GFP Is a Selective Non-Linear Optical Sensor of Electrophysiological Processes in *Caenorhabditis elegans*

Artium Khatchatouriants,* Aaron Lewis,* Zvi Rothman,* Leslie Loew,† and Millet Treinin‡

*Division of Applied Physics, The Hadassah Department of Ophthalmology, The Center for Neural Computation and the ‡Department of Physiology, The Hebrew University of Jerusalem, Jerusalem, Israel; and the †Department of Physiology, University of Connecticut Medical School, Farmington, Connecticut USA

ABSTRACT Electrophysiology of the nematode *Caenorhabditis elegans* has the potential to bridge the wealth of information on the molecular biology and anatomy of this organism with the responses of selected cells and cellular neural networks associated with a behavioral response. In this paper we report that the nonlinear optical phenomenon of second harmonic generation (SHG) can be detected using green fluorescent protein (GFP) chimeras expressed in selected cells of living animals. Alterations in the SHG signal as a result of receptor ligand interactions and mechanical stimulation of the mechanosensory cells indicate that this signal is very sensitive to membrane potential. The results suggest that this approach to membrane potential measurements in *C. elegans* and in other biological systems could effectively couple data on selective locations within specific cells with functional responses that are associated with behavioral and sensory processes.

INTRODUCTION

Caenorhabditis elegans is a soil dwelling non-parasitic nematode. It is a very well established model organism due to the ease of genetic molecular analysis and the ability to perform investigations at the single cell level (Brenner, 1974). The self-reproducing hermaphrodite has 302 neurons of a total of 959 somatic nuclei population in a completely transparent organism that is 1 mm in length and tens of micrometers in width.

The *C. elegans* nervous system has been reconstructed by serial imaging with electron microscopy (White et al., 1986) and there is a lot of information about the function of individual neurons from the analysis of animals with laser ablated cells and specific mutants (Chalfie and White, 1998). *C. elegans* is the first animal to have its entire genome sequenced (*C. elegans* Sequencing Consortium, 1998) and thus, there is a great deal of information about gene expression and function in nerve cells. It would be significant if one could bridge the behavior of *C. elegans* with this wealth of information. One approach to form this bridge would be through the measurement of electrophysiological alterations associated with specific behavior and selected mutations in this animal. In the past, because of the size (2–5 μm) and the inaccessibility of the neurons in this nematode, electrophysiological studies have been extremely difficult (Goodman et al., 1998).

Recently it has been shown that the nonlinear optical phenomenon of second harmonic generation (SHG) is a highly sensitive monitor of membrane potential (Bouevitch et al., 1993; Ben-Oren et al., 1996; Peleg et al., 1999). SHG employs infrared or near-infrared photons from a short pulsed laser to interact with asymmetrically distributed molecules to produce light at a frequency that is double the incident fundamental laser frequency. Thus, infrared photons at a 1 μm fundamental wavelength would produce a second harmonic (SH) signal in the green at 0.5 μm. The intensity of the SHG depends on the dipole that is induced in asymmetrically distributed molecules by the pulse of laser light. Therefore, if a chromophore is asymmetrically associated with a membrane and this chromophore has a large induced dipole relative to the surrounding membrane lipids and proteins then this probe molecule will be selectively observed in the SH image (Huang et al., 1989).

The membrane potential sensitivity of SHG also is related to this induced dipole since alterations in membrane potential would alter the magnitude of the induced dipole and thus, the SH signal. As would be expected this alteration with membrane potential is correlated with the direction of the induced dipole relative to the direction of the membrane potential and this has been shown in an earlier investigation (Bouevitch et al., 1993).

In order to apply SHG and its membrane potential sensitivity to the investigation of behaviorally related electrophysiological phenomena in intact animals it is necessary to be able to target, in an asymmetric fashion, a specific SH probe to the membrane of selected neurons. Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used extensively in *C. elegans* for monitoring the localization of specific proteins, some of which are membrane bound (Chalfie et al., 1994). Its chromophore has a phenol group at one end of the molecule conjugated to an imidazolinone group at the other. Upon light excitation the highly polarizable electrons in this π conjugated system are transferred from the phenol to the imidazolinone group. In the native chromophore this electron transfer results in deprotonation of the neutral phenol group to a phenolate anion. In fact in native species there is a predominant form

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Address correspondence to Aaron Lewis, Division of Applied Physics, The Hebrew University of Jerusalem, Bergmann Bldg., Jerusalem 91904, Israel. Tel.: 972-2-6798243; Fax: 972-2-6798074; E-mail: lewisu@vms.huji.ac.il.

