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The Response of Na^+/K^+-ATPase of Human Erythrocytes to Green Laser Light Treatment

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Summary

The objective of this study was to investigate the response of Na^+/K^+-ATPase of human erythrocytes to green laser irradiation. Effects of green laser light of fluences 9.5-63.3 $J.cm^{-2}$ and merocyanine 540-mediated laser light treatment were studied. Isolated erythrocyte membranes (protein concentration of 1 mg/ml) were irradiated by Nd:YAG laser (532 nm, 30 mW) and then incubated in a medium with 2 mM ATP for 30 min. Activity of ATPase was determined colorimetrically by measuring the colored reaction product of liberated inorganic phosphate and malachite green at 640 nm. Contribution of Na^+/K^+-ATPase to overall phosphate production was determined using ouabain. A positive effect of green laser light on Na^+/K^+-ATPase activity was observed. The dependence of enzymatically liberated inorganic phosphate on light fluence showed a linear correlation (R^2=0.96, P=0.0005) for all fluences applied (9.5-63.3 $J.cm^{-2}$). On the other hand, MC 540-mediated phototreatment caused a suppression of enzyme activity.

Key words

Na^+/K^+-ATPase activity • Biostimulation • Erythrocyte membrane • Merocyanine 540 • Photodynamic processes

Introduction

Lasers, as highly stable sources of coherent and monochromatic light, have been used extensively in technical applications and for medical therapy. The effect of laser irradiation on biological objects depends on experimental conditions, such as the type of cells irradiated, wavelength and intensity of light, etc. High-energy laser irradiation causes destruction and vaporization of tissues, which has been exploited in surgery. On the other hand, a positive effect of low-energy laser irradiation with red light on regeneration has been found in various tissues, such as skin (Conlan *et al.* 1996), bone (Yaakobi *et al.* 1996), nerves (Assia *et al.* 1986) and skeletal muscles (Weiss and Oron 1992). However, the molecular mechanisms of laser-induced changes in cell structure and function remain unclear. Efforts have been focused on studies of light-dependent changes in various biological objects, such as fibroblasts, mitochondria and blood components (Conlan *et al.* 1996). Among the latter, erythrocytes are most prominent because of their simplicity, availability and physiological importance. Several studies reported significant hemolysis and osmotic fragility caused by He-Ne laser

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irradiation (630, 670 nm) with powers over 200 mW/cm^{-2} (Fisher *et al*. 1998, Siposan and Lukacs 2000, Zavodnik *et al.* 2002). Erythrocyte deformation and platelet aggregation as well as an approx. 50 % drop in membrane microviscosity were reported after irradiation with 633 nm laser light (Makropoulou *et al.* 1995, Olban *et al.* 1998).

Enzymes are the natural focus in studies of laser light effects on biomembrane function and stability. Changes in enzyme activities can reflect shifts in the conditions both in and outside the cell.

Three ATPases can be found in erythrocyte membranes (Ca^{2+}-ATPase (EC 3.6.3.8), Mg^{2+}-ATPase (EC 3.6.3.1) and Na^+/K^+-ATPase (EC 3.6.3.9)) in various amounts (Drickamer 1975). The Na^+/K^+-ATPase is considered the most important of the three. This enzyme consists predominantly of two types of subunits: the catalytic α subunit (110-115 kDa) that spans plasma membrane ten times, and the β subunit (35 kDa) with a single transmembrane segment regulating conformational stability and activity of the α subunit. In association with the αβ heterodimer, a third small polypeptide, the γ subunit (10 kDa), has been found. It does not seem to be necessary for the Na^+/K^+-ATPase to be functional and may play a regulatory role (Ziegelhöffer 2000, Yu 2003). All three subunits form a complex membrane-bound ion pump that transports 3 Na^+ ions out and 2 K^+ ions into the cell and thus keeps the transmembrane potential balanced. The importance of this transporter system is given by the fact that it has been identified in virtually all animal tissues (Schwinger *et al.* 2003). This fact makes the enzyme interesting for biostimulation studies. So far, the effect of laser light on ATPase activity has been studied using red and infrared laser light sources (Kilanczyk *et al.* 2002, Kujawa *et al.* 2004).

