A POSSIBLE TWO-PHOTON EFFECT IN VITRO USING A FOCUSED LASER BEAM

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ABSTRACT A probable two-photon effect is described as a result of focusing an intense pulsed laser beam onto chromosomes of living cells. The effect was suggested after the derivation of a two-photon action spectrum and the demonstration of a lack of reciprocity.

INTRODUCTION

With the advent of lasers, it has become possible to attain monochromatic photon densities many orders of magnitude greater than those obtainable with classical sources (Joussot-Dubien, 1975). Furthermore, these intense beams can be focused to near-diffraction-limited spots. The resulting effects produced in the focused spot could be due to any number of uncommon phenomena, such as plasma formation, ionization, pressure, acoustic shock, electric fields, two-photon absorption, etc. Two-photon absorption has already been demonstrated in solution of NADH (Rounds et al., 1966).

One of the major areas of biology in which focused lasers are being employed is in microirradiation (Berns and Salet, 1972; and Berns, 1974*a* and 1974*b*). In these studies, a pulsed laser beam is focused to an area of less than 1 μm within a single living cell. The beam has been focused onto structures such as single chromosomes, nucleoli, and mitochondria. In the case of mitochondrial irradiation, the primary damage is produced because of visible light absorption by the respiratory pigments (Rounds et al., 1967). In the case of organelles, such as the chromosomes and nucleoli, there are no known visible absorbing chromophores. Effects can easily be produced by laser microirradiation after binding a selective vital stain, such as acridine orange to chromosomal DNA (Berns et al., 1969) and quinacrine hydrochloride to nucleoli (Berns et al., 1970). However, it has also been possible to produce distinct effects by laser microirradiation of nonstained chromosomes (Berns et al., 1971) and nucleoli (Meredith and Berns, unpublished data). In the case of the chromosomes, the effect is a phase "paling" of the irradiated chromosome segment. Cytochemical staining indicates that basic proteins (histones) have been damaged (Berns and Floyd, 1971).

The approach of laser molecular dissection of the chromosome is being used in studies on gene mapping, mitosis, and chromosome structure. However, the lack of understanding of the basic mechanism of laser light interaction with the biological molecules

poses severe limitations in both application of the technique and interpretation of the results. In this communication, evidence is presented that implicates two-photon absorption in the production of chromosome lesions.

MATERIALS AND METHODS

Individual chromosomes of mitotic PTK_2 (*Potorous tridactylis*) cells were selectively irradiated in Rose culture chambers following the procedures described elsewhere (Berns et al., 1972). The laser beam was focused to a spot approximately 0.5 μm in diameter according to the method described previously (Berns, 1971). The effect on the chromosome was designated either as "paling" (+) or "no paling" (−). If no effect was observed within 30 s of irradiation, the negative (−) designation was assigned.

The laser employed in this study was the Chromatix no. 1050 flash-lamp, pumped dye laser (Chromatix, Mountain View, Calif.) equipped with intracavity doubling optics. The wavelengths employed were the second harmonics of the infrared lines: 473, 526, 532, 540, 556, and 562 nm. Laser output was monitored in both direct energy (ergs) and in power (watts). Energy was monitored with an Eppley thermopile (Eppley Laboratory, Inc., Newport, R. I.) and power

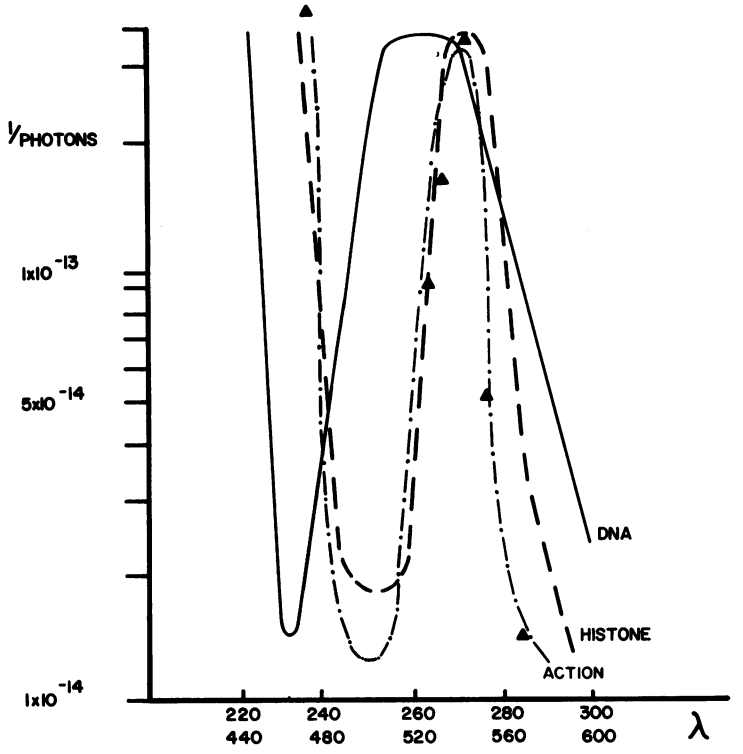

FIGURE 1 Plot of action spectrum of paling response using visible wavelenths and ultraviolet absorption spectra of DNA and histone. The action spectrum is expressed in terms of the reciprocal $(1/x)$ of the minimal number of photons needed to produce the paling. Note that if the action spectrum for the paling is viewed as a two-photon spectrum (one-half the visible wavelengths), then the action spectrum matches the ultraviolet absorption spectrum of histone.

