Changes in Calcium Transport in Mammalian Sperm Mitochondria and Plasma Membranes Caused by 780 nm Irradiation

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Background and Objective: Regulation of intracellular Ca\textsuperscript{2+} concentrations are very important in control of sperm motility and acrosome reaction. It was shown previously that low-power lasers in the visible and near-infrared range alter Ca\textsuperscript{2+} uptake by sperm cells. In the present work the effect of a 780 nm diode laser on Ca\textsuperscript{2+} uptake by sperm mitochondria and isolated plasma membrane vesicles is investigated.

Study Design/Materials and Methods: Digitonin-treated spermatozoa and plasma membrane vesicles were irradiated with a 780-nm diode laser at various powers and energy doses, and Ca\textsuperscript{2+} uptake was measured by the filtration method.

Results: It was found that 780-nm irradiation inhibits Ca\textsuperscript{2+} uptake by the mitochondria but stimulates Ca\textsuperscript{2+} binding by sperm plasma membrane vesicles. The effect of light on Ca\textsuperscript{2+} uptake by plasma membrane vesicles in the absence of ATP was much larger than that measured in the presence of ATP. Addition of Ca\textsuperscript{2+} ionophore decreased the Ca\textsuperscript{2+} uptake by the irradiated membranes in the presence of ATP but enhanced it significantly in the absence of ATP.


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Key words: Ca\textsuperscript{2+} binding; spermatozoa; plasma membrane vesicles; 780 nm diode laser

INTRODUCTION

Intracellular calcium plays a vital role in cell proliferation, and in mammalian spermatozoa it has a pivotal role in control of sperm motility [1] and acrosome reaction [2]. Therefore, the alteration in calcium levels in response to exposure to light may have considerable biological and clinical significance.

It was found that irradiating various cells with certain wavelengths at specified energy doses in the visible and in the far-red range resulted in acceleration of the Ca\textsuperscript{2+} uptake by the cells [3,4].

Light in the visible and far-red range can be absorbed by mitochondrial enzymes [5]. Therefore, it was suggested [6,7] that HeNe laser radiation activates the redox reactions in the respiratory chain by exciting mitochondrial porphyrins or cytochromes. This activation could lead to changes in [Ca\textsuperscript{2+}], in the cell. We have fixed our attention on the effect of light on Ca\textsuperscript{2+} transport in sperm cells. The systems which regulate intracellular Ca\textsuperscript{2+} concentration in spermatozoa in-

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Accepted 24 July 1997
volve the mitochondria. [8,9]. the plasma membrane ATP-dependent Ca\textsuperscript{2+} pump [10,11], the Na\textsuperscript{+}/Ca\textsuperscript{2+} antiport [12,13], and the Ca\textsuperscript{2+} channel [14]. In a previous work [3], we measured Ca\textsuperscript{2+} uptake by sperm mitochondria irradiated with HeNe (633 nm) light and found acceleration or inhibition of Ca\textsuperscript{2+} uptake, depending on the energy doses of the light. Irradiation of isolated plasma membrane vesicles with HeNe laser did not change their Ca\textsuperscript{2+} uptake ability.

In the present work we have examined Ca\textsuperscript{2+} uptake by sperm mitochondria and isolated plasma membrane vesicles irradiated with 780 nm light. At powers and energy doses used in our experiments, we found that Ca\textsuperscript{2+} uptake was inhibited in the mitochondria and increased in the plasma membrane vesicles. In the case of isolated plasma membrane vesicles we have investigated the nature of this Ca\textsuperscript{2+} uptake in the presence and absence of ATP and Ca\textsuperscript{2+}-specific ionophore A23187. We found that the elevation in Ca\textsuperscript{2+} uptake by the plasma membrane was a consequence of Ca\textsuperscript{2+} binding to the membrane after irradiation.

MATERIALS AND METHODS

Frozen ejaculated bull sperm cells (from Hashomer Artificial Insemination Center) were thawed at 37°C in medium comprising 150 mM NaCl, 10 mM histidine (pH 7.4). The cells were washed by three centrifugations at 600g, at 25°C for 10 min. The washed cells were resuspended in buffer containing 110 mM NaCl, 5 mM KCl, and 10 mM sodium morpholinopropanesulfonate (MOPS), pH 7.4.

