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ScienceDirect
Journal of Photochemistry and Photobiology B: Biology 89 (2007) 22–28
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

Biostimulation of Na,K-ATPase by low-energy laser irradiation (685 nm, 35 mW): Comparative effects in membrane, solubilized and DPPC:DPPE-liposome reconstituted enzyme

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Received 7 May 2007; received in revised form 25 July 2007; accepted 30 July 2007
Available online 7 August 2007

Abstract

Objective: The aim of the present work was to investigate the effect of low-energy laser irradiation (685 nm, 35 mW) on the ATPase activity of the different forms of the Na,K-ATPase.
Methods: Membrane-bound and solubilized $(\alpha\beta)_2$ form of Na,K-ATPase was obtained from the dark red outer medulla of the kidney and proteoliposomes of DPPC:DPPE and Na,K-ATPase was prepared by the co-solubilization method. Irradiations were carried out at 685 nm using an InGaAIP diode laser.
Results: The ATPase activity of the membrane fraction was not altered with exposition to irradiation doses between 4 and 24 J/cm^2. However, with irradiation doses ranging from 32 to 40 J/cm^2, a 28% increase on the ATPase activity was observed while when using up to 50 J/cm^2 no additional enhancement was observed. When biostimulation was done using the solubilized and purified enzyme or the DPPC:DPPE-liposome reconstituted enzyme, an increase of about 36–40% on the ATPase activity was observed using only 4–8 J/cm^2. With irradiation above these values (24 J/cm^2) no additional increase in the activity was observed. These studies revealed that the biostimulation of ATPase activity from different forms of the Na,K-ATPase is dose dependent in different ranges of irradiation exposure. The stimulation promoted by visible laser doses was modulated and the process was reverted after 2 h for the enzyme present in the membrane and after about 5 h for the solubilized or the reconstituted in DPPC:DPPE-liposomes.

Keywords: Na,K-ATPase; Bioestimulation; ATPase activity; Low-energy laser irradiation; Solubilized enzyme; Membrane enzyme; Proteoliposome

1. Introduction

"Biostimulation" or "biomodulation" are processes induced by low-intensity (the output power of laser devices in the mW range) irradiation that is characterized by its ability to induce stimulatory effects [1–4].

The explanation of the photobiological effects of laser light is based on the light absorption by primary endogenous chromophores (mitochondrial enzymes, porphyrins, flavins, cytochromes). Many studies *in vivo* and *in vitro* showed the influence of laser irradiation on cellular functional state [2–4]. At the same time, other groups of work found no detectable effects from low visible light exposure [1].

Primary changes in the structure of the lipid bilayer of a red cell membrane in response to visible laser irradiation and activation of an antioxidant system were observed [5]. It was established that laser irradiation of blood from patients with exertion stenorcadia was accompanied by the increase of the activity of erythrocyte membrane ATPases, as an indicative of erythrocyte deformability and

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doi:10.1016/j.jphotobiol.2007.07.007

positive changes of cardial function [5,6]. It was suggested that optimization of structural–functional organization of the erythrocyte membrane as a result of laser irradiation may be the basis for the observed improvement of the cardiac function in patients under a course of laser therapy [2].

Laser light processes present some special features, which allow it to selectively and precisely influence some targets, including biological ones [1,4,7]. The biological effects of irradiation depend on wavelength, power, time of exposure and irradiation dose or fluence [7,8]. Small doses induce stimuli, large doses depress physiologic processes, while extreme ones cause destruction of biological structures (as for example, a lipoperoxidation or heating which causes protein denaturation) [7,9–12]. Laser light biostimulation is mainly a photobiological phenomenon, but the response of biological membranes and their components to low-power laser irradiation has not been fully elucidated [2].

In our group we have been working with the $(\alpha\beta)_2$ form of Na,K-ATPase from the outer medulla of rabbit kidney [13–16]. Na,K-ATPase is a member of the P-type family of active cation transport proteins. It is found in the plasma membrane of virtually all animal cells and has been isolated in membrane-bound form from tissues rich in protein, such as mammalian kidney [17]. The enzyme complex undergoes a conformational change between two main reactive states, E_1 and E_2, in the multi-step reaction sequence in which 3 Na^+ ions and 2 K^+ions traverse the membrane [18–20].

