hemolytic holes (100–1000 Å [16]) and their small number per RBC at relatively high osmolality of the hypotonic buffer [8,20], this problem is simplified and reduces to Fick's law.

A priori, one cannot completely rule out the possibility that the time dependencies of the conductance are controlled by the time of unsteady-state ion diffusion through hemolytic holes. If so, the time required to equalize the intra- and extracellular ion concentrations will depend on ion concentrations in the external medium before and after RBC lysis. Other conditions being equal, these concentrations depend solely on the blood/external solution ratio. Therefore, if $G(t)$ curves reflect the ion transport through holes, the time required to reach a steady-state conductance must depend on this ratio. However, the experimental results (Fig. 4a) show that this time remains approximately unchanged at blood/hemolytic solution ratios from 0.09 to 0.33. Therefore, one may conclude that other factors affect the kinetics of the conductance.

Assuming that the flux of the ith ionic species is not affected by fluxes of other ions, first Fick's law for the passive ion leakage through holes formed in the membranes of all RBCs in a population, is written as follows:

$$\sum_i \mathrm{d}c^i_{\mathrm{ext}}/\mathrm{d}t = \pi\langle N\rangle\bullet\langle r_{\mathrm{hole}}\rangle^2\bullet\mathrm{Hct}\bullet[(1-\mathrm{Hct})\bullet\langle V_{\mathrm{RBC}}\rangle\bullet l]^{-1}\bullet \sum_i D_i\bullet(c^i_{\mathrm{cyt}}-c^i_{\mathrm{ext}}) \quad (10)$$

where c, $\langle N\rangle$, $\langle r_{\mathrm{hole}}\rangle$, $\langle V_{\mathrm{RBC}}\rangle$, l and D are the ion concentration, the average number of holes per RBC and their average radius, the average RBC volume, the membrane thickness and the ion diffusion coefficient, respectively; subscript i stands for the ith ionic species.

Inserting the equation for the material balance:

$$c^i_{\mathrm{ext}}\bullet(1-\mathrm{Hct}) + c^i_{\mathrm{cyt}}\bullet\mathrm{Hct} = \mathrm{const}^i \quad (11)$$

into Eq. 10 and integrating it, yields:

$$-\sum_i \ln(\mathrm{const}^i - c^i_{\mathrm{ext}}) = \pi\bullet\langle N\rangle\bullet\langle r_{\mathrm{hole}}\rangle^2\bullet\sum_i D_i\bullet t\bullet [(1-\mathrm{Hct})\bullet\langle V_{\mathrm{RBC}}\rangle\bullet l]^{-1} - \sum_i \ln[\mathrm{const}^i - c^i_{\mathrm{ext}}(t=0)] \quad (12)$$

where const^i is the total amount of ion i in the external and internal media.

Decomposing $\Sigma_i \ln(\mathrm{const}^i - \mathrm{c_{ext}}^i)$ in the Taylor series, this equation may be re-written in the following form:

$$\sum_i \sum_{n=1}^{\infty} (c_{ext}^i/\mathrm{const}^i)^n = \pi \bullet \langle N\rangle \bullet \langle r_{hole}\rangle^2 \bullet \sum_i D_i \bullet t \bullet [(1-\mathrm{Hct}) \bullet \langle V_{RBC}\rangle \bullet l]^{-1} - \left\{ \sum_i \ln[\mathrm{const}^i - c_{ext}^i(t=0)] - \sum_i \ln(\mathrm{const}^i) \right\} \quad (13)$$

Since low Hct values and low conducting hypotonic solutions were used in this study, the external media are dilute solutions of strong electrolytes even if the cells are completely hemolyzed. In this case, the ion mobility approaches that at infinite dilution and therefore the conductivity of these solutions equals to the product of $F \bullet \Sigma_i \mu_\infty{}^i \bullet c_{ext}{}^i$, where F and $\mu_\infty{}^i$ are the Faraday constant and the mobility of the ith ionic species at infinite dilution. Since the limiting mobilities of the main current carriers (K^+ and Cl^-) differ within $\pm 2\%$ [21] and taking into account that $\Sigma_i c_{ext}{}^i/\mathrm{const}^i = G_{ext}(t)/[G_{ext}(t=0)+\Delta G_{hem}]$, Eq. 13 may be rewritten as follows:

