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Application of a low-angle light scattering technique to cell volume and cell signaling studies on Ehrlich ascite tumor cells

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Abstract. A method for studying cells based on low-angle light scattering was applied to cell volume and cell signaling studies on Ehrlich ascite tumor cells (EATC). Changes in the volume of EATC were measured in hypotonic medium, as well as after activation with exogenous ATP, ionomycin and thimerosal. Increase of $[Ca^{2+}]_i$ under ATP and ionomycin action induced reversible changes of cell volume: fast shrinking was followed by swelling. Thimerosal caused a reversible change in EATC volume with high amplitude; endoplasmic reticulum played the key role in this response. Having obtained kinetic parameters of changes in cell volume under activation of the cells, quantitative measurements of K^+, Na^+ and anion flows responsible for this process can then be obtained. In spite of some fundamental differences in the behavior of cells of different dimensions there are many similarities, and there is a good theoretical background for dealing with both small and large cells.

Keywords: Low-angle light scattering, cell volume, cell signalling, Ehrlich ascite tumor cells

1. Introduction

The theoretical and experimental foundations of a low-angle light scattering technique, as well as applications in experimental toxicology and clinical pathology studies on platelets, have been described in our recent papers [1,2]. The advantages of the new method in comparison with other light scattering techniques were highlighted. The method was used as a diagnostic tool in experimental low-level intoxication by organophosphates and for interpretation of the pathogenesis of delayed effects. In human patients with ischemic heart disease or with prosthetic heart valves, significant changes in the functional state of platelets were observed. A model for cooperative binding of receptors with ligands on platelets from pregnant women with pre-eclampsia was developed.

In the present work we report on another application and further development of the low-angle light scattering technique, to provide quantitative measurements of the cell volume transitions which reflect operation of intracellular signalling systems and ion channels. Changes in the volume of Ehrlich ascites

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tumor cells (EATC) were measured in hypotonic medium, as well as after activation with exogenous ATP, ionomycin or thimerosal. The data show that, in spite of some fundamental differences in the behavior of cells of different dimensions, there are many similarities and that there is a good theoretical background for dealing with both small and large cells.

2. Materials and methods

The intensity of the scattered light was measured with a Laser Particle Analyzer, described in [1]. Experiments were carried out with EATC, which were obtained from NMRI mice. The cells were isolated on the 7th day after transplantation as described in [3]. The registration was carried out at 37°C in Hanks' medium consisting of (mmol l^{-1}): NaCl, 140; KCl, 5.4; $CaCl_2$, 1.3; $MgSO_4$, 1; KH_2PO_4, 1 Na_2HPO_4, $NaHCO_3$, 1; glucose, 6; HEPES, 20; pH = 7.2. Concentration of cells in the experimental cuvette of 3 ml volume was about 3×10^5 cells ml^{-1}. The $[Ca^{2+}]_i$ was measured with Indo-1 AM [4]. The following reagents were used: ionomycin, digitonin (Calbiochem); Indo-1AM, EGTA, HEPES, medium H199, Hanks' medium (Sigma); ATP (ICN). Viscum album agglutinin (VAA) was a kind gift of Dr. A.V. Timoshenko (Institute of Photobiology, Minsk).

3. Results and discussion

3.1. Some important notes for dealing with cells of a large diameter

In contrast to cells of a very low diameter (about 3 μm for platelets), the measurement of cells of a larger diameter with the low-angle light scattering technique has an important feature: all the measurements are carried out far away from the first diffracting ring and on the leveled curve. In these circumstances, the mode of dependence of light scattering intensity (LSI) upon cellular dimensions has a strong relation to the initial distribution of the cells and is also dependent on the measuring device. Figure 1 shows dependence of LSI for particles of 10 and 12.5 μm diameter, for sigma 1.0. It can be seen that the increase in dimensions (from 10 to 12.5 μm) causes increased LSI at low angles (4–6 degrees), but lower LSI at angles of >6 degrees.

For particles with diameter of more than 10 μm, the dependence of LSI upon the angles of registration should be described by the approximation of Fraunhofer, in contrast to the approximation of Rayleigh–Debye–Gans suitable for small particles (such as platelets). Figure 2 shows the angle diagrams for particles of 12.5 μm diameter (approximate dimensions of EATC), with various distribution of the particles (normal distribution; sigma shown in figure). It can be seen that for particles of this diameter the minimal diffraction corresponds to an angle of 2 degrees. In the range of angles from 4 to 14 degrees, there are 4 minima of diffraction. The wider the distribution of particle dimensions, the smaller the amplitude of these minima. In the distant ranges of angles to be measured these minima are principally leveled. It should be noted that for studies of platelet aggregation, the signalling behavior is similar at both low and high degrees and has a different theoretical foundation than that for larger particles. For large particles, it is a leveled diffraction picture at the higher angles that is considered.