The effect of other wavelengths and, in particular, of green light on the activity of the enzyme has not been studied yet. The main objective of our work was to study the effect of green laser light irradiation (532 nm) on the activity of Na^+/K^+-ATPase in erythrocyte membranes for fluences in the range 9.5-63.3 J.cm^{-2}, corresponding to the expected biostimulation effects. As green laser light is prospective in combination with a photosensitizer merocyanine 540 (MC 540) in applications such as photosterilization of blood in transfusions from viruses (Sieber *et al.* 1992a), bacteria (Dune and Slater 1998, Bednarska *et al.* 2003) and cancer cells (Sieber *et al.* 1992b), we also examined combined effects of MC 540 and 532 nm laser light on the activity of Na^+/K^+-ATPase.

Methods

Reagents

All standard reagents obtained from Polskie Odczynniki Chemiczne (Liwice, Poland) were of analytical grade (Tris, SDS, NaCl, HCl, Na_2HPO_4, NaH_2PO_4). TCA (trichloroacetic acid), ATP (adenosinetriphosphate) and EDTA were from Sigma-Aldrich (St. Louis, MO, USA). Merocyanine 540 (MC 540) was purchased from Fluka (Buchs, Switzerland).

Membrane preparation

Fresh human blood was collected under the guidelines of the Helsinki Declaration for human research at Central Blood Bank of Łódź from anonymous adult healthy donors. Erythrocytes were obtained by centrifugation at 916 x *g* for 10 min at 4 °C. Excess plasma and platelets were removed and erythrocytes were washed three times with ice cold PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4). To obtain a suspension of isolated erythrocyte membranes (ghosts) in 5 mM Tris-EDTA-HCl (pH 7.4) buffer, approximately one volume of erythrocytes was hemolyzed in five volumes of 20 mM Tris-EDTA-HCl buffer (pH 7.4) and centrifuged at 19621 x *g* for 10 min at 4 °C. Double washing with 20 mM, 10 mM and 5 mM Tris-EDTA-HCl buffer followed (Hanahan and Ekholm 1974). For unification, protein concentration in ghosts was estimated according to the standard method of Lowry *et al.* (1951) using 3 % sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) as a standard. For all experiments, ghosts were resuspended to a final concentration of 1 mg of protein per ml of (5 mM Tris-EDTA-HCl) buffer (pH 7.4). Adding EDTA as a calcium ion chelator caused inactivation of Ca^{2+}-ATPase during preparation and incubation of membranes.

Irradiation procedure

Second Harmonic Generation of Nd:YAG laser (Raise Electronics, Taiwan) with a constant light power of 30 mW was used as a source of green light (532 nm). A volume of 110 µl of each sample was irradiated from 100 to 680 seconds to attain fluence (i.e. light energy per unit area of sample surface received by irradiated sample) of 9.5, 19.0, 28.4, 38.0, 47.5, and 63.3 J.cm^{-2}. In a separate experiment, the sample was incubated for

30 min with MC 540 (final concentration 1 μM) in the dark and irradiated with a fluence of 47.5 J.cm^{-2}. Samples neither irradiated nor treated with MC 540 were used as controls.

ATPase activity measurements

Activity of ATPases in erythrocyte membranes was determined in terms of liberation of inorganic phosphate during enzymatic ATP hydrolysis, and was expressed in nmol phosphate per mg of protein released during 30 min incubation (Pi). Calibration was based on KH_2PO_4 as a standard (Bonting and Canady 1964). Briefly, 15 μl of ghost suspension was incubated with 55 μl of medium (100 mM Tris-HCl, 10 mM $MgCl_2$, 15 mM KCl, 85 mM NaCl, 1 mM EDTA, 2 mM ATP, pH 7.4) at 37 °C for 30 min to observe both non-enzymatic and enzymatic ATP hydrolysis. In parallel, 15 μl of the ghost suspension from the same samples were mixed with 55 μl of medium and incubated at 4 °C for 30 min. These served as a control for non-enzymatic hydrolysis of ATP. Both reactions were stopped by adding an equal volume of 15 % TCA. Inorganic phosphate liberated during ATP hydrolysis formed a colored product with malachite green and absorbance values were estimated spectrophotometrically at 640 nm as described by Baykov *et al.* (1988) using a Spekol 11 spectrophotometer (Carl Zeiss, Jena). Na^+/K^+-ATPase activity was evaluated from the difference between the total (Mg^{2+}, Na^+/K^+) ATPase activity and Mg^{2+}-ATPase activity (incubated in the presence of 0.2 mM ouabain to inhibit the sodium/potassium pump). Unless stated otherwise, all experiments were done at room temperature 22±2 °C.

