Biophysical Journal Volume 65 August 1993 672–679

Probing Membrane Potential with Nonlinear Optics

Oleg Bouevitch,* Aaron Lewis,* Ilan Pinevsky,* Joseph P. Wuskell,‡ and Leslie M. Loew‡

*Division of Applied Physics, Hebrew University of Jerusalem and Hadassah Hospital Laser Center, Jerusalem, Israel; ‡Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030 USA

ABSTRACT The nonlinear optical phenomenon of second harmonic generation is shown to have intrinsic sensitivity to the voltage across a biological membrane. Our results demonstrate that this second order nonlinear optical process can be used to monitor membrane voltage with excellent signal to noise and other crucial advantages. These advantages suggest extensive use of this novel approach as an important new tool in elucidating membrane potential changes in biological systems. For this first demonstration of the effect we use a chiral styryl dye which exhibits gigantic second harmonic signals. Possible mechanisms of the voltage dependence of the second harmonic signal are discussed.

INTRODUCTION

Nonlinear optics is an area of considerable current interest. Nonlinear optical phenomena are formally described by the optically induced polarization density, P, which characterizes the optical response of materials to electromagnetic radiation. P can be expanded as a power series in the electric field. The second order term in this expansion, which is composed of the square of the incident electric field and the second order susceptibility, governs the best understood of those optical processes that are nonlinear in the electric field. This second order term is responsible for such nonlinear effects as second harmonic generation, sum and difference frequency generation, the rectification of light and the electrooptic (Pockells) effect.

There have been relatively few direct applications of such second order processes to understanding the structure and function of biological systems. The only investigations to date have used second harmonic generation (SHG). One set of these investigations used the characteristics of SHG and second harmonic microscopy from rat tail tendon to understand details of the structure of this biological system (1, 2). Another set of such studies was aimed at both structural (3) and functional (4) questions that were related to the nature of light-induced proton pumping in the membrane protein bacteriorhodopsin. In this paper we demonstrate that SHG can be used as a monitor of membrane potential and this could be of considerable significance as a new tool in the arsenal of biophysicists interested in investigating the electrical processes that govern biological function.

Optical techniques for monitoring membrane potential were introduced 20 years ago by L. B. Cohen and his colleagues (5) and are now used in a wide variety of research problems (6). Presently, the most popular optical mode for monitoring membrane potential is fluorescence. In this linear spectroscopic approach the fluorescence intensity is monitored as a function of membrane potential. It has been shown that the fluorescence intensity of a dye can be altered by membrane potential primarily in one of three ways: first, by a reorientational mechanism in which a membrane-bound dye tilts in response to an electric field (7); second, by a voltage-dependent redistribution mechanism in which the fluorescent dye partitions into the membrane as a function of the electrical potential (8); and third, by an electrochromic mechanism in which the electric field directly perturbs a dye's electronic transition (9, 22). Such an optical methodology has been found to be very useful in answering a variety of biological questions which require parallel detection of membrane potential over a defined area with relatively high spatial resolution.

In view of the availability of an optical methodology for monitoring alterations in the potential of a membrane, what is the importance for biology of probing membrane potentials with SHG? First, it should be noted that the fundamental laser beam that is used to elicit the SHG can be in the infrared and the infrared nature of the light source should be specifically significant in preventing damage to photosensitive biological systems and to the fluorescent voltage-sensitive probe which readily bleaches as a result of the visible illuminating light source. In addition, unlike the case of fluorescence, in SHG the molecules endocytotically internalized into the cell will not contribute to the observed signal. This results from the fact that symmetry considerations forbid second harmonic generation in an isotropic medium in the electric dipole approximation. Therefore, only those asymmetrically distributed molecules in the membrane will be responsible for the signal without any of the background normally seen when fluorescence is used as the probe. Moreover, because the second harmonic signal is generated instantaneously the only limitation to the kinetic detection of membrane potential with this technique, besides the signal-to-noise considerations to be discussed below, is in the time response of the dye and that is very fast (less than femtoseconds) for the electrochromic molecules that we have chosen for the measurements reported in this paper. Furthermore, in terms of microscopy, second harmonic microscopy will allow the interrogation of

Received for publication 22 January 1993 and in final form 10 May 1993.

Address reprint requests to Aaron Lewis.

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0006-3495/93/08/672/08 $2.00

thick tissue slices, such as networks of neurons in brain slices, that can be monitored with the added three-dimensional spatial resolution normally associated with nonlinear optical processes (10). Of special importance in this regard is the dramatic reduction in scattering that occurs with the infrared light that is used to elicit the second harmonic signal. Finally, it should be noted that surface enhancement phenomena that have been used very effectively in such linear processes as Raman scattering have only a marginal effect on fluorescent signals. In terms of nonlinear optical phenomena however, these enhancement factors can occur, and, the enhancements observed for nonlinear processes are at least four orders of magnitude greater than those observed in Raman scattering (11). Thus, one can conceive of an experiment in which a nanometer-sized silver particle, that is chemically directed to a specific subset of membrane proteins, can be used to enhance selectively the SHG of dye molecules in close proximity to this subset of proteins. Such an approach, together with our demonstration of the membrane potential sensitivity of SHG, holds promise of eventually developing an optical, noncontact, analog to the extremely successful method of patch clamping for measuring the electrical properties of membranes around specific protein channels. Therefore, for all of these reasons the study of alterations in the surface SHG as a function of changes in cell membrane potential should be most interesting.