3.2. The dependence of LSI on cell concentration

Figure 3A shows changes in LSI at various angles (4, 6, 8, 10, 12, 14 degrees). Under increasing concentrations of the cells, the LSI is firstly increased then decreased. Thus the change of LSI in the

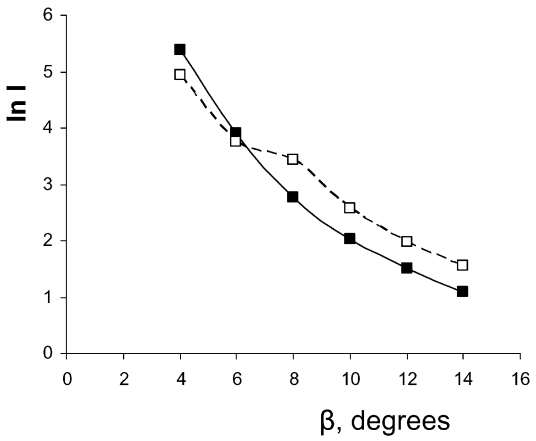

Fig. 1. Calculated angular diagrams for particles 10 (□) and 12.5 μm (■) (sigma = 1.0). Theoretical curves can not be exactly described by linear trend, but since there are photosensors with identical aperture and intensity (I) is determined by the solid angle which decreases in far angles, then the experimental dependence is much better described by the linear trend.

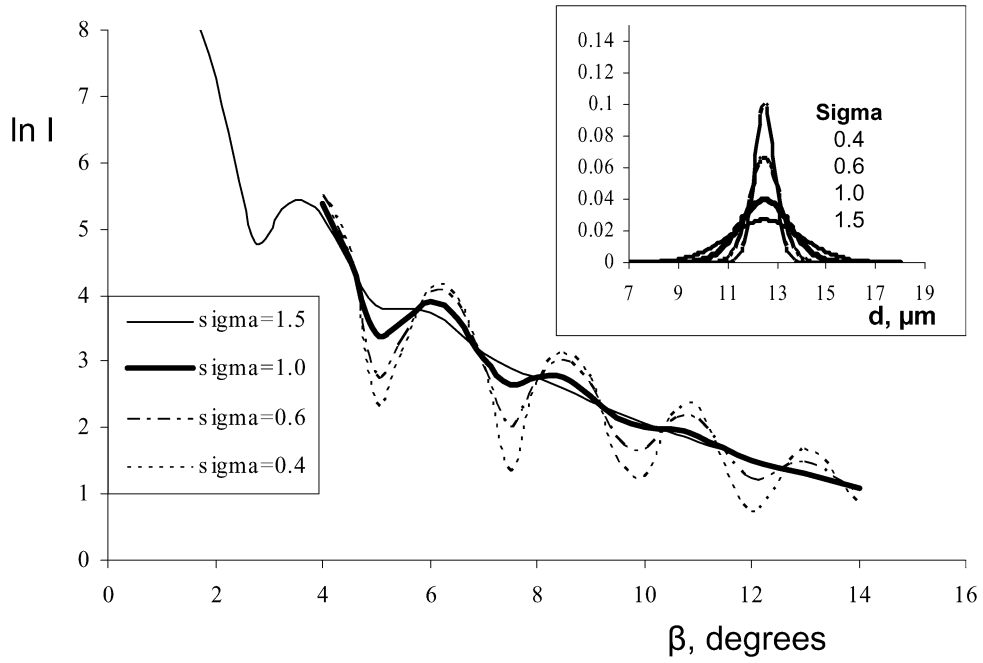

Fig. 2. Calculated angular diagrams for particles 12.5 μm with the assigned characteristics of particle distribution (sigma: 1.5 to 0.4).

low angles is mostly sensitive to cell concentration, with linear increase of the intensity being up to 3×10^{-5} cells ml^{-1}. For the calculations to be simple and correct, the measurements are carried out under conditions of single light scattering, when there is a linear dependence between LSI and concentration of the cells [5]:

