Depolarization increases cellular light transmission

Minoru Tomita[1], Norihiro Suzuki[1], Yutaka Tomita[1,2], Haruki Toriumi[1], Takashi Osada[1], Miyuki Unekawa[1], Jemal Tatarishvili[1],

[1]Department of Neurology, School of Medicine, Keio University

[2]Department of Preventive Medicine for Cerebrovascular Disease, School of Medicine, Keio University

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Correspondence to:

Minoru Tomita, mtomita@sc.itc.keio.ac.jp

Department of Neurology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Application of optical methods to human brain tissue *in vivo*, e.g., measuring oxyhemoglobin and deoxyhemoglobin concentration changes with near-infrared spectroscopy (NIRS), requires the *a priori* assumption that background optical properties remain unchanged during measurements[1,2]. However, fundamental knowledge about light scattering by brain cells *per se* remains sparse; many factors influence light transmission changes through living brain tissue, bringing into question what is being measured. We have observed slow wave-ring spreads of light transmission changes on the rat cerebral cortex during potassium-induced cortical spreading depression (CSD) and ascribed them to squeezing-out of blood from capillaries by swollen brain cells[3,4]. However, in rat hippocampal slices, where no blood components were involved, similar light transmission changes were observed during K^+-induced CSD and ascribed to cell swelling and dendritic beading[5,6,7]. Here we show that two-dimensional light scattering changes occur through suspensions of osmotically swollen (depolarized) red blood cells, apparently arising from light scattering changes at the less curved, swollen surface of the steep electrochemical gradient coupled with water activity difference across the plasmic membrane. These optical property changes are likely to be relevant to interpretation of photometry or spectroscopy findings of brain tissue *in vivo*, where neurons are polarizing and depolarizing during brain

function.

The above findings imply that cell membrane depolarization/polarization during brain activity would contribute to the optical transmission changes of the brain tissue. Considering the complexity of the *in vivo* situation, we decided to use a simple experimental model to investigate the relationship between membrane depolarization and light transmission changes through the plasmic membrane. We previously observed that glutamate-induced swelling of cultured C6 cells (rat astroglioma) was associated with a membrane potential decrease from -49.7 mV to -23.0 mV[8], employing an intracellular microelectrode developed by T. Tomita[9]. However, we could not confirm light transmission changes through the cell suspension because of the inhomogenous distribution of the cells in the medium due to aggregate or sludge formation. In the present paper, we report an examination of the relationship between osmotic shock-induced cell swelling (depolarization) and light transmission changes using red blood cells (RBCs) as representative mammalian cells, since RBCs lack a nucleus and yet are known to be polarized with membrane potential at the plasmic membrane, which contains numerous channels and a sodium pump[10]. Further, it is easy to measure the isotropic diametric changes of RBCs, and to measure the light transmission through

RBC suspensions.

Figure 1 shows a continuous recording of changes in light intensity when the photodetector (PD) was rotated around the surface of the tube containing the suspension A (control). The rotation was done rapidly in a stepwise manner at intervals of 10°. As shown, the light intensity was low at around 0°, but became high at around 80° indicating that 1ight was more scattered to the side than transmitted. Microscopic observation revealed that the RBCs were swollen in hypotonic media (Figure 2: bottom). The leftmost picture is a microphotograph of an RBC having the diameter of 7.2 μm, taken with a video-enhanced contrast differential interferential contrast microscope[11]. As described in Materials and Methods, the RBC suspension was made successively more hypo-osmolar from A to E. Above the symbols for suspensions A-C are superimposed lines representing the edges of RBCs in those suspensions. RBCs in suspensions D and E were hemolyzed, so that the edges could not be traced. In A, RBCs exhibited a typical concave appearance. In diluted suspension B, RBCs showed slight swelling, and in C, RBCs showed marked swelling with an anisotropic convex appearance, but also some hemolysis. Although RBC swelling was heterogeneous and individually irregular, so that the values can not be considered precise, there was a clear trend for increasing diameter of RBCs with increasing dilution: 7.2 ± 0.9 μm (mean