$$\sum_{n=1}^{\infty} \{G_{ext}(t)/[G_{ext}(t=0)+\Delta G_{hem}]\}^n/n = \pi \bullet \langle N\rangle \bullet \langle r_{hole}\rangle^2 \sum_i D_i \bullet t \bullet [(1-\mathrm{Hct}) \bullet \langle V_{RBC}\rangle \bullet l]^{-1} - A \quad (14)$$

where $G_{ext}(t=0)$ and ΔG_{hem} are the conductance of the suspension at $t=0$ and the change in the conductance of the external medium caused by the complete hemolysis of all RBCs in the suspension. The value of ΔG_{hem} equals the difference between the conductances measured before and after complete RBC hemolysis in the suspension with the lowest osmolality of the external solution; $A = \Sigma_i \ln[\mathrm{const}^i - c_{ext}{}^i(t=0)] - \Sigma_i \ln(\mathrm{const}^i)$. Obviously, A is a constant parameter for a certain RBC suspension.

For treatment of the experimental data it is convenient to write this equation in a simplified form:

$$\Delta\varphi[G_{ext}(t)] = \varphi[G_{ext}(t)] - \varphi[G_{ext}(t=0)] = \langle N\rangle \bullet \langle r_{hole}\rangle^2 \bullet t \bullet B \quad (15)$$

where $\varphi\,[G_{ext}(t)] = \Sigma_{n=1}^{\infty}\{G_{ext}(t)/[G_{ext}(t=0)+\Delta G_{hem}]\}^n/n$ and $B = \pi \bullet \Sigma_i D_i \bullet [(1\text{-Hct}) \bullet \langle V_{RBC}\rangle \bullet l]^{-1}$.

To compute values of φ (G_{ext}) with a high accuracy (the remainder term $< 10^{-4}$), the 5th-order approximation of the Taylor series was used. Since RBC morphology has no measurable effect on the conductance of dilute RBC suspensions [11,12], lysis-induced changes in the product of $\langle N\rangle \bullet \langle r_{hole}\rangle^2$ (i.e., the hole formation) is the only factor affecting $\Delta\varphi$ [$G_{ext}(t)$].

Eq. 15 predicts that the kinetics of the conductance should depend on both the time-dependent number of holes and the rate of hole expansion. However, studies of RBC membrane electroporation show that the formation of hydrophilic pores takes ~ 100 μs [22], thus suggesting that the observed kinetics of the conductance cannot be accounted for by hole expansion. It follows that $\Delta\varphi$ [$G_{ext}(t)$] $\propto N(t) \bullet \langle r_{st}\rangle^2$, where $\langle r_{st}\rangle$ is the average steady-state radius of hemolytic holes.

A priori, however, one cannot rule out that the $\langle r_{st}\rangle$ value is affected by the external ion concentration. Indeed, reported data obtained for erythrocyte ghosts point out that the steady-state size of hemolytic holes increases drastically as the external concentration of cations is reduced below 30–40 mM [20]. If this is also the case for erythrocytes and the steady-state size of hemolytic holes is controlled by the external ion concentration, the time dependence of $\Delta\varphi(G_{ext})$ must be affected by the hypotonic solu-

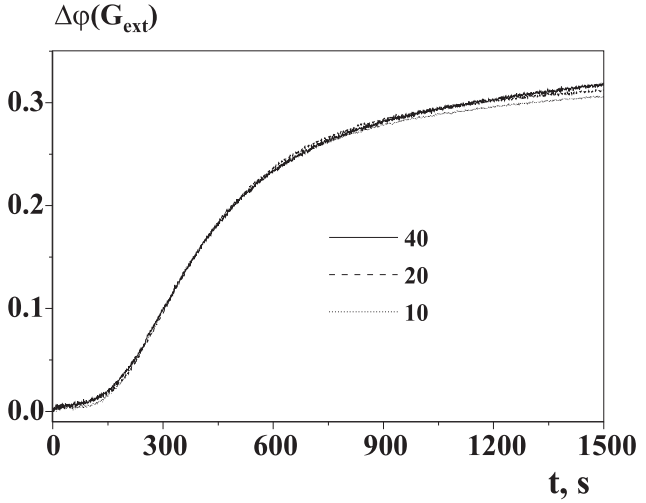

Fig. 9. $\Delta\varphi(G_{ext})$ versus time curves for different hypotonic solution/blood ratios (shown in the figure). The water to LIS ratio in the hypotonic solution (1.67) is the same for all cases.