$$I_{\mathrm{i}} - I_{\mathrm{o}} = k \cdot [C_{\mathrm{i}}], \tag{1}$$

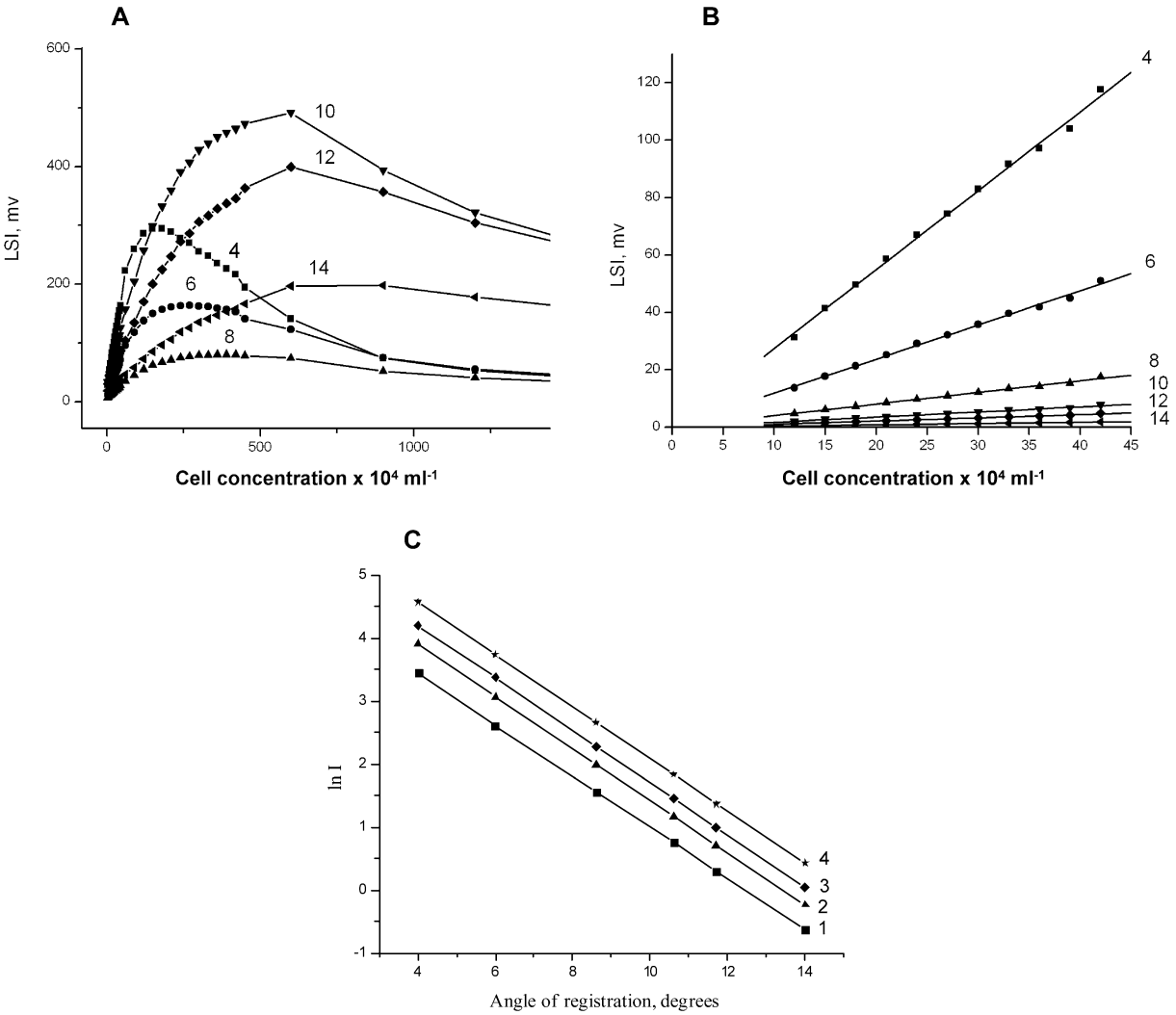

Fig. 3. (A) Dependence of the LSI, which are registered in the angles of 4–14 degrees, upon concentration of EATC. Multiplication factor of the channels at 4, 6, 8 degrees is 1, and that of the channels at 10, 12, 14 degrees is 10. (B) Linear range of the LSI dependences upon the cell concentrations for various angles of registering (4 to 14 degrees). (C) Dependences of logarithm of LSI upon the angle of registration for different concentrations of the cells. Cell concentrations for plots 1 to 4 are 12, 18, -24 and 36×10^4 cells ml^{-1} respectively.

where:

I_i = LSI caused by cells being in concentration C_i in a given angle;
I_o = LSI of the incubation medium without cells;
k = constant of proportionality.

Figure 3B shows linear dependence of LSI changes upon cells concentration for the angles mentioned above. Because of cell size heterogeneity, the dependence of logarithm of the intensity ($\ln I_\mathrm{i}$) upon the scattering angle (β) also has a linear character (Fig. 3C). The constancy of the slope angle of the indicatrix follows from the linear dependence of LSI upon the cell concentration, as shown in Fig. 3C.

3.3. Changes of LSI with cell swelling in hypotonic medium

Changes in cellular volume are usually coupled with induction of an $[Ca^{2+}]_i$ increase and activation of Ca^{2+}-dependent K^+ and anionic channels and Na^+/K^+-ATPase [6]. The transport of these ions and the concurrent flow of water cause the volume changes, which are registered by the reciprocate LSI changes. It is well known that if cells are surrounded with a hypotonic medium, they at first swell as a more or less ideal osmometer, but then reduce the initial volume by a regulatory volume decrease mechanism. Therefore the regulatory volume decrease in a hypotonic medium is a good model for investigation of the LSI dependence upon the cell volume.

Figure 4A shows that swelling of the cells in the hypotonic medium is accompanied by increase of LSI at low angles and decrease of LSI at high angles. After 30 seconds the swelling is followed by shrinking, the LSI being returned to the initial state. Figure 4B shows that the slope of indicatrix increases with swelling and decreases with contraction of the cells. Under conditions of single light scattering, the tangent of the slope angle is proportional to cell diameter. To obtain the tangent in routine experiments it is sufficient to register the LSI at two angles, then the cell size (diameter) can be calculated according to equation:

$$D = K \cdot \operatorname{tg}\beta = K \cdot \left(\ln I^4 - \ln I^{14}\right)/10 = K \cdot \ln\left(I^4/I^{14}\right)/10, \tag{2}$$

where: I^4, intensity of LS at 4 degrees; I^{14}, intensity of LS at 14 degrees; D, cell diameter; K, coefficient.