In this paper we present the first steps in this new approach to membrane potential measurements. For this demonstration of the inherent sensitivity of SHG to membrane potential we chose dye molecules that undergo internal charge transfer and can bind and orient in a lipid bilayer. Such molecules fall in the category of dyes that respond to membrane potential by an electrochromic mechanism (9, 22) and large second harmonic signals that have already been detected for monolayers of these compounds (12). In this work, a chiral group is covalently incorporated into the structure since this helps assure a noncentrosymmetric environment for the dye molecules (13). Indeed, our investigations establish an enhancement in the second harmonic signal upon introduction of a chiral center. Furthermore, we demonstrate for the first time the sensitivity of SHG to membrane potential using a hemispherical lipid bilayer stained with a potentiometric dye.

MATERIALS AND METHODS

All the experiments were performed on dyes that were inserted at a concentration of approximately 1% in a hemispherical bilayer membrane of oxidized cholesterol. The hemispherical bilayer is a bubble of approximately 3-mm diameter with walls consisting of a bilayer membrane. The bubble, which is filled with a 0.1 M KCl solution in which there is between 0.3–0.5% ethanol, sits at the tip of a Teflon pipette. It is inserted into a 1- × 1-cm glass cuvette that is filled with the same solution as is in the bubble. The detailed procedure of forming such hemispherical bilayers is described elsewhere (14). This model membrane system has been frequently used to characterize the potentiometric responses of fluorescent dyes (7, 9, 22).

The dyes/molecular voltage-sensitive probes used in these experiments (Fig. 1) were synthesized by procedures adapted from Hassner et al. (1984) (15) and added to the 0.1 M KCl bathing solution from 1 mM stock solutions in ethanol.

For the measurements (see Fig. 2) a Coherent Antares Q- switched mode-locked Nd:YAG laser was used at a repetition rate of 400 Hz. The laser

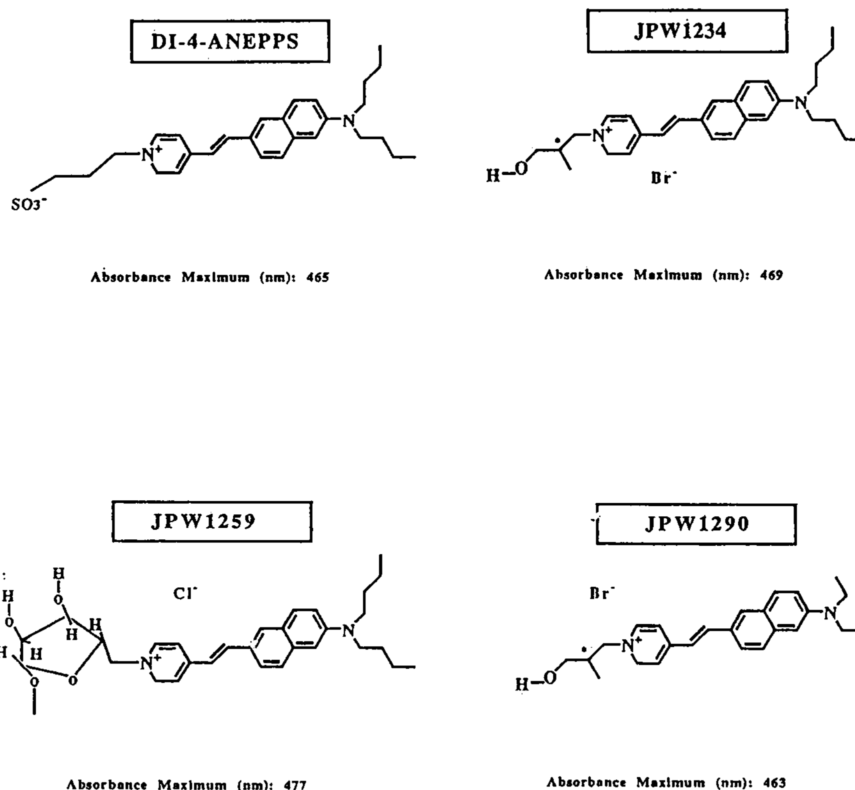

FIGURE 1 Structures of the chiral dyes used in this study. The structure of di-4-ANEPPS (9, 12) is also included for comparison. The wavelength corresponding to the absorbance maximum of dye associated with lipid vesicle membranes is noted below each structure.

