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DISCLAIMER

LASER ACTION SPECTRUM OF REDUCED EXCITABILITY IN NERVE CELLS

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ABSTRACT

The change in excitability of unstained nerve cells from neonatal rat cerebellum was measured as a function of energy density and wavelength of incident laser light. The energy density was in the range of 0 to 30 $\mu J/\mu m^2$; six wavelengths between 490 and 685 nm were used. Laser pulses above a threshold energy density significantly reduced the cells' excitability. The sensitivity of the cells, defined as the inverse of this threshold energy density, increased by an order of magnitude toward the shorter wavelengths. These results are consistent with primary absorption of the light by mitochondrial enzymes, resulting in local heating followed by mitochondrial calcium release into the cytoplasm.

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Laser radiation, focused to subcellular dimensions, is known to produce lesions at a prescribed location within a single cell (1). A large increase in the selectivity of the irradiation to the intended target can be achieved by using vital dyes which selectively bind to cellular organelles such as nucleoli (2), mitochondria (3), lysosomes (4), and chromosomes (5). However, the stains themselves often produce physiological alterations which must be considered along with the laser microdissection. In some cases light may be absorbed by the dye at some other locus than the one intended. In the absence of dyes, light is absorbed by endogenous chromophores within the cell such as chlorophyll (6), hemoglobin (7), and the cytochromes (8). By proper selection of laser wavelength, damage may be restricted to a particular organelle or enzyme (9). Thus, the laser may be used to alter the characteristics of a single cell without the complicating factors introduced by added substances.

We previously reported the results of preliminary experiments which indicated a reduction in the rate of spontaneous activity of unstained nerve cells as a result of high intensity ruby laser irradiation (694 nm) (10). No change in rate occurred unless the incident laser energy density exceeded a certain threshold value. In our attempt to correlate the functional change produced by the laser with absorption by a particular constituent of the cell, we obtained an action spectrum of a reduced electrical

excitability using a liquid dye laser. The functional end point is the change in voltage needed to elicit an action potential when stimulated by current injection through an extracellular microelectrode. The results presented here corroborate our earlier experiments in that the laser absorption leads to a reduced electrical excitability, and that low doses of laser energy may be delivered with no apparent effect.

Cerebellar tissue culture was chosen as the object of our investigation because it provided a system of easily accessible neurons that were morphologically, pharmacologically, and physiologically similar to those in vivo (11). The ability to measure electrophysiological parameters of these cells within minutes before and after a laser pulse enabled a direct assessment of the immediate changes produced by the laser.

Explants of rat cerebella were obtained from two- to three-day old animals. The cerebellum was sliced parasagitally into six pieces and affixed onto a glass coverslip using a plasma clot. The explants were incubated in a roller drum at 37°C in an atmosphere containing 5% CO_2. The incubation medium, consisting of 25% Earle's balanced salt solution, 25% fetal calf serum, 50% minimum essential medium, and 5.5 mg/ml glucose, was changed twice weekly. After about two weeks a near monolayer of nerve cells and glia was obtained, permitting visualization of individual nerve cell bodies using phase contrast microscopy.

Electrophysiological recordings were made while the culture was bathed in Hank's balanced salt solution (without phenol red) and maintained at 33 ± 0.1 °C. Extracellular recording and stimulating micropipets were pulled to tip diameters of about 1 μm and filled with 2M NaCl. The micropipet resistance was in the range of 5 to 7 MΩ.

A liquid dye laser (phase-R model DL 1100), producing a 1 μsec-long pulse, was used for the irradiations. Broad-band laser emission (bandwidth approximately 10 nm) was obtained with maximum power centered at 480, 540, 580, 600, 650, and 685 nm using various dyes (12). The maximum focused laser energy available for irradiation varied from 15 μJ for 540 nm to 360 μJ for 685 nm. The energy of each laser pulse was measured with a photodiode and beam splitter arrangement. The response of this photodiode was standardized in a separate measurement against that of a calibrated photodiode placed in the focal plane of the microscope. The pulse energy was controlled by interposing neutral density filters in the path of the laser beam. The measured half-width of the focused laser spot was 2 μm. The laser pulses were delayed by 6 msec after a spontaneous action potential in order to study nerve cells in a defined state of polarization (13).

Large nerve cells (20 to 40 μm diam) of the type studied in this work show spontaneous electrical activity, which can be recorded with an extracellular electrode for several hours (14). Action potential

amplitudes and time intervals between action potentials were determined over several minutes prior to each laser pulse. Data (from approximately 10% of the cells) were discarded in those cases where the pulse amplitude did not remain stable or the firing pattern was not stationary.