tion/blood ratio. However, the similarity of $\Delta\varphi(G_{ext})$ versus time curves for different hypotonic solution/blood ratios (Fig. 9) suggests that the steady-state size of the hemolytic holes is unaffected by the external ion concentration. Therefore, it may be concluded that the value of $\langle r_{st}\rangle$ remains unchanged during RBC lysis and consequently, the time dependencies of $\Delta\varphi(G_{ext})$ plotted in Fig. 9, reflect the kinetics of hole accumulation.

In order to clarify whether RBC lysis occurs immediately after the cells become spherical, the kinetics of RBC swelling and of the hole formation should be compared. The time-dependent effective radius of RBCs was calculated (Eq. 8) from the capacitance data obtained for the suspension with a relatively high osmolality of the hypotonic solution using $C_{RBC} = 1.3$ μF/cm^2 [15]. The maximal r_{RBC} value of 3.70–3.75 μm (Fig. 10) which is characteristic for spherical RBCs, testifies that the overwhelming majority of the cells is completely swollen. Provided that once a RBC is completely swollen, its membrane is ruptured without delay, the same time will be required for $\langle r_{RBC}\rangle$ and $\langle N\rangle$ to reach maximal values. However, the data shown in Fig. 10 contradict this assumption. A comparison of the kinetics of the swelling (e.g., $r_{RBC}(t)$) and of the hole formation clearly indicates that the stretched membrane of completely swollen RBC retains the integrity for a rather long time, which will be termed as the membrane lifetime, t_{memb}.

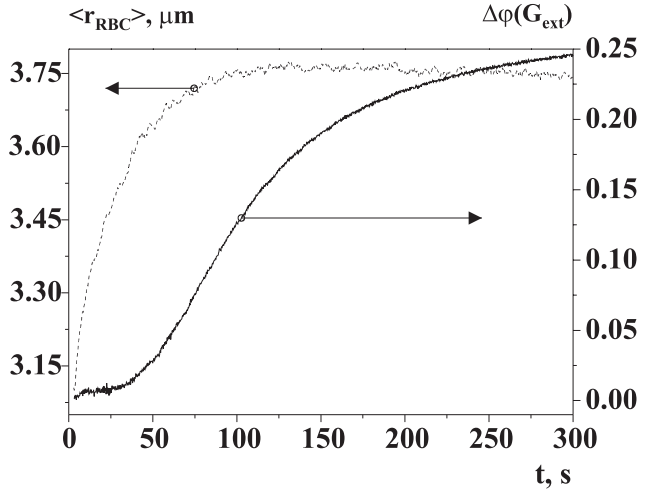

Fig. 10. Time-dependent increase in the mean effective radius of RBCs (dashed line) and the kinetics of hole accumulation (solid line) in the suspension with the water/LIS ratio of 1.5.

If resealing of erythrocyte membrane does not play an appreciable role in the hole formation and only a single hole is formed in the membrane, RBC transformations in hypotonic media may be described by the following kinetic scheme:

$$RBC_{discoid} \xrightarrow{t_{swel}} RBC_{sp}(0) \xrightarrow{t_{memb}} RBC_{sp}(1) \qquad (16)$$

where the digit in parentheses indicates the number of holes in the membrane of a spherical RBC.

