Infrared Nonlinear Optical Measurements of Membrane Potential in Photoreceptor Cells

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ABSTRACT In the past it has not been possible to measure optically the membrane potential of cells and collections of cells that are either naturally photosensitive or that can be activated by photolyzable caged transmitter molecules. This paper reports on a unique application of nonlinear optics that can monitor the potential of cellular membranes with a near-infrared source. Among many other singular advantages, this nonlinear optical approach to measuring membrane potential does not activate light sensitive cells or cell suspensions and cellular networks surrounded with photolyzable molecules. To demonstrate this capability we show that the technique can be applied to living photoreceptor cells that are very sensitive to visible light. These cells are ideal for characterizing such a new technique, not only because of their unmatched sensitivity to light, but also because their electrical responses have been extensively characterized (Minke and Selinger, 1992).

INTRODUCTION

The present standard approach to optically measure membrane potential is based on changes in the absorption or the fluorescence of appropriate potential sensitive dyes. There is an enormous amount of literature in this area (Waggoner and Grinvald, 1977; Loew, 1994), but in all the published work it has been impossible to measure optically the membrane potential of photoexcitable cells or associated cellular neural networks surrounded by photoexcitable excitatory or inhibitory molecules. In this paper we demonstrate that the membrane potential of living photoexcitable biological systems can be measured optically using the nonlinear optical phenomenon of second harmonic generation (SHG).

One photon absorption and fluorescence measurement on which previous optical techniques to measure membrane potential are based would excite the photoactivatable molecules, which can trigger cellular physiology. In addition to this obvious and important disadvantage, these linear, one photon techniques suffer from several other limitations in their applicability for monitoring cellular membrane potentials. These limitations include: 1) the poor sensitivity of the linear absorption and fluorescence of potential sensitive dyes to membrane potential; 2) the background contribution of the nonmembrane bound dye molecules; 3) the high cross-section for bleaching the dyes in normal oxygenated physiological conditions; 4) the damaging photoreactions of some potential sensitive dyes such as merocyanine 540; 5) the highly scattering nature of visible light that prevents penetration into the depths of biological media; and 6) the limitations on microscopic resolution that can be obtained with conventional visible light microscopy without signal limiting confocal techniques.

Many of these problems are overcome in this paper by the application of a nonlinear optical process to monitor membrane potential. Specifically, the second-order nonlinear optical process, second harmonic generation, is very sensitive to membrane potential (Bouevitch et al., 1993). In addition, second-order processes require symmetry breaking surfaces (Shen, 1989), such as cell membranes (Huang et al., 1989), and thus, only dye molecules with a distribution between the two layers of a bilayer membrane that lack a center of inversion will produce the directed signals associated with second harmonic generation. Furthermore, the infrared excitation does not affect photoexcitable molecules in and around cells when their one or two photon absorptions occur out of the wavelength regimes that can be significantly excited by the 1.06-μ wavelength of this laser. Also, this radiation can penetrate deeper into cellular media without causing destructive photodamaging effects. Finally, the use of nonlinear processes in this context results in naturally high resolution in x, y, and z and, as has recently been suggested, this resolution can approach 75 nm for appropriate geometries of excitation and detection (Hell, 1994).

Recently, we have been able to demonstrate that appropriately configured molecules could be inserted into model membranes as sensitive nonlinear optical monitors of membrane potential (Bouevitch at al., 1993). In this paper we extend these model system experiments to investigate the light-induced changes in membrane potential of living photoreceptor cells of the fly. The sensitivity of SHG to the electric field in the area of the probing molecular species can originate, in principle, from several molecular processes that have been shown to occur as a result of changes in membrane potential. These processes include alterations in the orientation of the probing molecular species, the Nernstian partitioning of dye molecules between the membrane

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and its surrounding solution, electrochromic changes in the probing dye molecule due to Stark effects on the energy of the electronic transition of the molecule or electrophoretic internalization of the dye molecules from the membrane. It has been shown (Bouevitch et al., 1993) that by way of an electrochromic charge shift mechanism, the membrane potential affects the second harmonic of the dyes that have been designed for monitoring membrane potential and are used in this work.