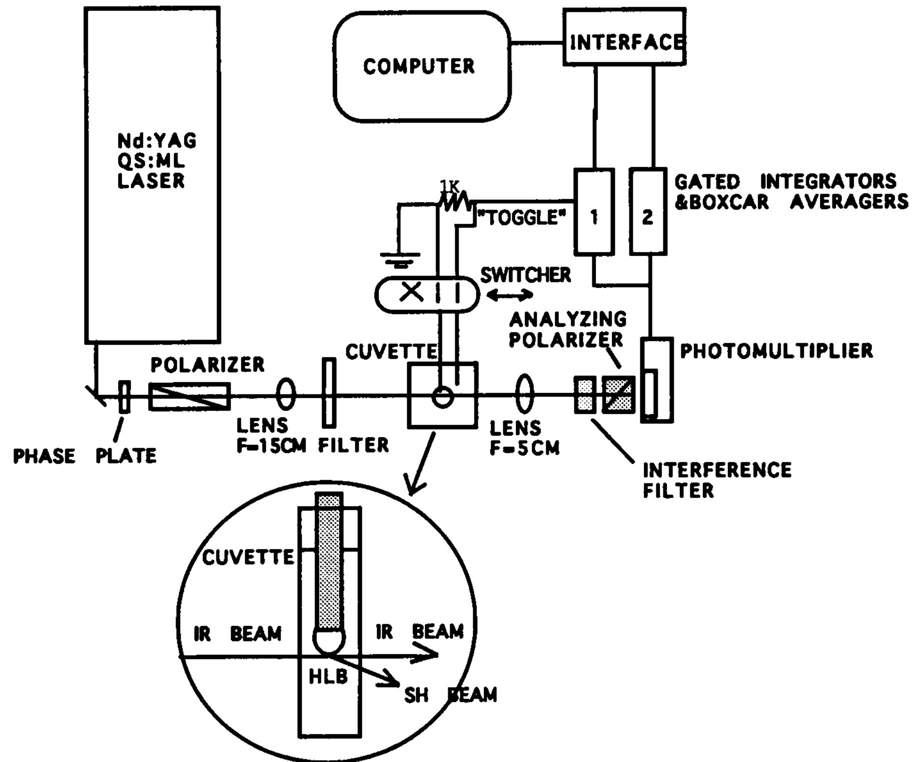

FIGURE 2 A diagrammatic representation of the experimental arrangement used to determine the voltage dependence of the second harmonic response from a dye/molecular probe-labeled HLB.

output was a 300-ns envelope which contained approximately 30 pulses of 120-ps pulse width. The laser pulses were passed through a half-wave plate and a Glan-Thompson laser prism polarizer. The 1064-nm laser power was adjusted to give <100 mW on the sample in a spot size of 180 μm on the bottom of the hemispherical bilayer after the laser had been filtered through a colored glass filter which blocked emissions at wavelengths above 600 nm. The second harmonic signal at a wavelength 532 nm was reflected from the bottom of the bilayer and was passed through an interference filter with a bandwidth of 1 nm followed by a Glan-Taylor prism polarizer. Finally, the signal was directed onto a Hamamatsu R1477 photomultiplier. In some of the preliminary experiments a 30-cm monochromator was used to select the second harmonic wavelength from that of the fundamental. The angle θ of the incident fundamental beam relative to the surface normal was chosen to be $\sim 60°$ to maximize the second harmonic signal within the constraints of our experimental geometry and in accordance with the expectation that $I(2\omega) \sim \sec^2(\theta)$ (16). The Teflon tip was mounted on a motor-driven X-Y-Z translation stage which allowed the hemispherical bilayer to be freely translated in all three dimensions.

The second harmonic signal and its voltage dependence were detected as shown in Fig. 2 and schematically diagrammed in Fig. 3. The electronics