The excitability was measured using the cells' response to extracellular stimulation. The neurons were stimulated with trains of 100 square voltage pulses of 0.5 msec duration and constant amplitude, V, through a micropipet positioned about 100 μm from the cell body. The response of the nerve cells was measured with a recording electrode in apparent contact with the cell soma under microscope observation. The ratio of elicited responses to stimulating pulses was measured as a function of the stimulus voltage, V. The voltage at which this ratio was equal to one-half of the maximum possible, or the half-maximal voltage (HMV) was determined for each cell before and after delivery of a laser pulse. The excitability was taken as (1/HMV). The relative excitability was then defined as $(HMV)_{before}/(HMV)_{after}$.

The relative excitability of each neuron under study was measured for a single laser pulse of prescribed energy and wavelength. Ten cells were irradiated at each wavelength, using different laser pulse energies. The relative excitability as a function of laser energy is shown in Figure 1 for 580 nm. This dependence is qualitatively the same for all wavelengths used. Low energy pulses produce no noticable

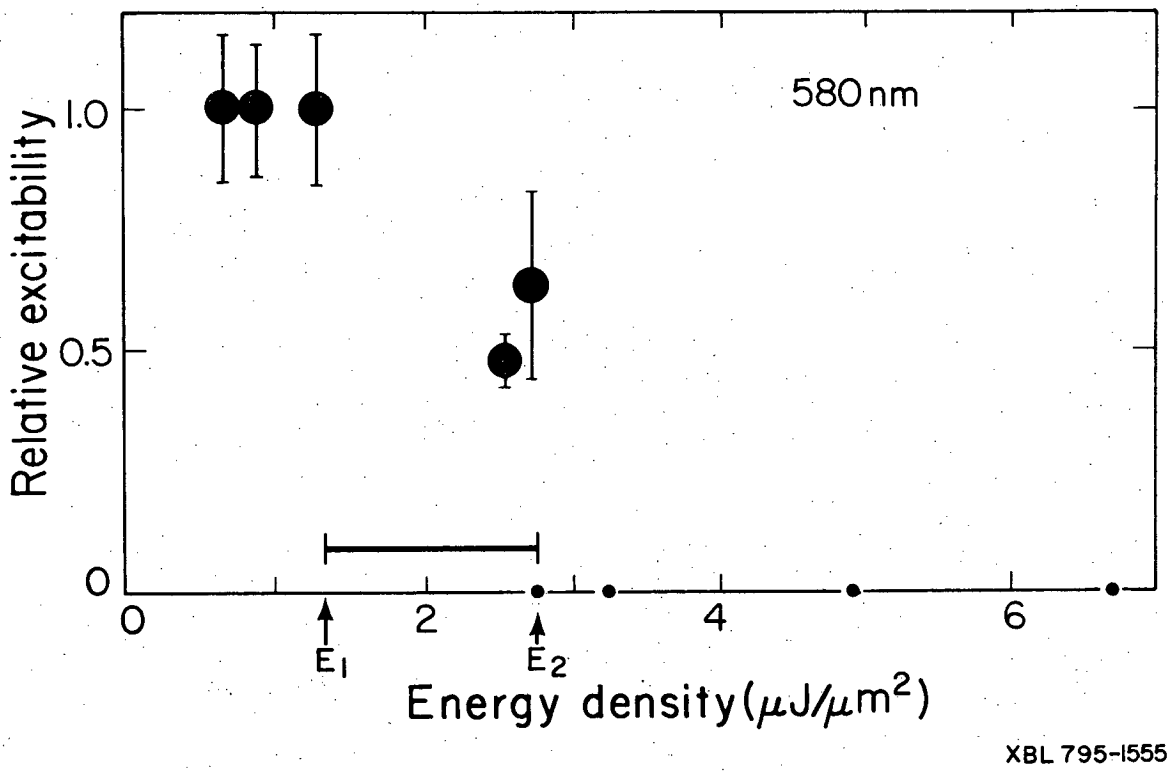

Fig. 1. The relative excitability after a single laser pulse as a function of laser energy density for irradiation wavelength equal to 580 nm. Each point corresponds to a determination made from a different cell. The excitability of the neuron is taken to be the inverse of the HMV as described in the text. The excitability after the laser pulse relative to the excitability before the pulse (or equivalently HMV_{before}/HMV_{after}) is plotted against the absolute laser energy density incident at the focus of the laser beam. The interval delimited by points E_1 and E_2 is the range of energy densities that characterizes the sensitivity of the neurons to the particular wavelength of light.

effect, while at high energies the cell no longer responds to stimulation. Over an intermediate range of energies, an increasing voltage is necessary to stimulate the cell.