Since there is no change in the slope angle with change in the cell number, introduction of a new parameter – relation of LSI at 4 degrees to that at 14 degrees – renders an opportunity to register the LSI changes that are caused only by cellular volume against a background of changes caused by aggregation of the cells. In a simple case, the coefficient K can be obtained experimentally by direct microscopic measurement of cell diameter. Figure 5 shows reversible changes of the EATC diameter under impulsive

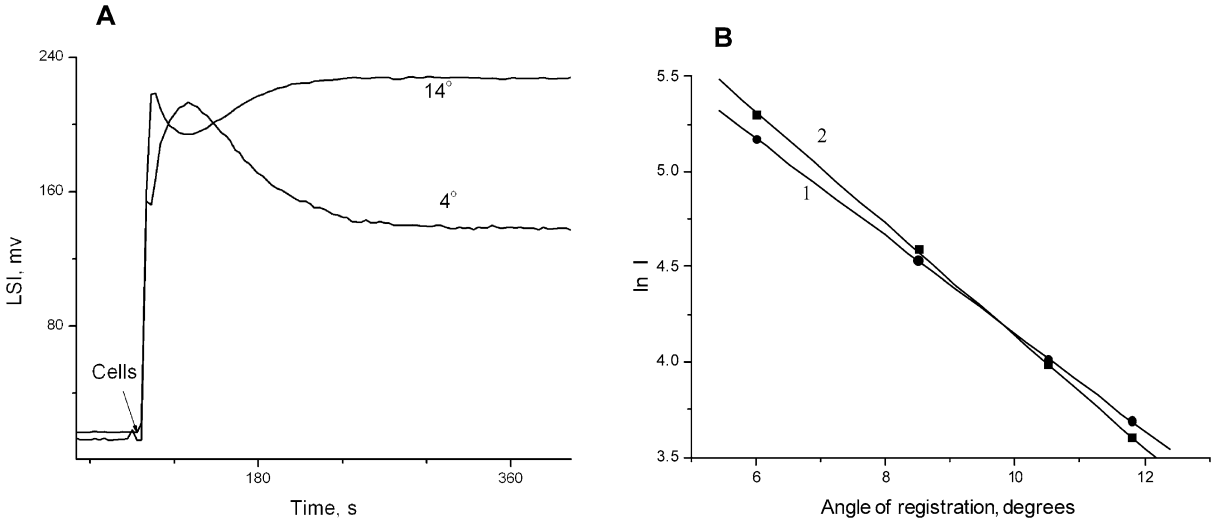

Fig. 4. (A) Change in LSI at 4 and 14 degrees upon swelling of EATC in hypotonic (50%) incubation medium. Cell concentration was 3×10^5 cells ml^{-1}. (B) Scattering indicatrixes upon EATC swelling in hypotonic (50%) incubation medium. 1 – Indicatrix of the cells before swelling; 2 – Indicatrix of the cells at 30 seconds of swelling.

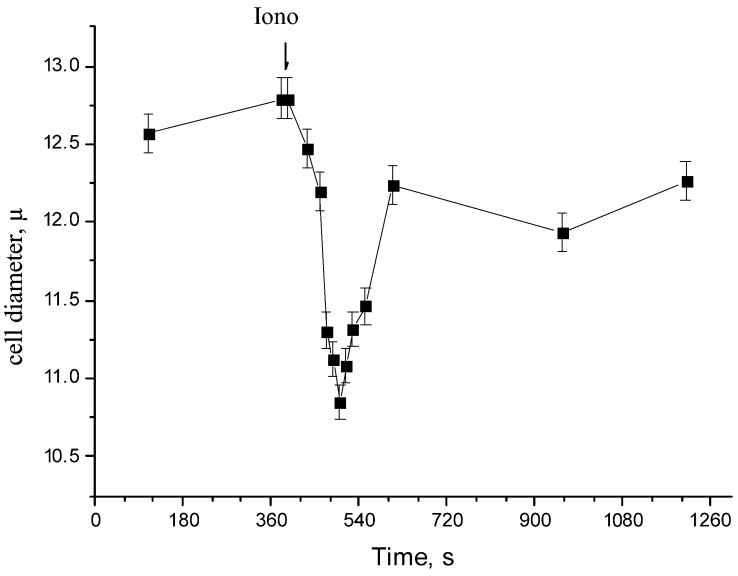

Fig. 5. Change of EATC diameter under action of ionomycin (3×10^{-7} mol l^{-1}), registered with a LSM 510 confocal microscope (Carl Zeiss Inc.).

Table 1
Dependence of EATC diameter upon tonicity of solution

Tonicity (%)	100	80	50	30
Cell diameter (μm)	11.8	13.0	14.4	16.6

increase of $[Ca^{2+}]_i$ induced by ionomycin and measured with confocal microscopy. These values of cell diameter entered into Eq. (2) returns $K = 3.75$. This meaning for K can be used for calculation of cell diameter in experiments. Test experiments demonstrated a coincidence of cell dimensions obtained by this method. The maximal values of cell diameter under swelling of cells in solutions of different tonicities are shown in Table 1; when re-calculate for volume, there is a linear dependence of volume upon tonicity of solution in the range of 50–100%.