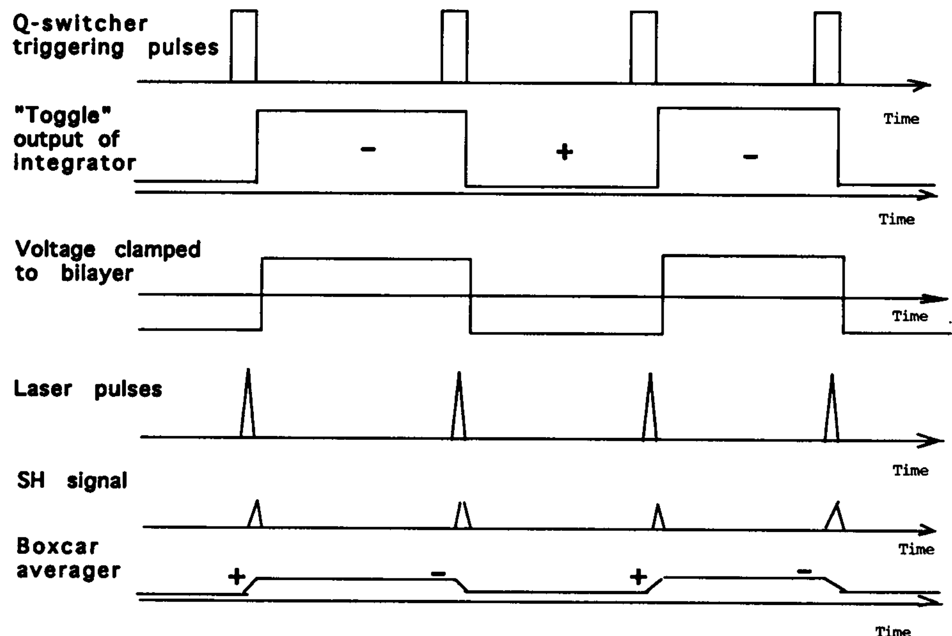

FIGURE 3 A digrammatic illustration of the sequence of steps from laser triggering to boxcar averaging of the signal from the photomultiplier that detects the second harmonic signal corresponding to the membrane potential change.

was synchronized by a triggering pulse from the Q- switcher of the laser as shown in Fig. 3. The photomultiplier output was connected to two Stanford Research (SR250) gated integrators and boxcar averagers. One of these, labeled 1 in Fig. 2, was used in "toggle polarity" mode. In this mode, the integrator reverses the polarity of the input signal after each triggering, adding it to the moving average of the integrator. The "toggle" transistor-transistor logic (TTL) output of the integrator follows this polarity and the TTL output is used to produce bipolar rectangular voltage of ± 20 mV which is applied to the hemispherical bilayer. This arrangement allows one to accumulate the difference between the energies of every two successive pulses (generated at membrane potentials of opposite sign) directly in integrator 1. This signal is stored in the internal capacitor of the boxcar. In other words the energy of a pair of pulses changes the charge of this capacitor. Integrator 2 simply measures the overall intensity of the second harmonic signal. Special care was taken so that the coefficients of boxcar amplification for both polarities were identical. In addition, shot-to-shot noise of the laser was reduced to 10–15% by careful adjustment of the laser Q-switcher and mode-locker. Second-order field dependencies, together with long-term instability of the membrane itself, led to noise of 40–50% in the second harmonic signal. Thus, in order to detect a second harmonic modulation due to membrane potential changes that are of the order of several percent of the total second harmonic intensity, at least 3000–5000 shots per measurement were accumulated. Furthermore, the dynamic range of the analog amplifiers used in the boxcar is big enough to follow the small changes expected in the signal. In our experiments, we were able to measure 3% modulation in second harmonic signal with an accuracy that was better than 50% of this modulation. To suppress zero drifts in the registering electronics and those caused by instability in the hemispherical bilayer voltage clamping electrodes were switched several times during the experiment. Every time the electrodes were switched the signal representing the second harmonic modulation [$dI(2\omega)$] increased/decreased by approximately the same value thereby giving us confidence that we indeed were observing the optical response to membrane potential modulation. Both signals representing the second harmonic response [$dI(2\omega)$] and second harmonic amplitude [$I(2\omega)$] were digitized and stored in an IBM PC computer. Relative second harmonic response was obtained by dividing the change in $dI(2\omega)$, due to the electrode switching, by $I(2\omega)$.

RESULTS

Previous experimental results had demonstrated that the potential sensitive dyes di-n-ANEPPS or di-n-ASPPS exhibit SHG with high efficiency when oriented in monolayer films (12). The results presented in this paper extend these results in two directions. First, we have prepared hemispherical lipid bilayers (HLB) stained with these dyes at a concentration of 1% as indicated above. With this dye-to-lipid ratio and based on the previous results obtained on these molecules we estimated a surface susceptibility of 2×10^{-16} esu. For such a surface susceptibility we should get approximately 40 photons/Q-switched pulse of our laser which emits 260 MW/cm^2 peak intensity at the fundamental emission of the laser at 1.06 μm. At first glance such a value seems rather small, however, at a repetition rate of 400 Hz, 16,000 photons accumulate in only 1 s and this should be readily detectable even with an ordinary noncooled photomultiplier using the technique described above which is based on gated integration and boxcar averaging. In spite of the fact that such a signal should be detectable in terms of detector sensitivities it could be easily overwhelmed, in our experimental arrangement, by the presence of the reflection of the fundamental of the laser. In fact, the infrared (IR) beam of the laser is not reflected by the tip of the hemispherical bilayer which was the region illuminated and, moreover, the second harmonic