The instrumental constraints made a continuous variation of laser pulse energy impracticable. However, as seen in Figure 1, a threshold energy density can be assumed to exist within the interval E_2 to E_1. This threshold interval was measured as a function of wavelength in order to obtain the neurons' action spectrum. A lower threshold energy implies a greater cell sensitivity, and accordingly, the cell sensitivity was taken as the inverse of this threshold energy. The results have been plotted in Figure 2, which shows the neuron sensitivity as a function of laser wavelength, with the sensitivity intervals corresponding to $1/E_1 - 1/E_2$.

The shape of the action spectrum is similar to that reported by Ham et al.(15) for threshold damage in Rhesus monkey retina following laser irradiation. Both curves show an increased sensitivity towards shorter wavelength with no indication of a peak sensitivity over the wavelengths studied (the spectrum in Ham et al. extends from 1064 to 441.6 nm).

Several investigations correlating the effect of visible laser irradiation with ultrastructural changes have concluded that the primary site of absorption within the neuron is likely to be the mitochondrion (8, 16). Although the action spectrum of Figure 2 has

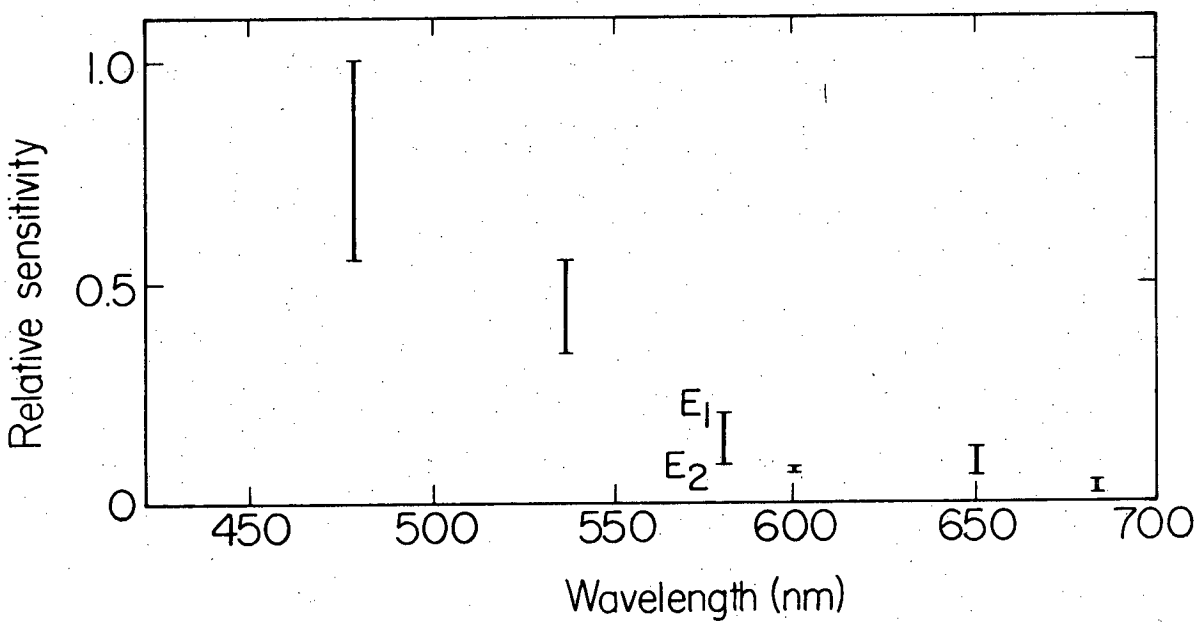

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Fig. 2. The action spectrum for producing a reduction in the excitability of the nerve cell. The points E_1 and E_2 are derived from the energy density values so labeled on Fig. 1. The ordinate is in relative units, which correspond to dimensions of inverse energy density.

relatively few points spaced over a wide range of wavelengths, its shape is consistent with this hypothesis. The increased sensitivity in the range of 490 to 540 nm can be ascribed to absorption by cytochrome-c (with absorption peaks at 415, 521, and 550 nm) (17), cytochrome-b (which absorbs maximally at 429, 532, and 563 nm) (17), cytochrome-a,a_3 (with maximum absorption around 440 and 600 nm) (17), and flavoproteins (which absorb near 450 nm) (17), all of which are found in high concentrations in mitochondria. Light absorption by the cytochromes has been suggested as the cause of various physiological responses (8,9,18). Of particular interest are the experiments of Rounds and Olson (9), who demonstrate by spectroscopic methods that the electron transport chain could be interrupted at the level of cytochrome-c by laser irradiation of 530 nm. Also of interest is the recent work of Pereira et al. (19) who demonstrate a photo-induced impairment of mitochondrial function with blue light, due to absorption by flavoproteins.

