THE EFFECT OF HeNe LASER (633 nm) RADIATION ON INTRACELLULAR Ca^{2+} CONCENTRATION IN FIBROBLASTS

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The increasing use of phototherapy (for example in wound healing) has led to a great interest in the mechanism of light-biosystem interaction. Therefore, recent observations on the enhancement of the proliferation of irradiated skin cells (fibroblasts and keratinocytes) by visible light are important to understand phototherapy at the cellular level. In the present communication we show that at the doses appropriate for enhanced fibroblast proliferation, HeNe irradiation produces transiently increasing calcium concentration in the target cells. Fibroblasts were irradiated with 1, 3 and 5 J/cm² HeNe (633 nm) laser and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in single cells was analyzed by Fluo-3/AM using a dynamic video imaging system with frame-grabbing software. Such observations can explain photobiostimulation because of the well-known vital role played by calcium in important biological processes such as proliferation and exocytosis.

Key words: Low energy lasers, fibroblasts, intracellular calcium concentration

Introduction

Low intensity laser irradiation (LILI) was introduced over 25 years ago in experimental treatments in medicine. LILI (mainly using HeNe lasers) has proven effective in promoting epithelization in full-thickness skin defects, as well as for gynaecological problems and aphthae of the oral mucosa.⁽¹⁻⁴⁾ Although reports on the effectiveness of HeNe laser in animals have appeared in the literature,^(5,6) contradicting reports have been accumulating concerning their effectiveness in humans.⁽⁷⁾ Some of the scepticism appears to arise from the absence of established mechanisms that might account for the photoeffects in traditionally non-photosensitive organisms exposed to visible light. Our previous studies on the effects of LILI on skin derived cultured cells,⁽⁸⁻¹⁰⁾ indicated that various parameters affect the extent of stimulation exerted by the lasers. These include the wavelength of irradiation, the intensity of the irradiation source, the energy dose and the model system used.

Irradiation at low energy densities of visible and near infra-red light 540 nm (4 J/cm²) (9), 633 nm (5 J/cm²) and 940 nm (0.4 J/cm²)⁽¹⁰⁾ were effective and significantly promoted proliferation of the cells. On the contrary, irradiation at higher energy densities of any of these sources inhibited the growth of the cells. Compa-

rable results were obtained by Colin et al.⁽¹¹⁾ with keratinocyte cultures irradiated with 633 nm coherent continuous light.

In order to affect the living cell, light has to be absorbed by some chromophores. It is generally assumed⁽¹²⁻¹⁴⁾ that the stimulating action of visible light is due to excitation of chromophores such as the flavins and the cytochromes in the respiratory chain (RC). As pointed out by Karu,⁽¹²⁾ there is a direct correlation between the action spectrum (the visible results of biostimulation) and the absorption spectrum of the pigments of the RC. This absorbed energy is transferred via photosensitization to molecular oxygen producing reactive oxygen species (ROS) as in photodynamic therapy.⁽¹⁵⁾ The ROS are powerful oxidants which amplify the redox activity of the RC. In particular, ATP production is increased, as shown in hepatocytes.⁽¹⁴⁾ This fuels the ATPase pumps in the cell membrane, thereby modulating Ca^{2+} concentration in the cytoplasm. As intracellular $[\text{Ca}^{2+}]_i$ oscillations play a vital rôle in cell proliferation, we have decided to measure free intracellular Ca^{2+} concentration changes in irradiated fibroblasts. $[\text{Ca}^{2+}]_i$ was analyzed by Fluo-3/AM using a dynamic video imaging system with frame-grabbing software. It was found that low energy HeNe light induces intracellular $[\text{Ca}^{2+}]_i$ variation in fibroblasts.

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Fig 1: Light induced intracellular calcium concentration ($[Ca^{2+}]_i$) variations. The cells were irradiated with 3J/cm² HeNe and the images of Fluo-3A/M calibrated $[Ca^{2+}]_i$ a: before irradiation; b:, c:, d:, and e: corresponding to 120 sec, 240 sec, 420 sec and 480 sec after irradiation are shown. The colour scale, representing fluorescence intensity (i.e. $[Ca^{2+}]_i$ levels, dark blue lowest, red highest) is given on the right hand side of 1a, and is the same for b, c, d and e.

Materials and Methods

NIH/3T3 fibroblasts grown on 25 mm glass coverslips were incubated with 2 μ M (final concentration) Fluo-3/AM (Molecular Probes Inc., USA) and 1.5 mM (final concentration) pluronic acid (Wyanodate Inc.) for 30 minutes in glucose enriched phosphate buffered saline (PBS) in humidified atmosphere of 5% CO₂ and 95% air at 37°C. After incubation, each dish was rinsed twice with glucose enriched PBS and the coverslip was placed in the microscope holder. Cells were covered with 1 ml glucose enriched PBS. Internal free Ca²⁺ concentration in irradiated cells was measured by using a fluorescence microscopic system. This system consisted of an inverted epifluorescence microscope (Axiovert 135M; Zeiss, Germany), an intensified couple-charge device C2400 (ICCD) camera (Hamatsu, Japan) and frame grabbing software (Galai, Israel). A 75 W Xenon lamp emitting light at 460-490 nm served as the source for excitation. A 510 nm long pass emission filter was used to select fluorescence emission.

The first three pictures were taken to estimate base levels of fluorescence. Following this, cells were irradiated in situ with a 6.7 mW HeNe laser (Meredith Instruments, USA).

The energy densities were 1.3 J/cm² or 5 J/cm². [Ca²⁺]_i in irradiated cells was measured every 30 sec,

for 10 min. Analysis of [Ca²⁺]_i was carried out using Scan Array 2 software (Galai, Israel).