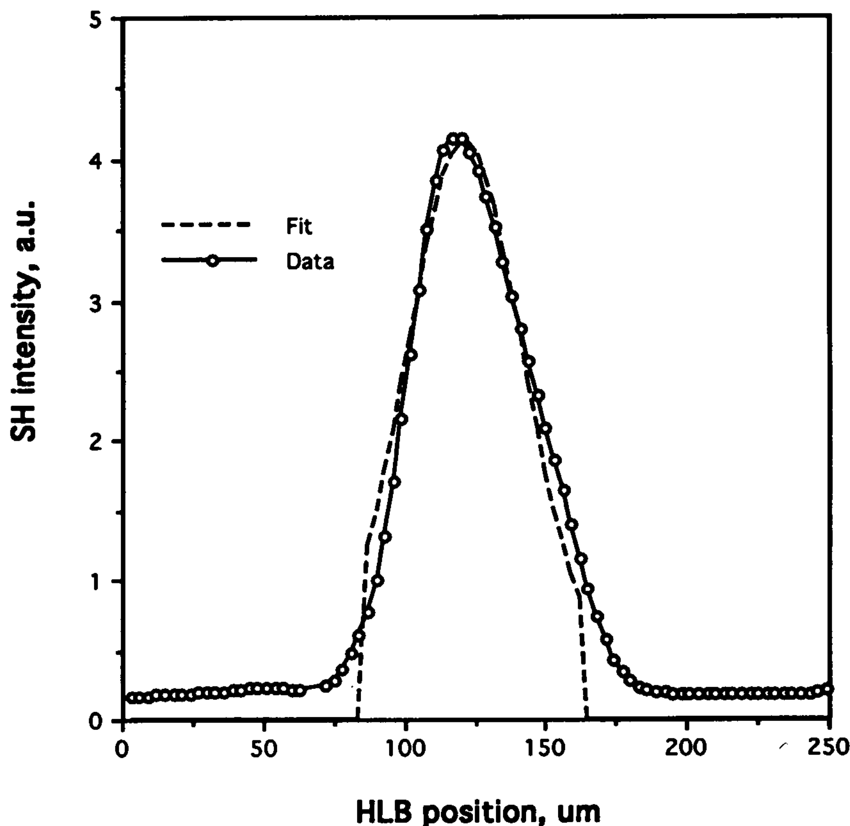

FIGURE 4 The result of slow (10 μm/s) vertical translation at constant velocity of HLB through the IR laser beam focused to 180 μm. The laser worked in a TEM_{∞} mode which has a Gaussian lateral intensity distribution. During the translation, the HLB bottom "probes" areas at different local intensity of the IR beam which leads to a corresponding change in the SH signal which is recorded as a function of time. Since the translation is at constant velocity, the form of the curve thereby obtained is close to Gaussian. This fact is illustrated by the solid curve representing the best Gaussian fit of the experimental data. The slight assymmetry of the peak is thought to be caused by the effects associated with the finite curvature of the HLB and the long-term instability of the probe-labeled HLB. The probe in this experiment was merocyanine 540. This probe was added to the HLB by introducing the dye into the surrounding 0.1 M KCl solution at a concentration of 3 μM. All measurements were performed at room temperature. The laser intensity was equal to 300 W/cm^2; peak intensity of the QS:ML pulse was about 260 MW/cm^2; the illumination area on the builayer was 3×10^{-4} cm^2 at the sample. In this experiment the fundamental beam was s-polarized and the SH beam was p-polarized.

was generated at an angle that was similar to what would be expected for the reflection angle. Thus, we essentially were able to detect the SHG without the presence of any background from the fundamental frequency of the laser.

The SHG from the tip of the dye stained hemispherical bilayer that was observed at 532 nm was well-defined both in terms of its spectrum and in terms of following the temporal nature of the laser pulse. To initially demonstrate the presence of SHG from the HLB we used the dye merocyanine 540 and slowly translated the HLB at a rate of 10 μm/s across a partially focused fundamental beam (FWHM 0.18 mm), while recording the SHG. Fig. 4 shows a typical result of such an experiment. The solid curve is the product of approximating the data by a Gaussian function which should effectively describe the square of the lateral intensity distribution of the fundamental beam. The relatively good fit that was obtained proves that the second harmonic signal is generated by the probe-labeled bilayer. The fact that the second harmonic signal does not go to zero when the bilayer is out of the beam probably results from dye molecules adsorbed to the walls of the cuvette in which the HLB was generated.

We tried to determine the threshold of the fundamental laser intensity at which the second harmonic signal of merocyanine is degraded. This occurred at an intensity of 1 GW/cm^2. At this intensity we succeeded to see an exponential decrease in the signal which is probably due to destruction of the dye molecules by laser heating since the voltage across the bilayer was still detected. In spite of these results we were unable to see any degradation of the second harmonic signal at such intensities when we used the chiral dyes shown in Fig. 1.