Theoretical description

When light strikes a molecule it generates a polarization in the electronic distribution that is expressed in Eq. 1.

$$\mathbf{P} = \chi^{(1)}*\mathbf{E}_1 + \chi^{(2)}*E_1*E_2 + \chi^{(3)}*\mathbf{E}_1*\mathbf{E}_2*\mathbf{E}_3 + \ldots \quad (1)$$

The linear spectroscopic methods are governed by the first order term in this expression namely the electric field from the photon $\mathbf{E}_1$ and $\chi^{(1)}$, which defines the polarizability change in the molecule with this electric field. The intensity of the SHG is principally determined by the second order term, $\chi^{(2)}$ in Eq. 1, which defines the second order correction to the polarizability change, and the two electric fields, $\mathbf{E}_1$* $\mathbf{E}_2$, of the two photons that are summed in SHG. Of principal importance to the experiments described in this paper is the fact that all processes defined by $\chi^{(2)}$ can only arise if the molecular species have a distribution that lacks a center of inversion. The intensity of the SHG that is detected is in principle proportional to the square of the incident laser intensity and the appropriate value of $\chi^{(2)}$ for the molecular species in question.

Calibration of the second harmonic signal with membrane potential

To effectively characterize our results on the visual photoreceptor system we have monitored the sensitivity of the SHG to membrane potential in a hemispherical bilayer (HBL) model membrane system. The HBL system has been described previously (Bouevitch et al., 1993). In this system a cholesterol bubble is formed at the tip of a Teflon pipette and electrodes are introduced across the membrane bilayer of the bubble. Several styryl dyes of the form used in the photoreceptor experiments were investigated.

To characterize that our signal was indeed arising from SHG we performed various tests. These included the square dependence of the SH signal on the intensity of the fundamental beam and the spectral characteristics of the SH emission, the time correlation of the SH signal with the infrared pulse, and the spatial characteristics of the SHG. In Fig. 1 the sensitivity of SH signal in an HBL to the amplitude of the membrane potential is shown for the dye JPW 1290. To eliminate from these results the contributions that are not linearly dependent on the electric field, the amplitude of the change for opposite polarities in the field was monitored.

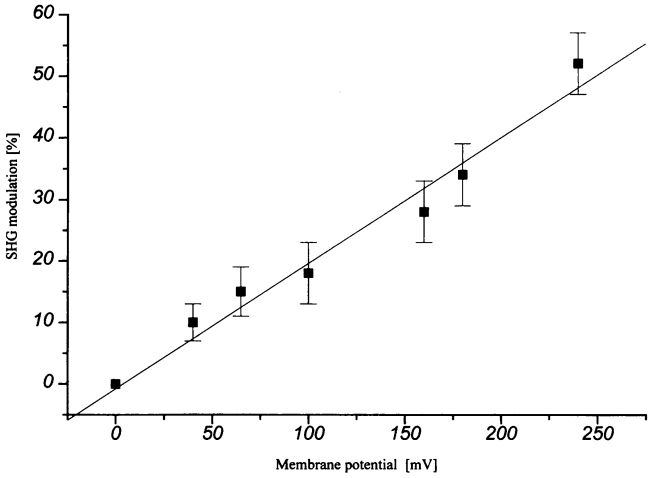

FIGURE 1 The alteration of the second harmonic signal versus the peak to peak amplitude of the modulating voltage with the dye JPW 1290 in a hemispherical bilayer.

The results shown in Fig. 1 clearly indicate the greater sensitivity (as determined from the slope of the linear dependence) of the method with respect to linear optical approaches to membrane potential measurements. In addition to these encouraging results, the data on cellular systems, for example the photoreceptor cells described in this paper or for a variety of cultured cell systems (data not shown), indicate that the sensitivity of the method as monitored in living cells is at least twofold higher from what is reported for the HBL system. In P19 neuronal cell cultures an ~10% change in the SHG with a 10-mV alteration in membrane potential was detected (data not shown). This is five times better than the HBL results reported in Fig. 1 and an order of magnitude better than the best linear fluorescence measurements with the same type of styryl dyes (Gross and Loew, 1984). The HBL result reported in this paper could arise from difficulties in interrogating regions of the HBL system in which the membrane has thinned to a single bilayer.

Experiments with visual photoreceptors and associated neural cells of the fly

The experiments were performed with the experimental arrangement schematically illustrated in Fig. 2. Musca eyes of a white-eyed mutant were partially cut to expose the photoreceptor and/or Lamina neural cells of the living fly, a morphological drawing can be seen in Minke and Selinger (1992). Each of the photoreceptors is connected to the laminar neural cells through an inverting sign synapse.