Irradiation

A schematic representation of the irradiation set-up is given in Figure 1. The light source was a 780 ± 5-nm, 25-mW diode laser (Lasotronics). An optical multimode fiber made of silica glass was connected to the laser and was inserted into a rotating tube, at 37°C, containing 2–5 ml cell suspension of 3 × 10⁶ cells/ml or 30 μgprot./ml of membranes. The light power at the end of the fiber ranged from 3 mW to 25 mW. (Neutral density filters were used to reduce the intensity of the laser.) The irradiation time was 1–20 minutes. All light intensities were measured with an Ophir (model PD2-A) power meter.

The experimental set-up in this work differs from that used in our previous experiments on intact spermatozoa [3]. With the intact spermatozoa the Ca\textsuperscript{2+} uptake was measured while the cells were transferred to 96-well culture plate at room temperature and the light was defocused on each well. As we preferred to measure Ca\textsuperscript{2+} uptake into plasma membrane vesicles at 37°C and not at room temperature, we had to transfer the light through a fiber into a rotating tube immersed in a 37°C water bath [15]. In such a set-up, time of exposure of the cells to the light beam is unknown, so the results can only be qualitatively compared with those obtained previously [3] on intact sperm cells.

 Digitonin treatment. Since it is impossible to isolate coupled mitochondria from spermatozoa, we have decided to follow calcium transport into permeabilized sperm. Under these conditions, the Ca\textsuperscript{2+} uptake is completely abolished by the mitochondrial uncoupler CCCP [2,7] (cytocholyl cyanide para trifluoro methoxy phenyl hydrzone), indicating that the transported Ca\textsuperscript{2+} is accumulated in the mitochondria. Thus, we could test the irradiation effect on mitochondrial Ca\textsuperscript{2+} transport. A suspension of 4 × 10⁶ cells/ml in 5-ml buffer A was mixed with 65.5 μl of 14.27 mM dig-
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Itonin in dimethylsulfoxide. When motility was completely stopped, the suspension was centrifuged at 4°C at 600g. The pellet was resuspended with buffer M composed of 250 mM mannitol, 70 mM sucrose, and 10 mM TEA-Hepes (pH = 7.4), centrifuged as above, and then suspended in cold buffer M and kept on ice.

**Preparation of sperm plasma membranes.** The sperm plasma membranes were prepared as previously described by us [10,11]. The sperm cells were pelleted by centrifugation at 1,500g for 10 min; then the cells were washed four times in buffer NKM (110 mM NaCl, 5 mM KCl, and 10 mM MOPS, pH 7.4). The washed cells were resuspended in hypotonic medium (10 mM histidine (pH 7.4)/0.5 mM EDTA) and disrupted by using the Ultraturrax homogenizer (Janke and Kunkel KG IKA WERK Typ TP18-10) in the following way: 10 s low rate, 3 s high, 7 s low, 3 s high, and 7 s low rate. Low and high rates represent 3,000 and 14,000 rpm, respectively. The suspension was centrifuged at 3,000g for 10 min, and the supernatant was removed and centrifuged at 6,000g for 10 min. The supernatant was removed and centrifuged at 35,000g for 30 min; then the pellet was resuspended in the hypotonic medium. The suspension was layered on a discontinuous sucrose gradient composed of 0.5, 1.0, and 1.5 M sucrose solutions prepared in 10 mM histidine (pH 7.4). The tubes were centrifuged at 30,000 rpm for 18 h at 4°C using a SW 41 rotor, in Spincos of (Beckman) ultracentrifuge. The membrane fraction located just above the 1.5 M sucrose layer was removed and diluted with 10 mM histidine (pH 7.4)/0.1 mM EDTA at 4°C. The protein concentration was determined by the method of Lowry et al. [16] using bovine serum albumin as the standard of reference. The membranes were stored at −20°C prior to analysis. These membranes showed a 45-fold enrichment of the plasma membrane marker (Na\(^+\) + K\(^+\)) ATPase and less than 4% of the mitochondrial marker cytochrome c oxidase-specific activity found in whole cell homogenates. When examined by transmission electron microscopy, the membranes were vesicular, and mitochondria were not identified [see ref. 10,11].