± SD) for A, to 7.9 ± 1.2 μm for B, and 9.4 ± 2.9 μm for C (volume-wise, by 1.3x in B/A and 2.7x in C/A). Light intensities of scattered light at various angles are summarized in Table 1 where the mean values of 5 measurements at each angle are listed for A, B, C, D, and E, respectively. Changes of the angular distribution of light scattering by these suspensions are presented on a polar chart in Figure 2. Light transmission (forward scattering) was increased more than 10 times by hemolysis, while sideward scattering was decreased to one-third. These changes occurred despite the presence of the same concentration of hemoglobin and the same amount of plasmic membrane components in all of the suspensions. Changes in total light energy with cell swelling were calculated by integrating light intensities from +120° to -120°. They were 16.6 a.u. (arbitrary units) for A, 18.1 a.u. for B, 19.4 a.u. for C, 23.9 a.u. for D, and 34.7 a.u. for E. This is consistent with the increases in total area of light scattering with cell swelling shown in Figure 2.

An important implication of the present results is that light is scattered by intact cells, and that increase of the membrane tension associated with swelling is not relevant to the light scattering changes, since in swollen cells, the membrane must have been tensioned, and yet the light was less scattered. This may imply that the site of light scattering is not at the hydrophobic surface of the phospholipid membrane, but rather

at the anionic gel layer where complex hydrogen bonding networks of membrane components and the ionic gradient create an electrochemical osmotic gradient between the extracellular fluid and intracellular colloid-rich fluid. If this is the case, then the extent of light refraction depends on the magnitude of the osmotic gradient, and therefore on the polarization/depolarization of the membrane. It is a well known fact that light is reflected/refracted at such a water activity difference interface, and the principle is practically used for determination of colloid concentration in aqueous colloid solutions. It should be noted that sialic acid modification of the cell membrane is common to both RBC and nerve cells[13]. A comment on the relationship between cell swelling and membrane depolarization is worthwhile here. In general, depolarization occurs upon sodium ion (Na^+) influx, which is accompanied by water movement across the cell membrane[12.13], resulting in more or less cell swelling[14.15]. Functioning ganglion cells were reported to swell[16]. In the present experiment, water moved into the cell along the osmotic gradient created and maintained by the sodium pump. The water movement dissipates thermodynamic potential at the membrane, naturally leading to loss of the potential and depolarization. We believe that mammalian cells of normal size are in a state of entropy-minimum imposed by the action of the sodium pump, and the membrane is unstable and responsive to even minimal stimuli[14]. Cell swelling is a

self-perpetuating process, not energy-requiring. Energy is required for recovery of depolarized cells to their previous small size[14]. Depolarization and cell swelling are thus two sides of the same coin.

Additional points derived from the present observations are that 1) the attenuation of light intensity at 0° (light transmission) in suspensions is strongly influenced by light scattering, even when the amount of hemoglobin (and therefore the absorbance by hemoglobin as a whole) remains unchanged. When the cells swell, the light transmission increases, even though the cell membrane must become tensioned during swelling. Even more notably, light transmission increases 10 times in the hemolyzed cell suspension, even though the same amount of membrane components (ghosts) is still present. Thus, the membrane structure *per se* is not relevant to light scattering as a reflector/refractor of light. When incident light passes through a suspension, light is absorbed and scattered, and the remaining light emerges as transmitted light from the opposite side of the suspension. Within the suspension, photons are scattered and mixed. Light energy as a whole is mostly preserved, since reflection at biological membranes is elastic[16], and light radiates from the surface of the suspension. The suspension as a whole would start to glow like a glow lamp[17]. This was practically illustrated when a brain model made of clear polystyrene encasing a Mercox cast of rat brain vasculature

was placed in a dark room and illuminated with a single narrow monochromatic NIR beam (λ = 650 nm). The whole brain glowed, since light was scattered (mixed) by the material[17].

On the other hand, absorption of light at a specific wavelength occurs with loss of light energy. Absorption can be a negative divergence of photon flow[19], and absorbers are a sink of light energy. The increase in total light energy with cell swelling (Table 1) may be explained by lessened absorption. In fact, when RBCs swell, they become hypochromic, though the reason is not known. Another possible explanation for the increase in total light energy with cell swelling would be the directional shift of light scattering, described by the so-called transport scatter coefficient g[18], since our integration data did not include changes in the angular distribution of backward light scattering between the angles of -120°— 180°— +120°.