With the confidence we developed in seeing the SHG from an HLB we proceeded to monitor this SHG signal as described above. A typical result is seen in Fig. 5 for the dye JPW1259. The absolute second harmonic signal as recorded in integrator 2 is seen in the top of this figure. In the bottom of this figure are the results obtained from reversing the phase of the bipolar voltage across the bilayer. The points at which the phase was reversed are marked with an asterisk. The measurements of SHG were performed with either *s*- or *p*-polarized laser light. The *p*-polarized second harmonic signal was at least one order of magnitude stronger than the *s*-polarized signal, and this was independent of the laser polarization. This means that the probe-labeled bilayer possesses reflection symmetry about any plane perpendicular to the plane of bilayer. Under such symmetry conditions, the only nonvanishing components of the second order surface susceptibility χ are χ_{ZXX} and χ_{ZZZ}. The second order surface susceptibility components of the probe-labeled hemispherical bilayer, χ_{ZXX} and χ_{ZZZ}, and their response to the transmembrane electric field, D_{sp} and D_{pp}, were determined from the measured second harmonic intensities, respectively I_{sp} and I_{pp}, which are proportional to the square of the modulus of the corresponding susceptibilities. (The values D_{sp} and D_{pp} are simply the relative changes in SH signal caused by membrane potential modulation.)

Table 1 provides coparative data for the three chiral dyes, JPW1234, JPW1259, and JPW1290. For the dyes JPW1234 and JPW1259, the value $|\chi_{ZXX}|^2/|\chi_{ZZZ}|^2$ is also given.

DISCUSSION

The molecules synthesized for these measurements have been designed with appropriate donor and acceptor groups and engineered to bind to and orient within a lipid bilayer in order to exhibit a direct electronic response to alterations in

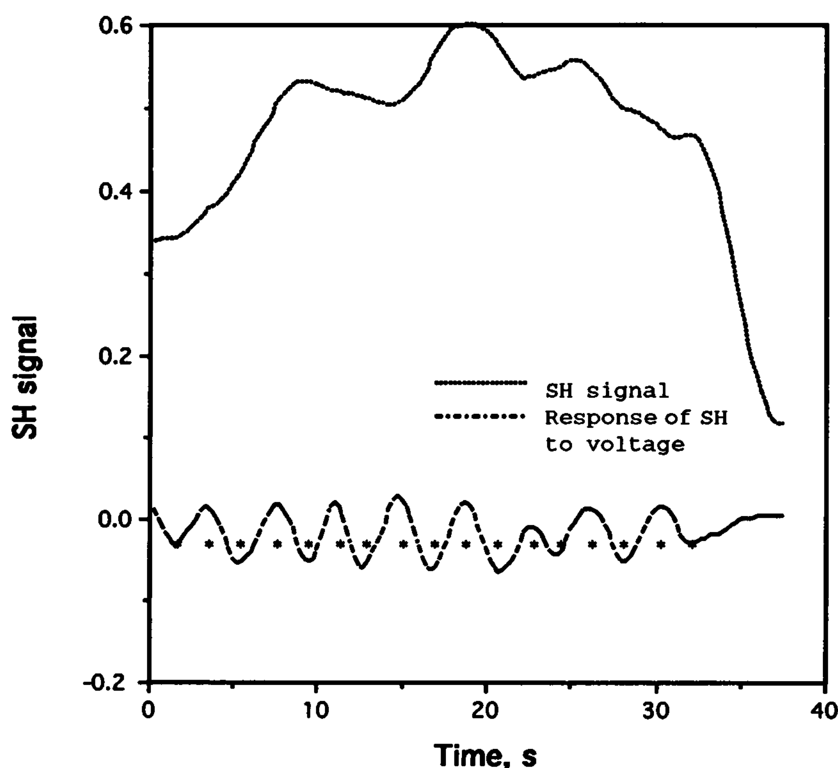

FIGURE 5 Determination of the second harmonic generation dependence on membrane potential. In this experiment, bipolar voltage pulses of ±20mV are clamped to the probe-bound HLB. The QS:ML laser pulses are synchronized with voltage pulses in such a way that QS laser pulse illuminates HLB bottom when positive or negative potential difference on the charging membrane reaches its maximum. Both second harmonic signal (*upper curve*) and its response to modulation of HLB potential (*lower curve*) are recorded simultaneously. To eliminate zero drift in the second harmonic response channel, voltage clamping electrodes are periodically switched. Moments of switching are shown in the figure by asterisks. The probe was JPW1259; bathing solution was 0.1 M KCl in water; concentration of the probe in sultion is 3 μM. All measurements were performed at room temperature. Fundamental intensity was equal to 300 W/cm^2; peak intensity of QS:ML pulse was about 260 MW/cm^2; the illumination area on the bilayer was 3×10^{-4} cm^2 at the sample. In this experiment the fundamental beam was *s*-polarized and the SH beam was *p*-polarized.