3.4. Changes of cell volume induced by ATP and ionomycin

Activation of EATC by exogenous ATP causes reversible changes of LSI, reflecting a $[Ca^{2+}]_i$-dependent cycle of cell swelling and shrinking (Fig. 6A), initial intensity of LS being reduced in low angles and enhanced in high angles. Average diameter of cells calculated according to the Eq. (2) is reduced with cell shrinkage from 12 to 10.5 μm (Fig. 6B). The reduction of cell volume is caused mainly by generation of Ca^{2+}-dependent K^+ and anionic channels. Efflux of these ions and the concurrent outflow of water cause the reduction of cell diameter. Decrease of cytosolic $[Ca^{2+}]_i$ causes a closing of Ca^{2+}-dependent channels and pumping of K^+ ions back into the cells by Na^+/K^+-ATPases. Low concentrations of ionomycin induce similar changes of LSI (Fig. 7A,B). Cellular dimensions measured by the low-angle light scattering technique are in close agreement with those obtained with confocal microscopy (Fig. 5). Thus, the new method described here is able to rapidly detect and quantify small cell volume changes on-line.

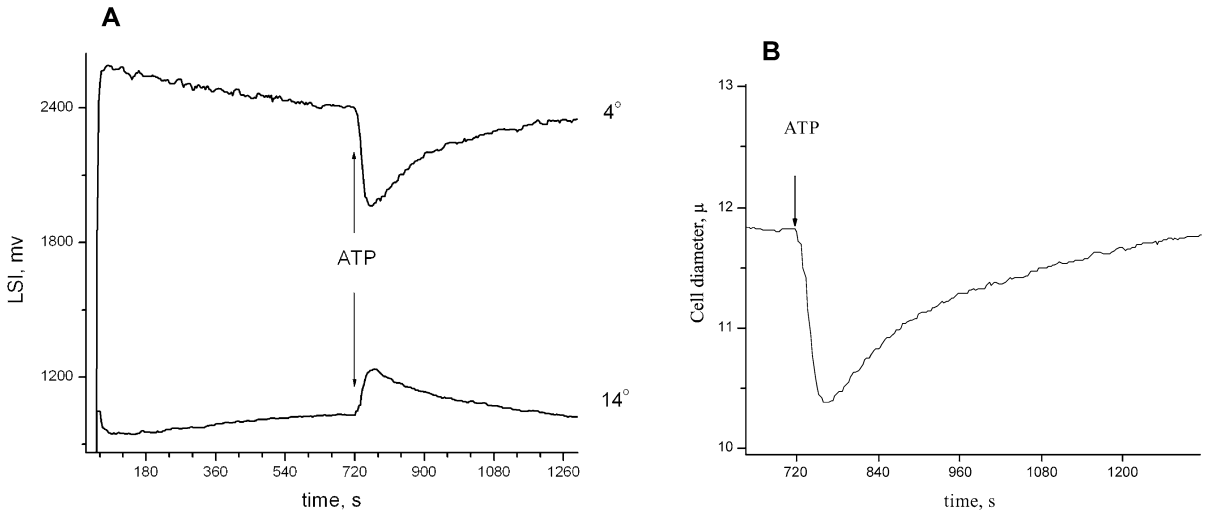

Fig. 6. (A) ATP-induced (10 μmol l^{-1}) changes of LSI of EATC measured at 4 and 14 degrees. For convenience, LSI at 14 degrees is given with another coefficient of amplification. (B) ATP-induced (10 μmol l^{-1}) changes of cell diameter calculated according to formula (2).