After staining with the JPW 1259 dye (Bouevitch et al., 1993), the SHG was detected at the reflection angle relative to the incident fundamental laser beam. In addition to the SHG we observed strong two photon fluorescence ~600 nm

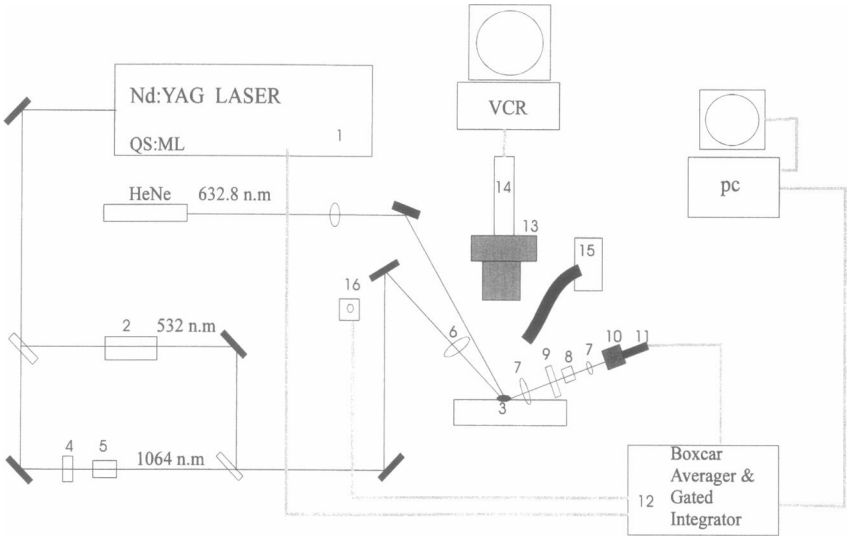

FIGURE 2 A diagrammatic representation of the microscopic second harmonic generation system used in the experiments. Instrumentation: The laser used was a Coherent Radiation Nd:YAG, which was modulated to generate Q-switched, mode-locked (QS:ML) pulses of 1064 nm (1). Each QS:ML pulse is structured from a 300-ns envelope that contains a series of 100-ps pulses. The laser could be doubled to 532 nm for alignment purposes by a Quantum Technology temperature controlled crystal (2), and this laser could be colinearly directed onto the sample (3) with the 1064-nm infrared beam that was appropriately controlled (4, 5, 6) with regard to its intensity, polarization, and spot dimension. The signal was collected from the sample and passed through appropriate lenses (7), polarizers (8), and filters (9) to a monochromator (10) equipped with a Hamamatsu R-1477 photomultiplier tube (11). The signal was recorded and analyzed by a Stanford Research Systems (Sunnyvale, CA), SR240 fast preamplifier, Boxcar Averager and Gated Integrator (12), and a PC. The sample was viewed by a surgical microscope (13) equipped with a charge-coupled-device camera (14) sensitive to the Nd:YAG laser irradiation. The sample could be modulated with light either from a HeNe laser or using a Zeiss (Oberkochen, Germany) fiber illuminator (15) with an orange filter. The infrared intensity was monitored with a fast EG&G Instruments (Princeton, NJ) photodiode (16).

from the labeled preparation. No SHG signals were detected without staining with the dye. We also determined that the visible excitation system used to modulate the photoreceptor physiology did not introduce any signal in the measurement system. In addition, we tested that the modulating visible light did not change the sensitivity of the detection