**Calcium uptake by permeabilized cells.** Uptake of \(^{40}\)Ca\(^{2+}\) was determined by the filtration technique. Cells (3 x 10\(^8\) ml) were incubated in a medium containing 10 mM lactate, 0.5 mM phosphate, 0.2 mM CaCl\(_2\), and 0.5 μCl \(^{40}\)CaCl\(_2\). The cells were irradiated with 780-nm light (4 mW, 9 mW, 24 mW) immediately after the addition of calcium. At various time intervals (up to 12 min) 0.1 ml was removed and immediately vacuum-filtered on GF/C filters. The cells trapped on the filter were washed three times with 5 ml of solution composed of 150 mM NaCl, 10 mM Tris (pH 7.4), and 2 mM EGTA. The dry filters were counted in scintillation vials with 5 ml Aquasol (DuPont). Calcium uptake was calculated as nmol Ca\(^{2+}\)/10\(^6\) cells.

**Calcium uptake by isolated plasma membranes.** Ca\(^{2+}\) uptake by spermatozoa plasma membrane vesicles was measured in a 0.8 ml medium containing 18 mM histidine/18 mM imidazole buffer (pH 6.8), 0.1M KCl, 3 mM MgCl\(_2\), 0.18 mM CaCl\(_2\) (10 μM free Ca), 1 μCl \(^{40}\)CaCl\(_2\), 0.2 mM EGTA, and 240 μg plasma membrane protein. In some experiments, 10 mM A23187 Ca-specific ionophore (Calbiochem) was added. After a 5-min preincubation at 37°C, Na-ATP was added to achieve a final concentration of 2 mM ATP. Part of the experiments were done without exogenous ATP. The reaction mixture was incubated at 37°C and irradiated with 780 nm light At appropriate time intervals, samples (100 μl) were removed and vacuum filtered on 0.45-μm millipore filters. The membrane vesicles trapped on the filter were washed with cold water and placed in scintillation vials for measurement of the β radioactivity.

The experiments were carried out in triplicate for a single preparation of ejaculated sperm and at least three different preparations, each taken from a different animal, were used. The percentage of Ca\(^{2+}\) uptake of each sample relative to the maximum Ca\(^{2+}\) uptake of a non-irradiated sample in the absence of ATP was calculated. The results of at least three preparations were analyzed statistically by Student’s t-test and analysis of variance.

**RESULTS**

Exposure of sperm mitochondria (permeabilized cells) to 780-nm light at 4 mW and 9 mW at various energy doses, 0.2–6.0 J, resulted in Ca\(^{2+}\) uptake inhibition (Fig. 2). We used permeabilized sperm cells because it is difficult to isolate coupled sperm mitochondria. Their separation requires the breakage of disulfide bridges [17]. Digitonin treatment disrupts the sperm plasma membrane while leaving the mitochondria functionally intact [18]. When digitonin-treated cells were washed free of phosphate and mitochondrial substrate, little Ca\(^{2+}\) uptake occurred. However, a
high degree of Ca\(^{2+}\) uptake can be observed with the addition of mitochondrial substrate and phosphate. In this work we used lactate as a sperm mitochondrial substrate. Ca\(^{2+}\) uptake into permeabilized cells is above 90% inhibited by mitochondrial uncoupler (data not shown), indicating that most of the absorbed Ca\(^{2+}\) is accumulated in the mitochondria.