The electrophysiological results can be understood in terms of calcium release by mitochondria. Mitochondria sequester large amounts of calcium in an energy-linked process (20), leaving the ionized calcium concentration in the cytoplasm to be 10^{-7} to 10^{-8} M as measured in squid axon (21). Rattner et al. have suggested that laser-induced calcium release from mitochondria may be the cause of the changes in contractility observed in rat myocardial cells after irradiation (22).

Changes in internal calcium concentration have been related to pacemaker rhythm in Aplysia neurons (23) and rat myocardium (24) as well as increased ionic conductances in nudibranch neuron (25) and cat motoneuron (26). It may be expected, therefore, that the release of calcium by mitochondria that are damaged by laser irradiation would reduce the excitability of these nerve cells.

Using reasonable approximations, the average increase in calcium concentration induced by the laser pulse can be estimated to be on the order of 30 μM (27). This value is within the range of concentrations that have been shown to have various physiological effects (28). In our case, this high concentration of calcium on the inner side of a portion of the plasma membrane may lead to a transient increase in the potassium conductance and effectively short out the soma membrane. This would result in reduced excitability until the remaining active calcium pumps in the undamaged mitochondria and plasma membrane return the intracellular calcium concentration to its normal level.

Various possible mechanisms for the interaction of laser light with biological material have been cited by several authors. These include purely thermal mechanisms (29), photodynamic action (30), two-photon absorption (31), and second harmonic generation (32). The present results, by themselves, do not allow a firm conclusion as to the

actual process whereby the cell is affected. However, in a series of related experiments (33) we have found that the sensitivity at 690 nm is dependent upon the power density of the laser pulse. This would seem to exclude a photochemical mechanism, because such a process would be sensitive only to the total energy of the pulse and not to the rate at which it is delivered (i.e., photon flux).

The local increase in temperature at the mitochondrion can be estimated to be 12°C (33,34). If the laser energy absorbed by the mitochondria is dissipated over the entire cell volume, the average temperature of the cell will increase by only 0.1°C (35). This increase in cell temperature is well within physiological ranges, whereas that of the mitochondria is assumed to be sufficient to induce calcium release. Such a mechanism would be consistent with our results.

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12. Dye solutions used for each wavelength are: 685 nm, oxaline 170 plus rhodamine 6-G; 650 nm, cresyl violet plus rhodamine 6-G; 600 nm, 500 μM rhodamine 6-G; 580 nm, 50 μM rhodamine 6-G; 540 nm, brilliant sulfaflavine; 480 nm, C6H.

13. Pilot studies in our laboratory have indicated a different energy dependence for functional modification if the laser pulse occurs during an action potential or is delayed to occur after one. To avoid technical problems of precise triggering during an action potential, we delayed all laser pulses 6 msec after a spontaneous action potential. For cells firing at the observed rates, the probability that a second action potential would be produced during this delay interval was calculated to be less than 5%.

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27. With 100 nMol Ca^{+2} sequestered per mg of mitochondrial protein [E. Carafoli, J. Mol. Cell. Cardiol. 7(2), 83 (1975)] and 40 mg of mitochondrial protein per gram of wet tissue [W. Spector, ed., Handbook of Biological Data. (W.B. Saunders Co., Philadelphia and London, 1956)] one calculates 4 μMol mitochondrial Ca^{+2}/gram of wet tissue. The irradiated volume is 3.4×10^{-11} cm^3 assuming a spherical distribution of energy deposition of radius 2 μm radius (the laser spot radius). If all sequestered calcium within this irradiated volume were released, it would be equivalent to 1.34×10^{-10} nMol Ca^{+2} (assuming unit-density tissue). In a spherical cell of 10-μm radius (4.2 picoliter), the laser pulse would be expected to raise the average calcium concentation on the order of 30 μM.

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35. Assuming all the irradiated volume is elevated by 12°C and that the specific heat of the cell is uniform, the increase in average cellular temperature relative to that of the irradiated volume will be equal to the ratio of these volumes (irradiated volume/cell volume). For a 10 μm radius cell this ratio is 8.1×10^{-3}. Hence the average cell temperature increase becomes 0.10°C.

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