Statistical analysis

Results were expressed as mean ± S.E.M. of 7-8 independent experiments. Data were examined for normal distribution by the Shapiro-Wilks W test. Statistical significance was evaluated by two tailed *t*-test and the level of statistical significance was set to $P<0.05$. Linear regression was used to prove the trend of the measurements.

Results

In this study, the effect of laser light (532 nm) on the activity of the Na^+/K^+-ATPase in the human erythrocyte membranes was assessed by the quantity of P_i released in the process of enzymatic hydrolysis of ATP. Figure 1 shows a significant increase in the concentration of P_i produced by Na^+/K^+-ATPase in all irradiated samples. The increase is directly proportional to radiation energy used. The least-squares fit through the experimental points (concentration of P_i *vs* energy fluence, Fig. 1) showed linearity (R^2=0.96, P=0.005) with a slope of 2.3 within the range of fluence values of 0- 63.3 J cm^{-2}. Thus, we observed a biostimulating effect of green light on ATPase activity, proportional to the energy of irradiation (9.5-63.3 J.cm^{-2}).

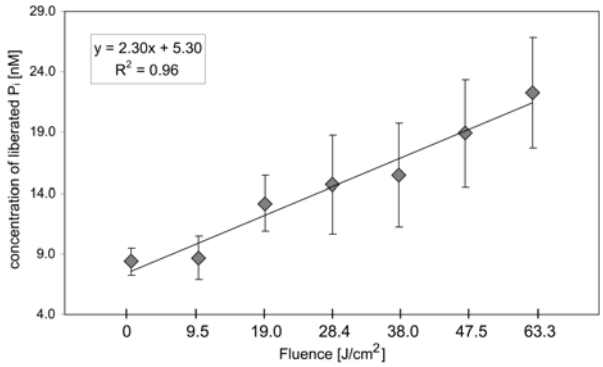

Fig. 1. Activity of Na^+/K^+-ATPase of red blood cells irradiated with Nd:YAG laser of various fluences. Results are presented as mean ± S.E.M. of the concentration of inorganic phosphate (n=8). Equation of the trend line and coefficient of determination (R^2) are shown.

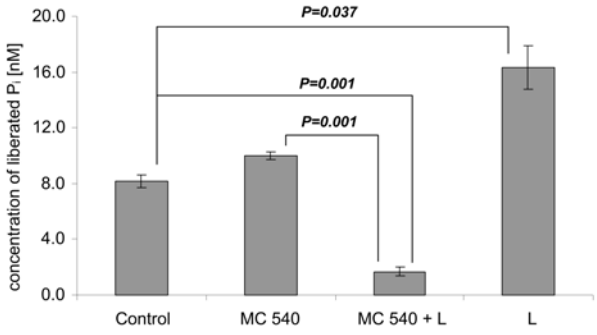

Fig. 2. Activity of Na^+/K^+-ATPase of red blood cells in the presence and in absence of MC 540 and irradiated with green laser light with fluence of 47.5 J.cm^{-2}. C - control; MC 540 - non-irradiated sample in the presence of MC 540 (1 μM); MC 540 + L - sample exposed to the combined action of MC 540 (1 μM) and light fluence of 47.5 J.cm^{-2}; L - sample irradiated by light fluence 47.5 J.cm^{-2}. Results are presented as mean ± SEM (n=7) of the concentration of inorganic phosphate.

In the second part of our experiments, incubation of ghost suspension with the photosensitizer MC 540 was followed by irradiation with 532 nm laser light of fluence 47.5 J.cm^{-2} and activity of Na^+/K^+-ATPase was determined as described above. Our findings show a

dramatic decrease of the Na^+/K^+-ATPase activity due to the combined action of MC 540 and 532 nm laser light, opposite to the biostimulating effect of laser light itself. Incubation of ghosts with MC 540 alone showed no significant changes in Na^+/K^+-ATPase activity (Fig. 2).

Discussion

The experiments presented here show a positive biostimulation of human erythrocyte membrane Na^+/K^+-ATPase activity by green light irradiation within the fluences in the range of 9-63 $J.cm^{-2}$, and a negative effect of irradiation on enzyme activity in the presence of the photosensitizer MC 540. We would like to stress that (i) due to the time scale of our experiments, these effects are long-lived or permanent, (ii) the range of light fluences used is well below those expected to produce significant photodamage.