Irradiation of plasma membrane vesicles with 25 mW 780-nm light at energy doses of 1.6–30 J enhanced Ca\(^{2+}\) uptake by the membranes (Fig. 3). A similar enhancement was achieved with a 9-mW 780-nm laser (data not shown). Comparison of the relevant pairs, namely irradiated versus non-irradiated membrane specimens, reveals clear-cut statistically significant differences and indicates that the effect of light in the absence of ATP (P < .01) is much larger than that observed in the presence of ATP (P < .05). Ca\(^{2+}\) uptake in the non-irradiated control specimens was significantly higher in the presence of ATP as compared to that in the absence of ATP (P < .003), while the difference in Ca\(^{2+}\) uptake in the irradiated samples was not significant.

Addition of Ca\(^{2+}\)-specific ionophore A23187 to the reaction mixture in the absence of ATP (Fig. 4) resulted in a very small decrease of Ca\(^{2+}\) uptake in the non-irradiated control membrane preparations (P < .003). At the same time, the ionophore did not eliminate the effect of 780-nm irradiation; moreover, Ca\(^{2+}\) uptake in the irradiated membranes was additionally and significantly (P < .002) increased in the presence of the ionophore.

In the presence of ATP, addition of the ionophore (Fig. 5) drastically and significantly decreased Ca\(^{2+}\) uptake in both the non-irradiated control membranes (P < .003) and the irradiated specimens (P < .03); in the latter case, Ca\(^{2+}\) uptake in the presence of ionophore dropped to the level of the non-irradiated control in the absence of ionophore (see Fig. 5). The effect of light on Ca\(^{2+}\) uptake was much higher (see Figs. 4, 5) in the presence of the Ca\(^{2+}\)-ionophore than in its absence. In Figure 5, the light effect in the presence of A23187 seems to be lower in comparison to laser alone. But, if we subtract the controls, we find 6 times enhancement of Ca\(^{2+}\) uptake by light in the presence of A23187, and only 1.6-fold increase in its absence.
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![Graphs showing Ca²⁺ uptake](image)

Fig. 3. Ca²⁺ uptake by irradiated (25 mW, 780 nm) plasma membrane vesicles from sperm cells in the presence or absence of ATP. The 100% represent Ca²⁺ uptake by non-irradiated membranes in the absence of ATP at 20 min uptake time and corresponds to the uptake of 1.48 nM Ca²⁺/mg membrane protein. The time in the graph represents time of irradiation and time of Ca²⁺ uptake. Each value represents the mean of three experiments performed in triplicate. In Figures 3-6 it was possible to perform a linear fit transformation of the results obtained because of the high values of the correlation coefficients which ranged from 0.88 to 0.98.

DISCUSSION

The mechanism by which light in the visible and far-red range interacts with the living cell is still being debated. As intracellular Ca²⁺ concentration ([Ca²⁺]), is responsible for various cell activities, several authors investigated the effect of light on [Ca²⁺]. Young et al. [4] found an increased ⁴⁵Ca²⁺ uptake by macrophages after 660-, 820-, and 870-nm irradiation. Karu [19] found an elevation of [Ca²⁺], in lymphocytes after HeNe irradiation. We measured changes in Ca²⁺ uptake by bovine sperm cells due to 633-nm and 780-nm irradiation [3]. We found that light in the visible and in the far-red range at a specified energy dose accelerated Ca²⁺ uptake by the various cells. In order to better understand the mechanism by which light affects the cell, we measured Ca²⁺ uptake by HeNe-irradiated sperm mitochondria (permeabilized cells) and by isolated plasma membranes [15]. We found that HeNe laser irradiation at low power (0.3 mW) and low energy doses (0.06–0.2 J), produced enhanced Ca²⁺ uptake, whereas at higher power (10 mW) and doses (0.6–7.2 J) the uptake was inhibited. No effect was observed when sperm plasma membrane vesicles were irradiated with HeNe laser.

In the present work the influence of far-red, 780-nm, laser diode radiation was examined. As described in Figure 2, 780-nm light at 0.2–6.0 J,
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This indicates that 780-nm light enhances \( \text{Ca}^{2+} \) binding to the membrane and not \( \text{Ca}^{2+} \) accumulation in the intravesicular space. The fact that A23187 stimulates the light effect on \( \text{Ca}^{2+} \) uptake indicates that \( \text{Ca}^{2+} \) binding to intravesicular sites is also enhanced by the light. The observed difference in \( \text{Ca}^{2+} \) uptake by the mitochondria and the plasma membranes under HeNe (633-nm) irradiation and 780-nm diode laser irradiation indicates the importance of the effect of specific wavelength on calcium transport in sperm cells.