MATERIALS AND METHODS

The method used here was reported in our previous communication[20]. Briefly, measurements of the intensity of 1ight scattering at various angles around a transparent tube containing blood were undertaken with an apparatus fabricated in our

laboratory. As shown in the center of Figure 1, the apparatus consisted of a light source (LED), a transparent vinyl tube (2.6 mm in ID and 3.2 mm in OD) to hold the RBC suspension and a photodetector (PD). The LED was a gallium arsenide diode which emitted an infrared beam with a maximum wavelength at 950 nm (Hamai Electric Co., Tokyo). The beam was collimated perpendicularly to the suspension. The PD consisted of a silicon photodetector (SPD-550, Sharp Electric Co., Tokyo) having a narrow sensitive surface (0.5 mm x 2 mm), which was arranged so that its long axis was along the longitudinal direction of the tube. In the apparatus, the PD was so mounted that it could be moved in an arc on the surface of the tube at the cross-sectional plane containing the incident beam. By this means, the intensity of light scattering by the blood was determined at angles between +120°— 0°— -120°around the tube. The light scattering at 0° was called forward light scattering or light transmission and that around at ± 90° was considered as sideways scattering. The materials used were 5 samples of 20 ml freshly drawn heparinized whole blood from healthy male volunteers, who had given informed consent. The whole blood was centrifuged to separate RBC and plasma. Five suspensions were prepared by mixing plasma, RBC and distilled water. Suspension A was RBC 40% + plasma 60% (hematocrit 40%), suspension B RBC 40% + plasma 40% + distilled water 20%, suspension C RBC 40% + plasma 30% + distilled

water 30%, suspension D RBC 40 % + plasma 20% + distilled water 40%, and suspension E RBC 40 % + distilled water 60%, so that all suspensions had the same hemoglobin concentration per suspension volume. The osmotic changes in suspensions B, C, D, and E were designed to cause osmotic cell swelling to different degrees. Microscopic observations of RBC swelling were made on the suspensions placed on a slide glass and covered with a cover slip. Microscopic photographs were enlarged and printed, and the diameters of RBCs were measured with a ruler. The cell shape changes were not similar, but anisotropic. The discoid shape became spherical, with bulging of the central part of the cell body. However, the volume changes were calculated simply as the cube of the diametric changes, as a representation of cell swelling and therefore the degree of the depolarization of the cells. To avoid the flow effect due to RBC aggregation[17] the measurement was made within a short period of less than 20 s. Practically, after gently shaking a suspension in the container, the suspension was introduced into the tube gravitationally. The output of the SPD was continuously recorded on a DC recorder while the SPD was rotating around the tube. The magnitude of the intensity change read off in mV from the record was plotted against angle in polar coordinates. To estimate absorption loss of light intensity, the total light energy in A, B, C, G and E was broadly calculated as $\sum_{-120}^{+120} I$, and the values obtained were compared.

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END NOTE

M. Tomita and N. Suzuki planned and supervised the experiment. Y. Tomita and H. Toriumi conducted the experiment. T. Osada obtained blood samples from subjects. M. Unekawa and J. Tatarishvili contributed to the preparation of figures and calculation of results.