TABLE I

PRODUCTION OF PALING

Wavelength	Duration of laser exposure	Total photons/s/cm^2 needed to produce threshold paling
nm	*s*	
473	4×10^{-7}	4×10^{21}
526	3×10^{-7}	1.7×10^{22}
532	1×10^{-7}	3×10^{23}
540	1.6×10^{-7}	2.15×10^{23}
556	6×10^{-7}	6.3×10^{24}
562	8×10^{-7}	3×10^{25}

was measured with an internally mounted pin-photodiode attached to a Tektronix no. 510 oscilloscope (Tektronix, Inc., Beaverton, Oreg.). The laser power in the focused spot of the ×100 oil immersion microscope objective was determined by direct measurement of the amount of power passing through the objective. The duration of each individual laser pulse was measured with a vacuum photodiode. Pulse duration for each wavelength used was fixed by the pulse-discharging network of the laser and varied from 1 to 8 × 10^{-7} s.

An action spectrum of the threshold energy for the paling response was determined using the six available wavelengths. In at least five repeats for each wavelength, the minimum laser output in the focused spot needed for the paling was determined. This was determined by producing the paling effect with a relatively high amount of energy and then using calibrated neutral density filters to attenuate the output beam until the threshold for visible paling was attained. Each irradiation was made on a new chromosome in a different cell.

Reciprocity was tested for one wavelength, 532 nm. This was accomplished by determining the threshold for paling and then attenuating the beam by a known amount and, at the same time, giving proportionally more laser shots. For example, five laser shots were given at one-fifth the threshold energy for producing the paling effect. Time between repeated shots was also varied by using a standard high speed photographic shutter. Repeated laser pulses were given either at 1/2-s intervals or at 10^{-6}-s intervals.

RESULTS

The action spectrum for the paling response is presented in Fig. 1 and the raw data in Table I. The action spectrum points (▲) are expressed as the reciprocal ($1/x$) of the number of photons needed to produce a threshold paling effect. The curve fitted to

TABLE II

RECIPROCITY AT 532 NM

Power	Energy/shot	Laser shots	Time between shots	Paling
W	*ergs/cm^2/s*		*s*	
125	6×10^{13}	1	0	+
25	$1/5(6 \times 10^{13})$	5	1/2	–
6.25	$1/20(6 \times 10^{13})$	20	10^{-6}	–
25	$1/5(6 \times 10^{13})$	5	10^{-6}	–
12.5	$1/10(6 \times 10^{13})$	10	10^{-6}	–

these points is a third order polynomial. The wavelengths of the action spectrum are expressed as the actual visible wavelengths tested. On the same figure, the typical ultraviolet absorption spectra of chromosomal DNA and histone are presented (Jagger, 1967). It should be noted that if the visible wavelengths of the action spectrum are viewed as the two-photon wavelengths (one-half of the visible wavelengths), the action spectrum would match the absorption spectrum of histone.

A test for reciprocity (Table II) demonstrates that this law does not hold for the paling response and the photon densities used. Five laser shots at 1/5 the threshold energy and 20 shots at 1/20 the threshold energy do not produce the paling. This result was obtained when the time between shots was either 1/2 s or 10^{-6} s.

DISCUSSION

The data implicate two-photon absorption as the mechanism of laser interaction with the biological molecules. The photon densities vary from 10^{21} to 10^{25} photons/s/cm^2. These are densities approaching those necessary for two-photon effects. The action spectrum for the paling response is almost a perfect match for histone absorption if it is viewed as a two-photon action spectrum (i.e., one-half the visible wavelengths). In addition, reciprocity does not hold therefore indicating some kind of uncommon physical effect, such as two-photon absorption. Finally, the action spectrum of the response indicates an effect on histone. Earlier studies involving chromosome cytochemical analysis demonstrated an effect on histone and not DNA (Berns and Floyd, 1971). Electron microscope analysis has indicated a very specific and localized effect on the irradiated chromosome region (Rattner and Berns, 1974). These observations argue against a gross effect that might be expected if plasma formation, heat build-up, pressure, and acoustic shock were the mechanisms of laser effects.

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