In terms of the formal kinetics, the hole formation is a first order process and hence, the probability of hole formation, P, is equal to $1-\exp(-t/t_{memb})$. Since, in this case, t_{memb} must be substantially shorter than the time which is required for the total number of hemolytic holes to reach a steady-state, t_{st}, P essentially equals unity at $t > t_{st}$. If so, the steady-state number of spherical cells and hemolytic holes must be the same regardless of the osmolality of the external medium. The results (Fig. 11), however, disagree with this assumption. While the steady-state radius of RBCs ($\sim$3.70 μm) indicates that at these osmolarities of the external medium all cells acquire the spherical shape (Fig. 11a), the steady-state number of the hemolytic holes decreases with an increasing tonicity (Fig. 11b). Thus, the findings clearly show that the spherical shape is necessary but not a sufficient condition for the hole formation.

To explain the results presented in Fig. 11, it may be suggested that the hole in the stretched membrane of a spherical RBC is formed only if the intracellular pressure exceeds a threshold value. If that is the case, P for a certain spherical RBC in a population must change in a step-wise fashion: $P = 0$ at $t \le t_{memb}$ and $P = 1$ at $t \ge t_{memb}$. Since the membrane lifetime of an ensemble of spherical RBCs is characterized by a distribution function, $RBC_{sp}(t_{memb})$, the number of holes formed at a time longer than that which is required to reach the steady-state number of spherical RBCs, RBC^{st}_{sp}, equals $\int_0^t RBC^{st}_{sp}(t_{memb})dt$. Obviously, for the single-hole regime $\int_0^t RBC^{st}_{sp}(t_{memb})dt \le RBC^{st}_{sp}$, an increase in the osmolality shifts the distribution function of t_{memb} to longer times and consequently, decreases the value of $\int_0^t RBC^{st}_{sp}(t_{memb})dt$; In other words, the number of hemolytic holes formed at a time longer than that which is required to reach the steady-state number

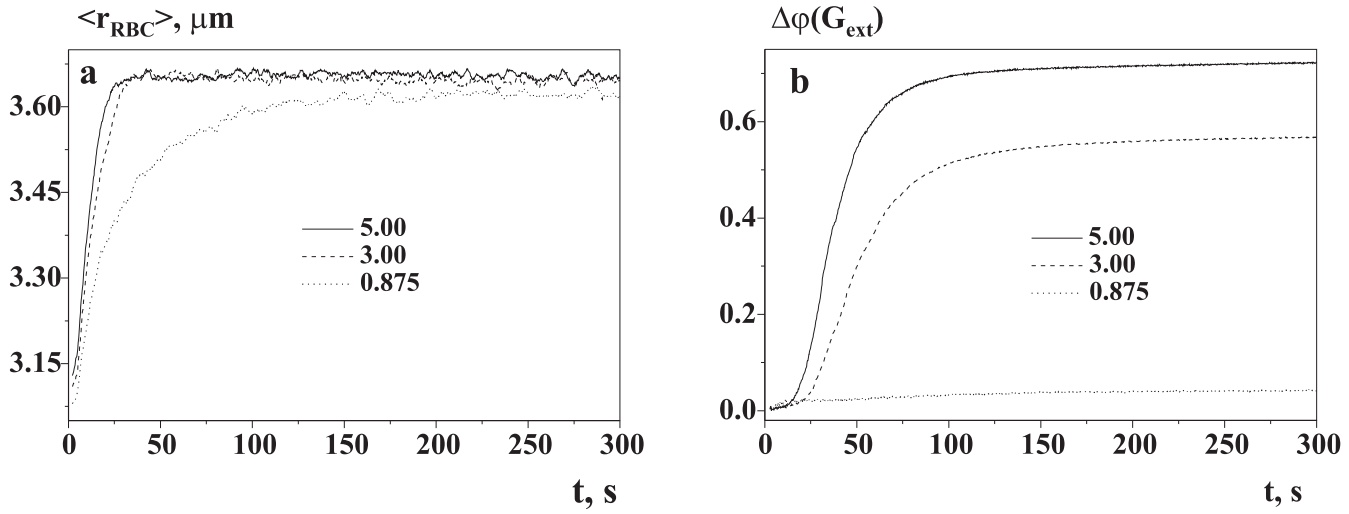

Fig. 11. Kinetics of RBC swelling (a) and of the hole formation (b) in suspensions with various osmolarities of the external medium. The ratios of ware to LIS volumes are shown in the panels.

of spherical RBCs, must correlate negatively with the osmolality. Moreover, one cannot completely rule out that above a certain tonicity of the hypotonic solution the value of $\int_0^t \mathrm{RBC}_{\mathrm{sp}}^{\mathrm{st}}(t_{\mathrm{memb}})\mathrm{d}t$ will approach zero. In this case, no measurable number of holes in the membranes of spherical cells will be formed. These predictions are in full agreement with the results shown in Fig. 11.