It is well established that the enzyme complex consists of two polypeptide chains: the α-subunit or catalytic chain (relative molecular mass ~110 kDa) that is responsible for the enzymatic activity of the complex [19,21], and the β-subunit (relative molecular mass 35–50 kDa) that has a structural function, and may also play a functional role in the catalytic reaction and ion-pumping mechanism [19,22]. A third γ-subunit (found only in the kidney) is a small hydrophobic proteolipid (relative molecular mass 7–12 kDa) that is associated with the Na,K-ATPase which, although not essential either for catalytic activity or for ion transport, does act as a kinetic regulator [23–25].

The Na,K-ATPase has been solubilized in several detergent systems and purified by different methods [13,15,26] to yield a form which permits reconstitution into phospholipid vesicles [14,26–29]. The complete solubilization of the phospholipid is critical for the success of the subsequent formation of proteoliposomes [30]. The best results for Na,K-ATPase reconstitution were obtained using a 1:1 (w/w) DPPC:DPPE mixture with a lipid:protein 1:3 (w/w) ratio resulting in proteoliposomes containing 89% of protein with average diameter of 140 nm with a 80% recovered ATPase activity. Integrity studies of the Na,K-ATPase in this vesicular system have demonstrated that the enzyme is reconstituted with the ATP hydrolysis site located at the external side of the lipid bilayer vesicle, called *inside-out* orientation [14,28,29].

The biostimulation and therapeutic effects of low-power visible irradiation of different wavelengths and light doses are well known, but the exact mechanism of action of the laser irradiation with living cells is not yet understood [1–4,31–33]. The role of membrane ATPases as possible targets was analyzed. The aim of the present work was to investigate its effect at 685 nm and radiant doses varying between 4 and 44 J/cm^2 in the structure of Na,K-ATPase purified, membrane fraction and reconstituted DPPC:DPPE-liposome.

2. Materials and methods

2.1. Materials

All solutions were made with Millipore DirectQ ultra pure apyrogenic water and all reagents were of the highest purity commercially available: trichloroacetic acid (TCA); tris[hydroxymethyl] aminomethane (Tris); *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (HEPES); adenosine-5′-triphosphate Tris salt (ATP); bovine serum albumin (BSA) and dodecyloctaethyleneglycol ($C_{12}E_8$), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetracetic acid (EDTA), KCl, NaCl and $MgCl_2$ were from Merck. Biobeads were from BioRad.

2.2. Preparation of Na,K-ATPase

Membrane-bound and solubilized Na,K-ATPase was obtained from the dark red outer medulla of the kidney of adult New Zealand white rabbits (*Oryctolagus cuniculus*) as previously described [15]. $C_{12}E_8$ solubilized enzyme was concentrated in a YM-10 Amicon filter, dialyzed overnight at 4 °C against 5 mM Tris–HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl and 0.005 mg/mL $C_{12}E_8$. The enzyme was purified by gel filtration at 4 °C on a Sepharose 6FF column (26 × 200 cm) equilibrated and eluted in the same buffer, using a flow rate of 1 mL/min, in an AKTA Purifier equipment. Finally, 1.0 mL aliquots were frozen in liquid nitrogen and stored at −20 °C. The molecular mass obtained for the purified enzyme was 320 kDa which is consistent with the $(\alpha\beta)_2$ form.

2.3. Reconstitution of Na,K-ATPase in DPPC:DPPE-liposomes

Proteoliposomes of DPPC:DPPE were prepared by co-solubilization of lipids, protein and detergent in a weight ratio of 1:1 lipid:lipid and 1:3 lipid:protein as previously described [14,29]. The lipid mixture was dissolved in chloroform and dried with nitrogen flow to deposit a thin lipid film on the wall of the glass tube. These films were then dried under vacuum for about 1 h. This lipid film was