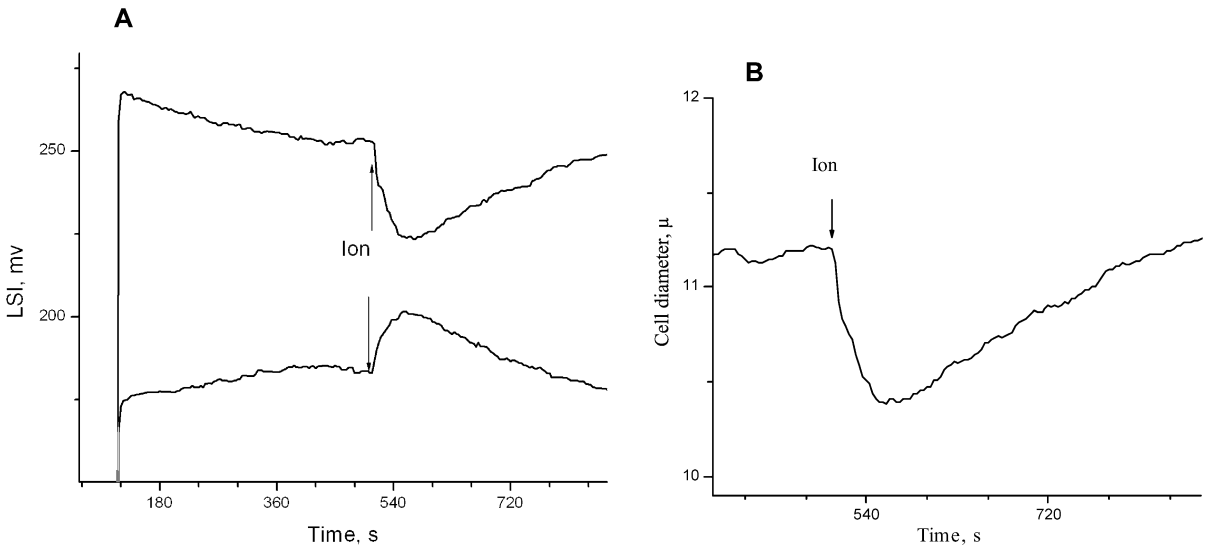

Fig. 7. (A) Ionomycin-induced (0.3 μmol l^{-1}) changes of LSI of EATC measured at 4 and 14 degrees. (B) Ionomycin-induced (0.3 μmol l^{-1}) changes of cell diameter calculated according to formula (2).

3.5. Thimerosal induces reversible high-amplitude changes of EATC volume

The structure and activity of most proteins, including calcium and potassium channels and Ca^{2+}-ATPases, are under the control of redox state of SH-groups [7]. Thimerosal oxidizes SH-groups in proteins. In different cells, thimerosal induces an increase in cytosolic $[Ca^{2+}]_i$ that is caused by activation of calcium channels and inhibition of Ca^{2+}-ATPases [8]. Thimerosal also stimulates mobilization of intracellular calcium through the IP_3-receptor [9]. As distinct from ATP and ionomycin which generate