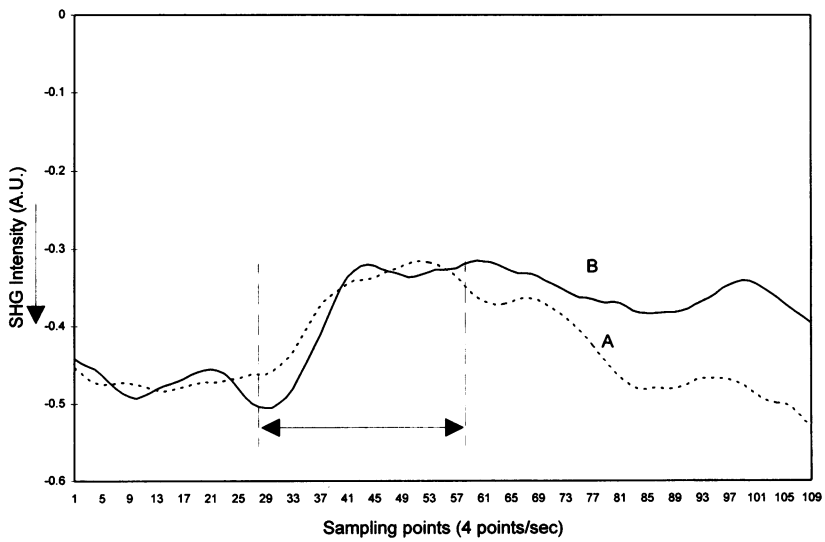

FIGURE 3 (*A*) Second harmonic generation before, during, and after illumination of the sample with orange excitation (*double-headed arrow*). (*B*) The same as *A* except for a sample after repetitive excitation. Methods: White-eyed Musca flies were grown, prepared and cut using standard procedures; the photoreceptor or lamina cells were very sensitive to ethanol in which the potential sensitive second harmonic dyes were dissolved. Thus, the staining of the cells was performed using a filtered Ringer's solution containing the dye JPW 1259. The resulting solution had an optical density of up to 0.3 at 480 nm. To prepare this final solution the dye was first dissolved in 0.25% dimethylsulfoxide, 0.25% ethanol, and 0.05% pluronic F127 (Sigma, St. Louis, MO) for more effective dissolution of the dye. After 5 min in the staining solution, the eyes were washed with the same Ringers solution without the dye present. The SHG signals were averaged using the gated integrator and PC system. This resulted in a response time of ~3 s.

system. The exposed eyes were viewed with a video infrared sensitive microscope to make sure that the fixed eye did not move during the measurement.

The membrane potential of the photoreceptor cells of the fly can be modulated with light. The wavelength of the modulating light is chosen with relation to the relative absorption of the two stable pigment states that are found in this photoreceptor. One of the pigment absorptions corresponds to 490 nm, which is the initial pigment form in the dark, rhodopsin (R_{490}). The other pigment absorption that is of importance to us in this work is the photochemically generated transformed structure of rhodopsin called acid metarhodopsin (M_{570}), which has an absorption at 570 nm. These two pigment forms can be switched optically and as a result a steady-state concentration can be maintained that defines a steady-state membrane voltage that can be monitored. This steady-state voltage is altered by the background level of the light, which causes a relatively rapid light adaptation process. Based on the absorptions noted above, orange light from a fiber optic lamp (<3 mW/cm^2) or a weak beam from HeNe laser (<0.5 mW) can induce steady-state voltage changes across the plasma membrane of the cell. These visible light-induced changes in membrane potential were monitored by the SHG. All experiments were performed in total darkness, except for the controlled exposure of the cells to the visible and infrared light sources mentioned above.

As a result of this visible light excitation of the photoreceptor a depolarization in the membrane potential occurs. This depolarization is seen in Fig. 3 *A*. This figure shows the response of a dark-adapted visual photoreceptor that was exposed to orange light. The light exposure is indicated by the double-headed arrow, which symbolizes the time of the exposure. When the modulating orange light that excited this photoreceptor was turned on, a decrease of ~30% in the signal level was detected. On the other hand, when the modulating light was turned off, a rapid return to the initial signal level is observed.

In Fig. 3 *B* the same procedure was repeated on the same cells after 10 min of repetitive excitations. This protocol resulted in a slower return to the equilibrium physiological voltage as can be seen by the slow return of the SH signal to its initial level. In addition to these results, when the modulation was performed by short sequential exposures to a red HeNe laser beam (see *arrows* 1, 2, and 3 in Fig. 4 *A*), the return to baseline was very rapid but the overall response of the cells was decreased due to desensitization. In contrast to these results, Fig. 4 *B* shows the SH response of the neuronal Lamina cells of a similar preparation as a result of a CW (continuous wave) HeNe laser excitation indicated by the double-headed arrow. As can be seen in this figure a small increase in the SHG level is now seen rather than a decrease as was observed when the fundamental laser beam was directed at the photoreceptor layer. An orange beam generates an undetectable response. The difference in the laminar cell behavior between the red HeNe laser focused excitation and the more diffuse orange excitation is understandable in terms of the known physiology of the system (Minke and Kivschfold, 1980). The laminar cells are connected to several photoreceptors and if the photoreceptors are excited uniformly only transient signals are observed in the laminar cells, whereas when the photoreceptor excita-