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FIGURES

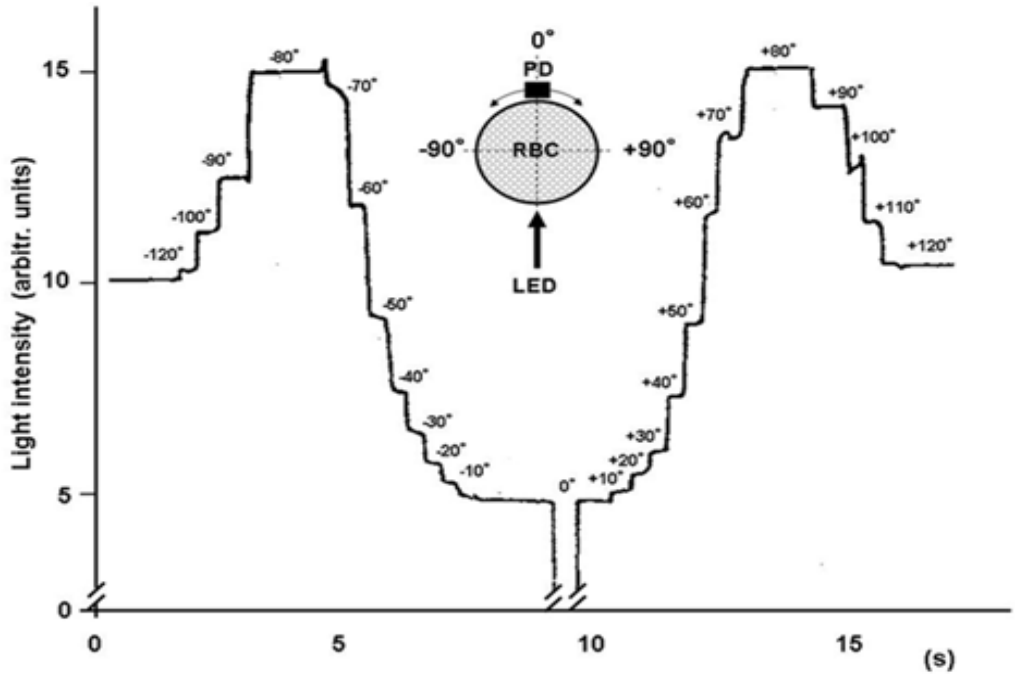

Figure 1. Schematic illustration of apparatus for continuous recording of light intensity by rotating the photodetector (PD) around the surface of the tube containing a suspension of red blood cells. The rotation was done rapidly in a stepwise manner at intervals of 10° within a period of about 16 s (see text for details). A typical output signal for suspension A (control) is shown (solid line).

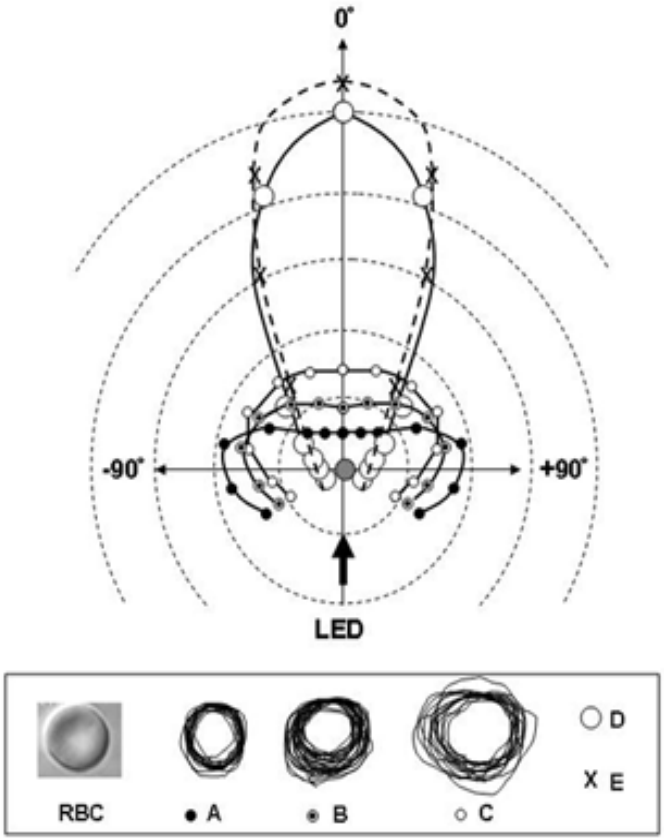

Figure 2. Light scattering changes diagrammed onto a polar chart. Closed circle is from suspension A (control); double circle, suspension B; small open circle, suspension C; large open circle, suspension D; cross, suspension E (hemolyzed blood). LED stands for light emitting diode. Underneath, on the left, is a microphotograph of a control RBC having the diameter of 7.2 μm. Above the symbols for suspensions A-C are superimposed lines representing the edges of RBCs in those suspensions. RBCs in suspensions D and E were hemolyzed, so that the edges could not be traced. For details, see the text.

TABLE

Table 1 Summary of results

	A	B	C	D	E
0°	1.2	1.8	2.8	9.2	10.6
20°	1.1	2	3	8.2	9.5
40°	1.4	2.5	3	2.5	6.6
60°	2.8	3.2	3.3	1.5	3.3
80°	3.8	3.5	3.2	1.2	1.9
100°	3.5	2.9	2.2	0.8	1.5
120°	2.8	2.2	1.9	0.5	1.3
	16.6	18.1	19.4	23.9	34.7

(in arbitrary units)