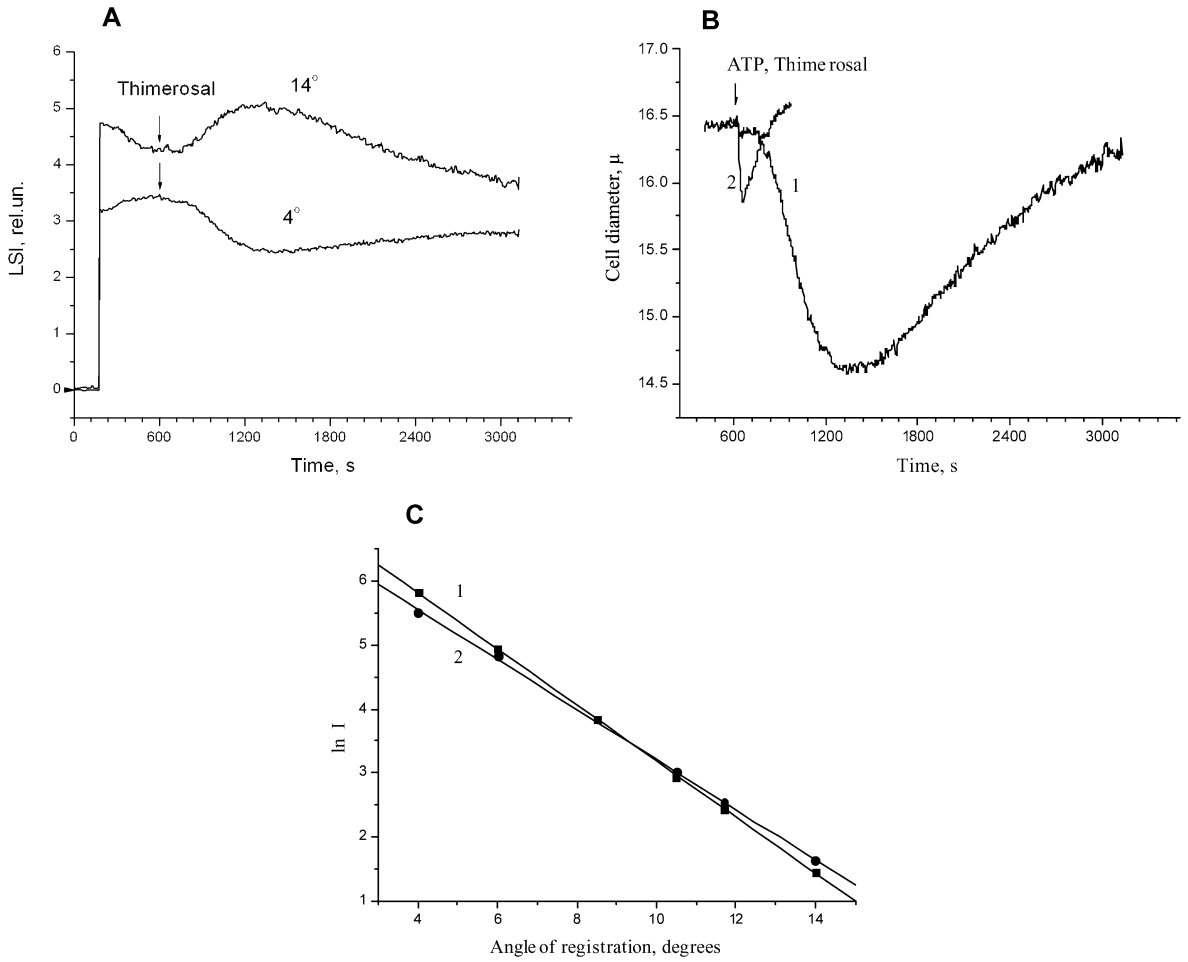

Fig. 8. (A) Change in LSI by EATC in response to thimerosal (40 μmol l^{-1}) at angles of 4 and 14 degrees. (B) Changes of cell diameter in response to thimerosal (40 μmol l^{-1}) (1), and ATP (5 μmol l^{-1}) (2). (C) Initial indicatrix of LSI by EATC (1) and indicatrix 10 min after addition of thimerosal (40 μmol l^{-1}) (2).

a cytosolic impulse of [Ca^{2+}], thimerosal causes a prolonged but low amplitude elevation of [Ca^{2+}]$_i$ due to SH-dependent opening of the store operated channels (SOC) of plasmatic membrane [8] and mobilization of Ca^{2+} from ER [9].

Figure 8A demonstrates that thimerosal induces a strongly pronounced and prolonged reversible change of LSI. The changes at angles of 4 and 14 degrees are opposite to each other – as was the case for cyclic swelling in hypotonic medium – and this corresponds with shrinking followed by swelling of the cells. Figure 8C shows two indicatrixes that correspond to initial state of the cells and to their state at maximum shrinkage. It is evident that for both cellular states there is a linear response but with different slopes. The point of intersection is the same as for the hypotonic medium (Fig. 4B), indicating the absence of any aggregation of the cells. Estimation of the cell size with the help of Eq. (2) revealed a much more pronounced contraction of the cells in comparison with that under action of ATP or ionomycin (Fig. 8B). A preliminary depletion of ER from Ca^{2+} by extracellular ATP addition completely inhibited the changes of cell volume in response to thimerosal (data not shown). Thus, the high ampli-

tude shrinking of the cells was probably caused by withdrawal of Ca^{2+} from ER as a result of inhibition of Ca^{2+}-ATPase and activation of calcium channels, the resultant elevation of $[Ca^{2+}]_i$ leading to the exit of K^+ and Cl^- through Ca^{2+}-dependent channels. The inverse process of swelling is probably caused by recovery of the intracellular ionic composition as a consequence of pumping Ca^{2+} out of the cells, closing of Ca^{2+}-dependent channels and the functioning of Na^+/K^+-ATPase.

3.6. Viscum album agglutinin (VAA) induced aggregation of EATC

Lectins are multidimensional glycoproteins that can bind with membrane hydrophilic carbohydrate residues, this binding being inhibited by complex oligosaccharides. It is well known that the lectin-induced aggregation of cells can occur in two different ways – with and without formation of inducible and durable contacts [10]. The formation of durable contacts is a consequence of aggregation or cooperation of cell receptors coupled with Ca^{2+}-dependent signaling which, as shown above, may cause the cell volume transitions. So there is a reduction of cell number, increase of cell size, and cell volume transitions can also take place under aggregation of the cells. Analysis of LSI at low angles makes it possible to estimate a contribution of each of these events to the LSI.

Aggregation of EATC was induced by addition of galactoside-specific lectin from white mistletoe, Viscum album agglutinin (VAA). The capability of VAA to induce aggregation is due to the presence of a carbohydrate-binding B-subunit in the lectin molecule. A particulate feature of VAA is that the corresponding cellular responses are blocked in the presence of galactose, which competes for the lectin binding sites with membrane galactosides. Figure 9A demonstrates that VAA, in contrast to ATP, causes decrease of LSI at all the angles of registering. Moreover, indicatrixes of the initial suspension of the cells and of those aggregated under action of VAA are parallel to each other (Fig. 9B). A conclusion that can be drawn from these data is that the change of LSI during aggregation of EATC under action of VAA is the result of reduction of cell quantity, but not the result of changes in their size. Thus, the scattering properties of the aggregated cells were not changed in comparison with the initial suspension. Studies of exogenous ATP-induced changes of the cells before and after their aggregation showed that EATC remained functionally relevant after aggregation, since addition of ATP caused the same cycle of shrinking-swelling as in the control cells (Fig. 9C). Thus, VAA-induced aggregation of the cells does not affect the mechanisms of Ca^{2+}-dependent activation; this aggregation proceeds without *macula densa* formation, and is not accompanied with elevation of $[Ca^{2+}]_i$ and the corresponding changes of cell volume.

4. Conclusions

The method of measuring the changes in cell volume by registering the indicatrix of LSI proved to be very sensitive and helpful in studies of intracellular signalling. In spite of some principal differences in the behavior of cells of different dimensions, there are many similarities and there is a good theoretical background for dealing with both small and large cells. For quantitative measurements of cell volume transitions, it is necessary to use cell concentrations that lie within the range of linear dependence of LSI upon cell concentration for the angles under consideration. This also allows use of LSI transition to monitor the cell concentration. In such conditions, the ratio of LSI at two angles can be used. Because of the exponential dependence of the LSI upon the angle of registration, the lower angles are preferable for increasing the signal-to-background ratio. However, very low angles should not be used in order to avoid indicatrix of the laser beam. In this research work we used the ratio of LSI at 4 and 14 degrees.