5. Concluding remarks

The following main conclusions may be drawn from the results reported herein:

1. The time record of the capacitance and conductance may be used to distinguish between RBC swelling and hole(s) formation in hypotonic media.
2. RBC swelling is largely controlled by water transport through aquaporin water channels.
3. The main factor affecting the hole formation in the membrane of initially biconcave-shaped RBC is the membrane viscoelasticity.
4. The stretched membrane of swollen RBC may retain its integrity for a certain time, t_{memb}. The resistivity of RBCs to a certain osmotic shock may be quantified by the distribution function of t_{memb}.

Future studies will focus on investigations of pathological RBCs, with the hope to clarify whether the proposed technique may be used for diagnosis of certain hematological diseases.

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References

[1] J.V. Dacie, S.M. Lewis, Practical Haematology, 6th Edition, Churchill Livingstone, New York, 1984, pp. 152–156.
[2] J.M. Steinke, A.P. Shepherd, IEEE Trans. Biomed. Eng. 33 (1986) 294–301.
[3] V. Twersky, J. Opt. Soc. Am. 60 (1970) 908–914.
[4] V. Twersky, J. Opt. Soc. Am. 60 (1970) 1084–1093.
[5] H.C. van de Hulst, Light Scattering by Small Particles, Wiley, New York, 1957.
[6] A. Sauer, T. Kurzion, D. Meyerstein, N. Meyerstein, Biochim. Biophys. Acta 1063 (1991) 203–208.
[7] P. Mazeron, J. Didelon, S. Muller, J.-F. Stolz, Photochem. Photobiol. 72 (2000) 172–178.
[8] Y. Sato, H. Yamakose, Y. Suzuki, Biol. Pharm. Bull. 16 (1993) 506–512.
[9] P. Seeman, D. Cheng, G.H. Iles, J. Cell Biol. 56 (1973) 519–527.

[10] D.A.G. Bruggeman, Ann. Physik. 24 (1935) 636–664.
[11] I.T. Ivanov, B.K. Popov, Gen. Physiol. Biophys. 12 (1993) 311–315.
[12] A. Pribush, D. Meyerstein, N. Meyerstein, Biochim. Biophys. Acta 1258 (1995) 187–193.
[13] H.P. Schwan, in: W.S. Adelman, D. Goldman (Eds.), The Biophysical Approach to Excitable Systems, Plenum Press, New York, 1981, pp. 3–24.
[14] H.P. Schwan, in: W.L. Nastuk (Ed.), Physical Techniques in Biological Research, Vol. VI, Academic Press, New York, 1963, pp. 323–416.
[15] A. Pribush, H.J. Meiselman, D. Meyerstein, N. Meyerstein, Biorheology 36 (1999) 411–423.
[16] S.T. Tsai, R.B. Zhang, A.S. Verkman, Biochemistry 30 (1991) 2087–2092.
[17] E.L. Gottfried, N.A. Robertson, J. Lab. Clin. Med. 83 (1974) 323–333.
[18] A. Caprani, L. Tamisier, in: H. Giesekus (Ed.), Progress and Trends in Rheology, Vol. II, Springer, Darmstadt, 1988, pp. 490–493.
[19] W.-H. Zhang, S.D. Tyerman, Plant Physiol. 120 (1999) 849–857.
[20] M.R. Lieber, T.L. Steck, J. Biol. Chem. 257 (1982) 11660–11666.
[21] Ya.A. Gerasimov, Physical Chemistry, Vol. 2, Mir Publisher, Moscow, 1974, p. 403.
[22] K. Kinozita, T.Y. Tsong, Biochim. Biophys. Acta 554 (1979) 479–497.