The stimulating action of visible and far-red light is assumed to be a consequence of light absorption by endogenous photosensitizers in the respiratory chain (RC) [6,19]. The electronically excited photosensitizers, cytochromes, or porphyrins produce reactive oxygen species (ROS) [20–22] which act as potent oxidizers stimulating the redox activity of the RC, enhancing the membrane potential (\( \Delta \Psi \)) across the inner membrane of the mitochondria and ATP production [23]. The enhanced \( \Delta \Psi \) and ATP production can modulate \( [\text{Ca}^{2+}]_i \) by enhancing \( \text{Ca}^{2+} \) influx into the mitochondria and by increasing the activity of the ATPase-driven pumps in the cell membrane [3,19]. However, in light of the results reported here, another possible mechanism should be considered, i.e., proteins may be the direct target of low-energy laser irradiation [24,25]. We assume that in the case of far-red light vibrational overtone excitation occurs, and not non-pigmented proteins can undergo a conformational transformation to either more activated conformation or to an inhibitory one. Thus, we ascribe the inhibition of \( \text{Ca}^{2+} \) uptake into the mitochondria irradiated by 780-nm light to inhibition of the enzymes of the RC [26] or the \( \text{Ca}^{2+} \) uniporter. On the other hand, the 780-nm radiation affects the conformation of proteins in the isolated plasma membrane in a way which increases the accessibility of \( \text{Ca}^{2+} \) to its binding sites. Alteration in \( \text{Ca}^{2+} \) binding undoubtedly can change intracellular \( \text{Ca}^{2+} \) concentration [27], leading to stimulatory effects. At high energy doses it can create the possibility of closing divalent ion channels, thus leading to cell death.

Since the effect of light is much smaller in the presence of ATP (Fig. 3), we assume that bound ATP might protect certain membranal proteins from undergoing light-induced conformational transformation. It is well known that binding of a substrate to an enzyme active site pro-

Fig. 5. \( \text{Ca}^{2+} \) uptake by irradiated (25 mW, 780 nm) plasma membrane vesicles from sperm cells in the presence of ATP and in the absence and presence of the \( \text{Ca}^{2+} \)-ionophore A23187, relative to maximum \( \text{Ca}^{2+} \) uptake by non-irradiated membranes (as designated in legend to Fig. 3). inhibits \( \text{Ca}^{2+} \) uptake by the mitochondria. On the other hand, 780-nm light at 1.5–30 J enhanced \( \text{Ca}^{2+} \) uptake by isolated plasma membrane vesicles (Fig. 3). The cell plasma membranes regulates intracellular \( \text{Ca}^{2+} \) concentration mainly by the ATP-dependent \( \text{Ca}^{2+} \) pump [10,11], the \( \text{Na}^+/\text{Ca}^{2+} \) exchanger [12,13], and by a voltage-gated \( \text{Ca}^{2+} \) channel [14]. Isolated plasma membrane vesicles can accumulate a significant amount of \( \text{Ca}^{2+} \) in the presence of added ATP. Without ATP, the uptake of \( \text{Ca}^{2+} \) is very low (Fig. 3). 780 nm light stimulates the \( \text{Ca}^{2+} \) uptake, and this effect is much higher in the absence of ATP. Also, the light effect is not canceled by adding the \( \text{Ca}^{2+} \) ionophore A23187 (\( \text{Ca}^{2+}/2\text{H}^+ \) exchanger), although the ATP-dependent \( \text{Ca}^{2+} \) accumulation was completely dissipated under these conditions (Fig. 4).
tects the enzyme from conformational transformations.

ACKNOWLEDGMENTS

We would like to thank the Israeli Ministry of Health for their support of this research and Lasotronic for providing us with the Med-140 780-nm diode laser. Many thanks to Avrille Goldreich for editing this manuscript.

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