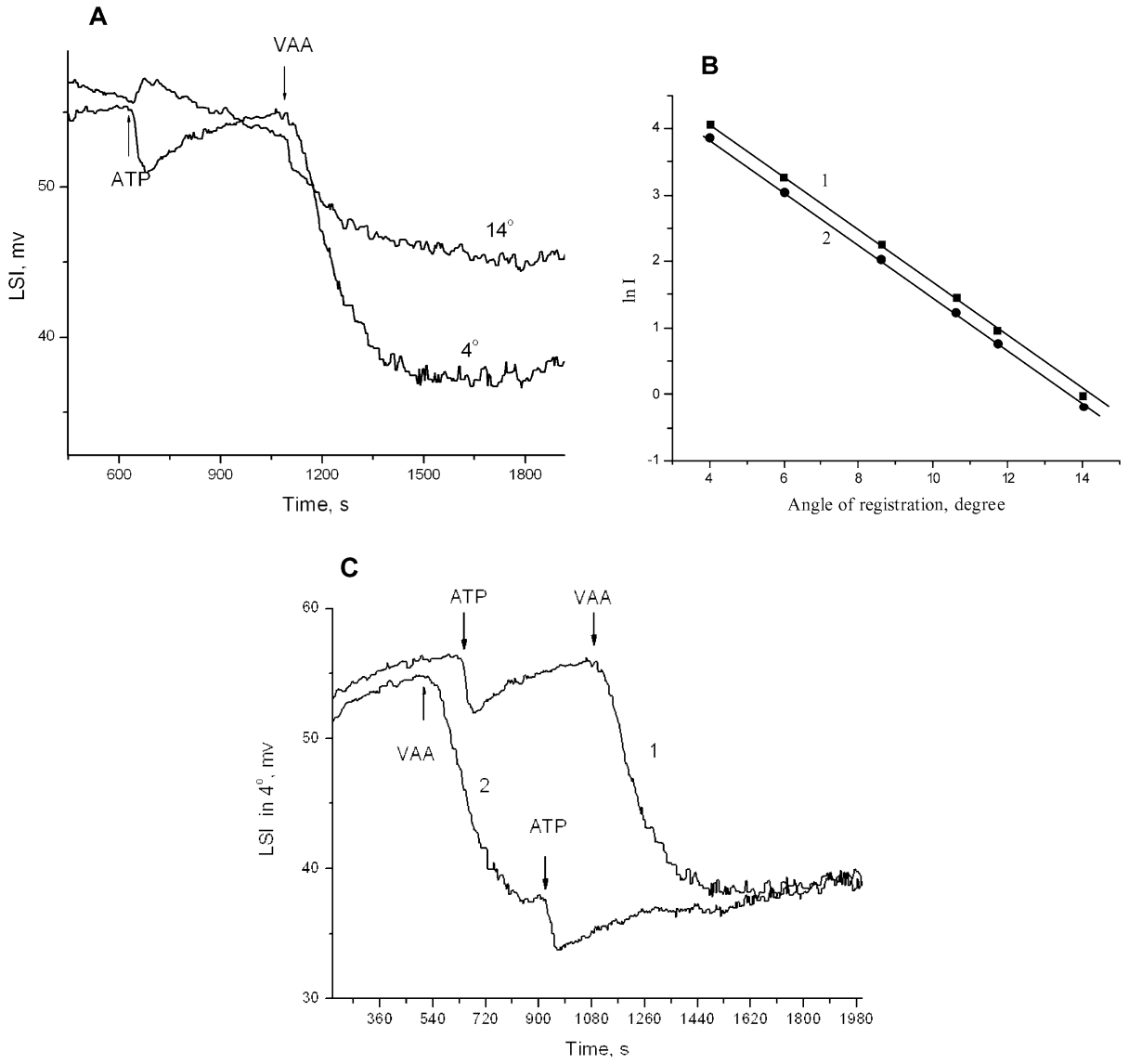

Fig. 9. (A) Change in LSI by EATC following addition of ATP (5 μmol l^{-1}) and VAA (2.5 μg ml^{-1}), registered at 4 and 14 degrees. (B) Initial LSI indicatrix of EATC (1) and indicatrix 6 min after addition of VAA (2.5 μg ml^{-1}) (2). (C) Change in LSI by EATC in response to ATP (10 μmol l^{-1}) before aggregation (1) and after aggregation in response to VAA (2). Registration at 4 degrees.

When dealing with small concentrations of the cells and low intensities of light scattering at the high angles, a ratio of LSI for measuring the cell diameter can be used at other angles, e.g. 6 and 10 degrees. Having obtained kinetic parameters of changes in the cell volume under activation of the cells, it is then possible to obtain quantitative measurements of K^+, Na^+ and anion flows that bring about this process, in order to understand causes and functional role of significant changes in cellular volume under their receptor-dependent activation.

Acknowledgement

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