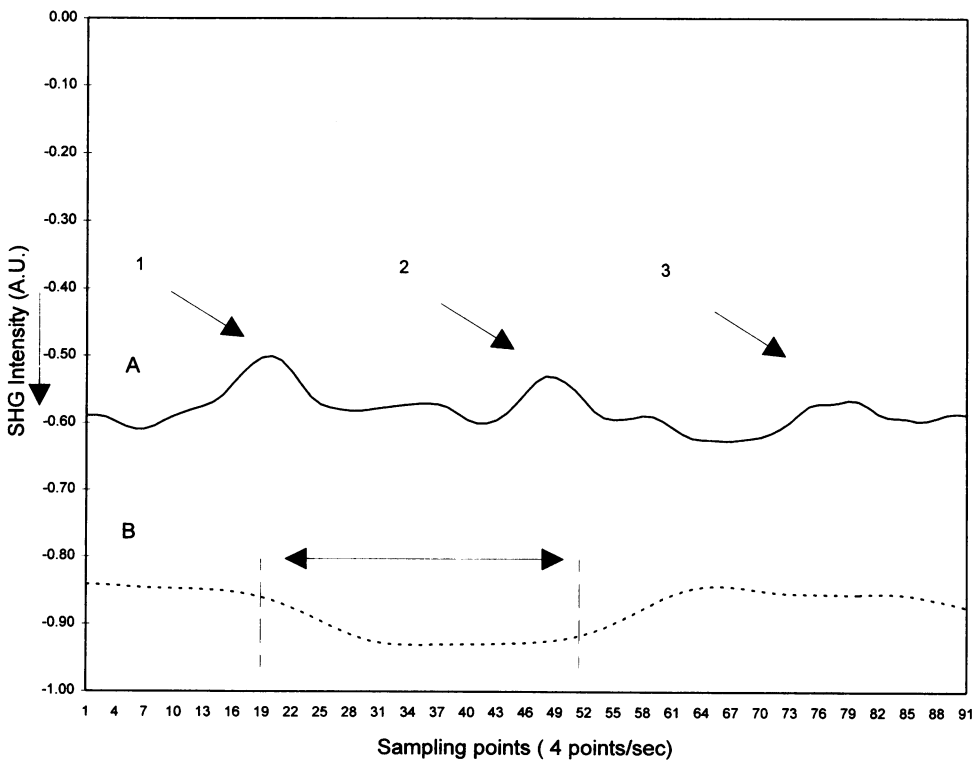

FIGURE 4 (*A*) Second harmonic generation from the photoreceptors cells in the presence of short pulses of excitation (*arrows*) from a HeNe laser. (*B*) The second harmonic generation from neural laminar cells before, during, and after excitation (*double-headed arrow*).

tion is not uniform, as is the case of the HeNe excitation, a direct-current hyperpolarization can be observed.

SHG was very sensitive to membrane potential with changes of at least 30% observed in photoreceptor cells. That such quantitative optical membrane potential measurements can be performed on samples that are highly sensitive to visible excitation is very significant. The photoreceptor cells that we have investigated in this report are archetypical systems of light sensitivity. The fact that such systems, which have extremely well-known physiological properties, can be studied with this optical method of monitoring membrane potential gives us considerable hope that we will be able to extend these measurements to other areas of neuroscience besides the obvious extensions to retinal neural networks.

One such possible extension that is of considerable importance in the field of neuroscience is the optical monitoring of SHG with infrared light in a neural network while a caged excitatory or inhibitory substance is released with visible or ultraviolet light. Not only will such an optical experiment allow for parallel monitoring of a variety of cells, but this can be performed with the excellent imaging capabilities that are inherent in nonlinear optical microscopies, which are experiencing an exponential growth phase in their development at the present time. Finally, it should be noted that the utility of these developments is not limited to problems in neuroscience, but is also of considerable significance in such areas as photosynthesis and other areas of membrane energy transduction. Thus, we believe that monitoring membrane potential with SHG will be a useful new tool not only in neuroscience but in a variety of interesting areas of biology.

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