Table 1

Dye	D_{sp}	D_{pp}	$\lvert\chi_{ZXX}\rvert^2/\lvert\chi_{ZZZ}\rvert^2$
JPW1234	2.2 ± 0.8	2.2 ± 0.8	1.0 ± 0.5
JPW1259	3.7 ± 0.8	2.8 ± 0.9	1.2 ± 0.5
JPW1290	3.2 ± 1.0	2.2 ± 0.9	

membrane potential. As a result of this design, a change in the wavelength of the absorption maximum of such molecules is detected as a function of membrane potential, and this translates into changes in the fluorescence intensity, which is the method that is presently used with such molecules to detect membrane voltage (9, 22). However, because the presence of strong donor and acceptor groups in these molecules can perturb π electron systems and result in large alterations in the molecular dipole in the vertically excited state, it is well known that these molecular species generally exhibit large nonlinear responses. Therefore, it seemed likely that the very same molecules, that have been shown to be effective in monitoring membrane potential using fluorescence methods, would also be large generators of second harmonic light. To additionally enhance SHG efficiency, chiral groups were incorporated into the polar head regions of the molecules. Furthermore, since the intensity of the second harmonic signal is related to the induced dipole, it was a likely possibility that this signal would be inherently sensitive to membrane potential.

In fact it is important to realize that a wide range of results have been obtained on the potentiometric responses of a variety of molecular probes (7–9, 17, 22), and these results indicate that the second harmonic signal dependence on membrane potential can be caused by one of several factors. These include the orientational response of the dye to membrane potential (7) which would be similar to the well known effect of electric field-induced second harmonic generation or EFISH (18), the possible redistribution of dye molecules with the electric field between different chemical environments such as the lipid membrane and the surrounding solution (8) and the direct electronic response of the dye which is described by the cubic polarizability $\gamma_{ijkl}(-2\omega, \omega, \omega, 0)$ (18). These factors can be expressed as shown below to give the χ(eff), the effective surface susceptibility that is observed.

$$\chi(\text{eff}) = \chi(\text{or}) + \chi(\text{env}) + \chi(\text{e}) \tag{1}$$

There is little doubt that, for different dyes and for various surroundings, the relative contribution of the terms in Eq. 1 will be different. For example, the first term in Eq. 1 is big for dyes which are known to rotate well in the membrane. Thus, if we had used merocyanine 540 in the experiments that measured alterations in the second harmonic signal with membrane potential, then this term would have a major contribution (7). Alternately, Nerstian dyes that partition into the membrane with alterations in membrane potential (8) would be expected to have a dominant contribution from the second term in Eq. 1. Finally, the charge shift probes (9, 22), like the ones that were used in detecting the alterations in the second harmonic signal with membrane potential, are interesting due to the anticipated direct electronic effect on the second harmonic response to membrane potential which should be exhibited universally in various environments (19). As noted above, these dyes are thought to undergo a large induced dipole after interacting with a photon. If the induced dipole is altered by the presence of membrane potential, then this alteration is directly correlated with the molecular hyperpolarizability on which the surface susceptibility and finally the second harmonic signal depends. Thus, one explanation for our observations of the voltage sensitivity of this class of molecules that we have investigated is a direct effect of the membrane potential on the magnitude of the induced dipole.

In support of the dominant contribution in our results of the last term in Eq. 1 is the fact that there is no evidence that such dyes undergo a voltage-dependent partitioning in the membrane and in addition, are not likely to change their orientation in the membrane with membrane potential. The lack of change in orientation is the result of the excellent binding of these molecules in the membrane due to the hydrophobic side chains covalently linked to the aniline nitrogen. The lateral diffusion coefficient of these dyes is equal to 10^{-8} cm^2/s (unpublished results), and this is much less than the square area of the hemispherical lipid bilayer divided by time interval between two successive QS laser pulses, 10^{-2} cm^2/s. This also indicates that migration of the probe molecules along the membrane is unlikely to contribute to the surface susceptibility alterations with membrane potential. Thus, the only term that can effectively describe such electric field induced alterations in the surface susceptibility is the last term in Eq. 1.

In a formal sense this external electric field dependence can be described by the electric field dependence of the β_{ZZZ} component of the second-order polarizability tensor. Using a nonresonant two-level model (20) β_{ZZZ} can be expressed as

$$\beta_{zzz} = \frac{3e^2\hbar^2}{2m_e} \frac{W}{[W^2 - (2h\omega)^2][W^2 - (h\omega)^2]} f d\mu_{ex}. \tag{2}$$

Here W is the energy of transition, $h\omega$ is the fundamental photon energy, f is the oscillator strength, and $d\mu_{ex}$ is the difference between the dipole moments in the ground and excited electronic states. The voltage-dependent alterations in $d\mu_{ex}$ probably result in a formal sense from the fact that the linear molecular polarizability in the excited state, P_{ex}, is as a rule bigger than in ground state, P_{gr}, for molecules with long conjugated π-electron systems (21). Thus we can write

$$\beta_{zzz} = \frac{3e^2\hbar^2}{2m_e} \frac{W}{[W^2 - (2h\omega)^2][W^2 - (h\omega)^2]} \cdot f\,[d\mu_{ex0} + (P_{ex} - P_{gr})E]. \tag{3}$$