Results

A single exposure of 3 J/cm² HeNe light resulted in a clear time dependent variation of intracellular Ca²⁺ concentration in a single fibroblast cell (see Figures 1, 2 and 3). Each curve in Figures 2 and 3 corresponds to a follow-up of a different cell. When irradiating the fibroblasts with 1 J/cm² HeNe light no change in the Ca²⁺ concentration was observed (Figure 4). Repeating these experiments with at least three other cell samples for each irradiation procedure essentially reproduced the same results.

After irradiation with 3 J/cm² or 5 J/cm² HeNe (633 nm) light, there is a peak in [Ca²⁺]_i. This peak, which lasts about 2 minutes, is followed by a latency period of about 4 minutes and new peaks are then observed (Figures 2, 3). Irradiation with 1 J/cm² HeNe light does not change intracellular Ca²⁺ concentration (Figure 4). It is interesting to note at this point that low fluences (1 J/cm²) which are unable to produce oscillations are also unable to produce proliferation.

No statistical evaluation of the fluorescence measurements was attempted since the [Ca²⁺]_i oscillations in different cells are not synchronous. However, the re-

Fig 2: Evolution of intracellular calcium concentration ([Ca²⁺]_i) following 3 J/cm² HeNe irradiation. The first three points in the graph indicated [Ca²⁺]_i in a single cell before irradiation. The (see arrow), the cells were irradiated during a period of 1.5 min (not shown in the graph), and immediately after irradiation [Ca²⁺]_i was monitored. Each curve corresponds to a follow-up of a different cell. It should be noted that the time scale is not linear! At t=0, there is a pause of 1.5 min in the imaging process which corresponds to the time needed for irradiation.

Fig 3: Evolution of intracellular calcium concentration ($[Ca^{2+}]_i$) before irradiation. Then (see arrow), the cells were irradiated during a period of 2.5 min (not shown in the graph), and immediately after irradiation $[Ca^{2+}]_i$ was monitored. Each curve corresponds to a follow-up of a different cell. As in Fig. 2 please note that the time scale is not linear. At $t=0$, there is a pause of 2.5 min in the imaging process which corresponds to the time needed for irradiation.

Fig 4: Evolution of intracellular calcium concentration ($[Ca^{2+}]_i$) following 1 J/cm^2 HeNe irradiation. The first three points in the graph indicate $[Ca^{2+}]_i$ in a single cell before irradiation. Then (see arrow), the cells were irradiated during a period of 30 sec (not shown in the graph), and immediately after irradiation $[Ca^{2+}]_i$ was monitored. Each curve corresponds to a follow-up of a different cell. As in Figs. 2 and 3 the time scale is not linear. At $t=0$, there is a pause of 30 sec in the imaging process which corresponds to the time needed for irradiation.

sults seem to indicate that the relative peaks of the $[Ca^{2+}]_i$ in irradiated cells are an order of magnitude larger than the uncertainties in the measurements.

Discussion

There have been many studies of Ca^{2+} oscillation using fluorescent imaging techniques. The most studied cells are cardiac cells, oocytes, hepatocytes, endothelial cells, fibroblasts and pancreatic acinar cells.⁽¹⁶⁾ Calcium oscillations arise either spontaneously^(17,18) or in response to stimulation by extracellular signals such as hormones or neurotransmitters.⁽¹⁹⁾ To our knowledge, the present work reports the first observation of low energy laser stimulation of $[Ca^{2+}]_i$ oscillations using a fluorescence imaging technique. Whereas in our work $[Ca^{2+}]_i$ variation in a single cell is monitored microscopically, previous work on laser induced effects were limited to bulk $[Ca^{2+}]_i$ variations.⁽²⁰⁻²²⁾ Thus, the effect of HeNe light on $[Ca^{2+}]_i$ in lymphocytes measured spectroscopically by Karu,⁽²⁰⁾ indicates that the kinetics of the variations of $[Ca^{2+}]_i$, after HeNe irradiation, resemble those obtained by adding the mitogen phytohemagglutinin (PHA). Young *et al.*⁽²¹⁾ found an increased bulk $45Ca^{2+}$ uptake by macrophages after 660, 820 and 870 nm irradiation. Similarly, we have measured bulk changes in $45Ca^{2+}$ uptake by laser-irradiated bovine sperm cells. We found an accelerated Ca^{2+} uptake with 6-18 J/cm² HeNe laser irradiation and inhibition in Ca^{2+} uptake with larger energy doses.⁽²²⁾

As was discussed previously,^(12,13,14) light in the visible range is responsible for photomodulation of the redox activity of the RC. We believe that the stimulating action of the visible light proceeds by exciting endogenous photosensitizers such as porphyrins or cytochromes in the RC, thereby producing ROS such as singlet oxygen or superoxide anions. the ROS act as potent oxidizers which stimulate the redox activity of the RC and lead to enhanced ATP production.⁽¹⁴⁾ We suggest the following mechanisms to explain how enhance ATP production produces the observed increased influx of Ca^{2+} into the cytosol. By virtue of the law of mass action an increase of ATP concentration accelerates the reactivation of the ATPase pumps in the cell membranes, increasing the activity of these pumps. As a result, membrane hyperpolarization and an increased $[Ca^{2+}]_i$ gradient can be expected across the cell membrane. Thus, the electrochemical gradient of Ca^{+2} is increased producing an increase influx of these ions through the cell membranes and thus a transiently increased $[Ca^{2+}]_i$ is obtained. It is well known that calcium ion oscillation are important intracellular signals⁽²³⁾ in virtually all cell types controlling many diverse processes.

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