Considering the biological action of laser light irradiation, it should be noted that only absorbed light energy can affect a biological object. Consequently, the primary question is, which molecules in the investigated sample have the potential to absorb the 532 nm light. In human erythrocytes, green light (532 nm) may be absorbed by heme-containing compounds, in particular hemoglobin, but also by the enzymes catalase and peroxidase. Actually, in the human erythrocyte ghosts prepared by the isolation method (Hanahan and Ekholm 1974), the residual hemoglobin content has been reported to be high enough (Dimitrov and Sowers 1990), to be responsible for absorption of green light. In general, after photon absorption and promotion of electrically excited states, energy transfer and energy transformation may initiate a cascade of biochemical reactions and signaling pathways that can lead to a measurable biological effect. Unfortunately, these processes are not yet understood, although some mechanisms of light action on cells and on cellular signaling have been discussed recently (Karu 1998). The proposed mechanisms are mostly related to specific mitochondrial structures, particularly to components of the respiratory chain (cytochrome c oxidase, flavins, dehydrogenases, cytochromes). As mitochondria are absent in red cells, direct activation *via* light absorption by components of the cellular respiratory chain in mitochondria cannot be considered.

In case of photostimulation of erythrocytes, we must take into account the "transient local heating hypothesis" (Karu *et al.* 1994, Karu 1998): Generally, a substantial fraction of absorbed light energy is inevitably converted to heat, which causes a local transient increase in the temperature of absorbing chromophores (Karu *et al.* 1995, Karu 1998). It should be noted that the local transient heating of absorbing molecules is quite different from average heating of the whole cell, tissue and organism. The local transient increase in temperature may cause structural (e.g. conformational) changes, and trigger biochemical activity. Changes in Na^+/K^+-ATPase activity may arise from conformational alterations, involving a rearrangement in the active site of the protein (Lumry 1959). In the membrane, Na^+/K^+-ATPase is surrounded by a ring of lipids, which can affect its function due to lipid-protein interactions (Brotheus *et al.* 1980). Thus, activity of the enzyme may be influenced by changes in the enzyme surroundings, which could be modified by absorbed light energy.

Another mechanism to be considered is related to the generation of reactive oxygen species (ROS) or free radicals as a result of absorption of light quanta by a photoacceptor molecule. ROS and free radicals can, in turn, initiate peroxidation of membrane phospholipids (Girotti 1998, 2001). However, lipid peroxidation has been shown to be responsible for a decrease of Na^+/K^+-ATPase activity (Rauchová *et al.* 1995) and we do not know how it could account for the reported positive biostimulating effect of green light in the range of fluences used in this study (9-63 J cm^{-2}). For higher light energies, oxidation followed by suppression of ATPase activity can occur. Besides this, laser light might affect several reactions involved in the signaling pathway.

Along with the positive biostimulating effect of irradiation alone, quite different results were obtained when we applied the 532 nm laser light (47.5 J. cm^{-2}) in combination with the membrane-directed photo-sensitising dye MC 540, which intensively absorbs green light. The combination of MC540 and green light has a clear photodynamic effect, which has been described extensively in the literature (Lagerberg *et al.* 1996, Girotti 1998, 2001). Therefore, it is not surprising that the activity of erythrocyte membrane Na^+/K^+-ATPase is drastically reduced (Fig. 2), while incubation of isolated erythrocyte membranes with MC 540 in the dark did not affect the enzyme activity. However, this has not yet been shown on membrane systems such as erythrocyte ghosts. Similar results were obtained by Feix *et al.* (1991). They found that irradiation of MC 540-sensitized erythrocyte membranes with white light had a negative effect on the enzyme activity. To elucidate the mechanism of this effect, the results of the well-documented experiments

showing that MC 540 activated with white or green light causes peroxidation of unsaturated membrane lipids may help (Kalyanaraman *et al.* 1987, Singh *et al.* 1992, Šikurová *et al.* 2001). This was reported to be a factor responsible for a decrease of enzyme activity (Rauchová *et al.* 1995). Feix *et al.* (1991) also found that MC 540-photomediated treatment resulted in a marked increase in protein rotational freedom, intermolecular cross-linking of proteins, and a loss of SH-groups. Thus MC540+light-induced structural perturbations in membranes may also cause a loss of Na^+/K^+-ATPase activity.

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