As a result we get a linear response of the second order polarizability to the electric field E. In this regard it is important to mention that the energy of the electronic transition W also depends on the external electric field due to electrochromism of a molecule (the Stark effect). It is in fact this

dependence which produces the electric field dependence of the optical response of these probes in conventional absorption/fluorescence measurement schemes. Furthermore, when either the fundamental or second harmonic photon energy is close to the electronic transition energy W, the electrochromic effect should be taken into account and Eq. 3 should be accordingly modified to account for damping (21). In our case however, the JPW membrane-bound dye absorption band is centered around 460 nm which is relatively far from second-harmonic resonance in our case (532 nm) and thus this effect should not be contributing significantly. In addition a reorientational mechanism is precluded by the data presented in Table 1 since both D_{sp} and D_{pp} have the same sign which would not take place if reorientation of these probes is taking place in the electric field. If reorientation of the dipole moment of the molecule was occurring it would lead to a response of opposite sign for these different polarization conditions. Thus all our data support a purely electronic effect as a major contributing factor of the electric field dependence of the surface susceptibility.

The probe molecules bind to the outer side of HLB by their hydrocarbon chain which favors orientation of the pyridinium ring near the aqueous interface. Calculations (22) predict that in the excited electronic state the positive charge of the molecule is shifted from the pyridinium end toward the hydrocarbon chain. In our case that means the shift of charge is occurring toward the center of the HLB. Since P_{ex} of a molecule is expected to be bigger than P_{gr}, a positive potential inside the HLB is expected to decrease the induced dipole moment, in accordance with Eq. 3. This leads to decreasing the second harmonic generation efficiency which we indeed have observed experimentally. It is striking that in the case of JPW1259 and JPW1290 dyes the value D_{sp} seems to be bigger than D_{pp}. This is presently not understood.

An important aspect of these studies is to arrive at an estimate of whether real-time measurements of living cell membrane potentials could be made with the unique aspects of second harmonic detection of membrane potential. In such living cellular systems the time response that would be required would be below 1 ms. Since the time response of the second harmonic signal in a purely electronic mechanism is very fast, $<10^{-15}$ s, the essential question is whether there is sufficient signal to noise to allow us to detect alterations in <1 ms. For this estimation, assume that the SHG measurement with a standard deviation 1% allows one to monitor the potential with sufficient accuracy. Based on this assumption and the results of this study, we believe that the best laser system for such measurements would be a cw mode-locked near IR laser source (e.g., a titanium sapphire system) working at a rate of 80–100 MHz. Such a laser system provides 1–5% pulse-to-pulse stability which is necessary for the 1% second harmonic detection accuracy we have assumed. Furthermore, the above-mentioned accuracy requires at least 10^4 photons/1 ms, or a 10^7 photons/s signal power. In view of the above the question that remains is whether such a SHG efficiency can be reached. Let us assume that we are limited by an average intensity of the fundamental laser light which is equal to 300W/cm^2, which was a typical value in our experiments. Such a near IR mode-locked tunable femtosecond laser system, operating at 300W/cm^2 average intensity at a 100-MHz repetition rate and a 100-fs pulse duration, yields about 30 MW/cm^2 peak intensity of the fundamental which is an order of magnitude less than the typical peak intensities of 260 MW/cm^2 employed in the present experiments. Based on the above estimate, of SHG from a probe-labeled lipid bilayer, only 1 photon at the second harmonic frequency is emitted per 100 fundamental femtosecond laser pulses which already gives a fluence of 10^6 photons/s. Moreover, tuning the fundamental laser to 920-nm wavelength will bring the second harmonic emission into resonance with the electronic transition of these probes which should increase the signal by a factor of 100 (12) and yield up to 10^8 photons/s. Thus, the results obtained clearly indicate that the SHG technique is readily applicable to detecting membrane potentials in living cell membranes if the most appropriate laser system that is readily available today is employed. It should be noted in this regard that, the enhancement factors mentioned in the introduction, that are applicable if surface enhancement with silver particles is employed has not been incorporated into these calculations. With such enhancements the time resolution of the method will be based solely on the pulse width of the laser source employed.

CONCLUSION

In conclusion, we have demonstrated that the electric potential of a lipid membrane can be monitored by surface second harmonic generation. A hemispherical bilayer of oxidized cholesterol stained by styryl dyes with chiral centers was used for this demonstration. A qualitative explanation, based on a two level model and a direct electronic response of the induced dipole of a molecule to an external electric field correlates well with our observations. The results obtained clearly indicate that direct measurements of membrane potentials in living cells are possible with this method and such measurements can be obtained with the required time resolution. The intrinsic sensitivity of the technique which promises excellent signal to noise and other important advantages portend extensive use of this new approach.

We are pleased to acknowledge the support of the Office of Naval Research which permitted the initiation of this line of research. Partial support from the U. S. Public Health Service (GM35063) is also gratefully acknowledged.

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