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of the chromophore in which there is a neutral phenol having an absorption of 395 nm and an additional component, red-shifted in its absorption to 475 nm, in which the phenol is in the phenolate form. Since both forms have excited states with the chromophore as the phenolate, the emission spectra of both molecular species are similar with maxima between 503 and 508 nm. Mutants can be devised in which all chromophores are in the phenolate form and the mutant used in some of the experiments reported in this paper, S65C, is such a mutant (Tsien, 1998). The important point for SHG is that the chromophore of GFP undergoes significant electron distribution in the presence of the electromagnetic field of light and this results in a large induced dipole which has the potential to be affected by the nature of the transmembrane field. Such induced dipoles and their alteration with membrane potential are directly related to the intensity of the SH signal. Thus, GFP was a candidate as a membrane potential sensitive SH probe that could be genetically coupled in a selective asymmetric fashion to specific cells and protein molecules in *C. elegans*. We have previously shown that SHG can be detected from *C. elegans* when either the animal is stained with the membrane probe, JPW1259 or is selectively labeled with GFP (Lewis et al., 1999). Animals without appropriate staining showed no detectable SHG under our conditions. In this paper we report that the SHG observable from GFP fusion constructs in *C. elegans* are sensitive to alterations in membrane potential that are associated with functionally relevant biological stimuli.

EXPERIMENTAL PROCEDURES

Nematodes growth and strains

Strains were grown as described by Wood et al. (1988). The strains of *C. elegans* used were *hmIS4* which has an integrated array expressing a DES-2 GFP chimera (Treinin and Chalfie, 1995), *hmIS4;4773*, which also carries a deletion eliminating most of DEG-3 and the last exon of DES-2 (Treinin, unpublished results), and *uIS9* which carries a MEC-2 GFP chimera (Huang et al., 1995).

SHG Observation

The Nd:YAG laser at 1.06 μm was run at a frequency of approximately 400 Hz and the signal from each pulse that was detected through a monochromator by a photomultiplier was averaged in a box-car averager as has been previously described (Peleg et al., 1999). For observation, single animals where transferred to a 2% agar pad on a 16 mm round coverslip with a second coverslip being used to cover the animal. The two coverslips were placed on a 3D flat scanning stage (Nanonics Ltd, Jerusalem, Israel) that was sitting on a Zeiss IM 35 inverted fluorescence microscope. To obtain the images in Fig. 1 the sample was scanned in x and y at a particular z extension of the 3D stage that can extended up to 100 μm. For the data in Fig. 2, only a single pixel from the cell body was scanned as a function of imposed pressure. Immobilization was achieved using 1mM levamisole in the agar pad. In the choline experiments a 15-μl drop of 0.1 M choline was added to the agar between the coverslips.

RESULTS

GFP generates a second harmonic signal

The SHG microscope has been described previously (Peleg et al., 1999). It employs a near infrared Nd:YAG laser with an emission at 1.064 μm. When a transgenic animal with an integrated DES-2-GFP (S65C) chimera (DES-2 is a subunit of the DES-2/DEG-3 nicotinic acetylcholine receptor (nAChR)) (Treinin et al., 1998) was illuminated in the region of the cells near the nervous ring that expressed this GFP chimera a signal was observed at 0.532 μm in the green. This signal was detected through a monochromator and when the monochromator setting was changed by as few as 4–5 nm around this frequency the signal disappeared as would be expected for SHG. Such an observation is inconsistent with the detection of the nonlinear optical phenomenon of two photon fluorescence of GFP whose intensity would not abruptly disappear over a few nanometers. In addition, the GFP absorption maximum is at 479 nm (Heim et al., 1995) and this is below the wavelength of two photon excitation with 1.064 μm. Thus, it is reasonable that we should be seeing no two photon emission with this type of excitation. In addition, in the same microscope we could excite the one photon fluorescence emission. This emission occurs at a wavelength that is similar to the two photon fluorescence. Changing the monochromator settings around 532 nm did not appreciably alter the one photon fluorescence intensity as would be expected from the published emission spectrum in this region. Therefore, our results are only consistent with the observed signal from the membrane associated GFP arising from SHG.

Membrane potential alteration affects the second harmonic signal

To investigate whether the SHG from GFP is sensitive to membrane potential we expressed DES-2-GFP (S65C) chimera using the *des-2* promoter (Treinin et al., 1998). In this chimera GFP is fused to the intracellular loop of a truncated DES-2 protein and is expressed in a number of cells including the PVD cells. These cells are the same cells that express the DES-2/DEG-3 receptor. The PVD cells were the focus of our investigation since they are spatially removed and easily identified from other neurons in which this receptor is expressed and should be directly stimulated by choline (Yassin et al., in preparation). Therefore, we focused on the PVD choline response to verify in a controlled manner, by the use of mutants, in which the DE2–2/DEG-3

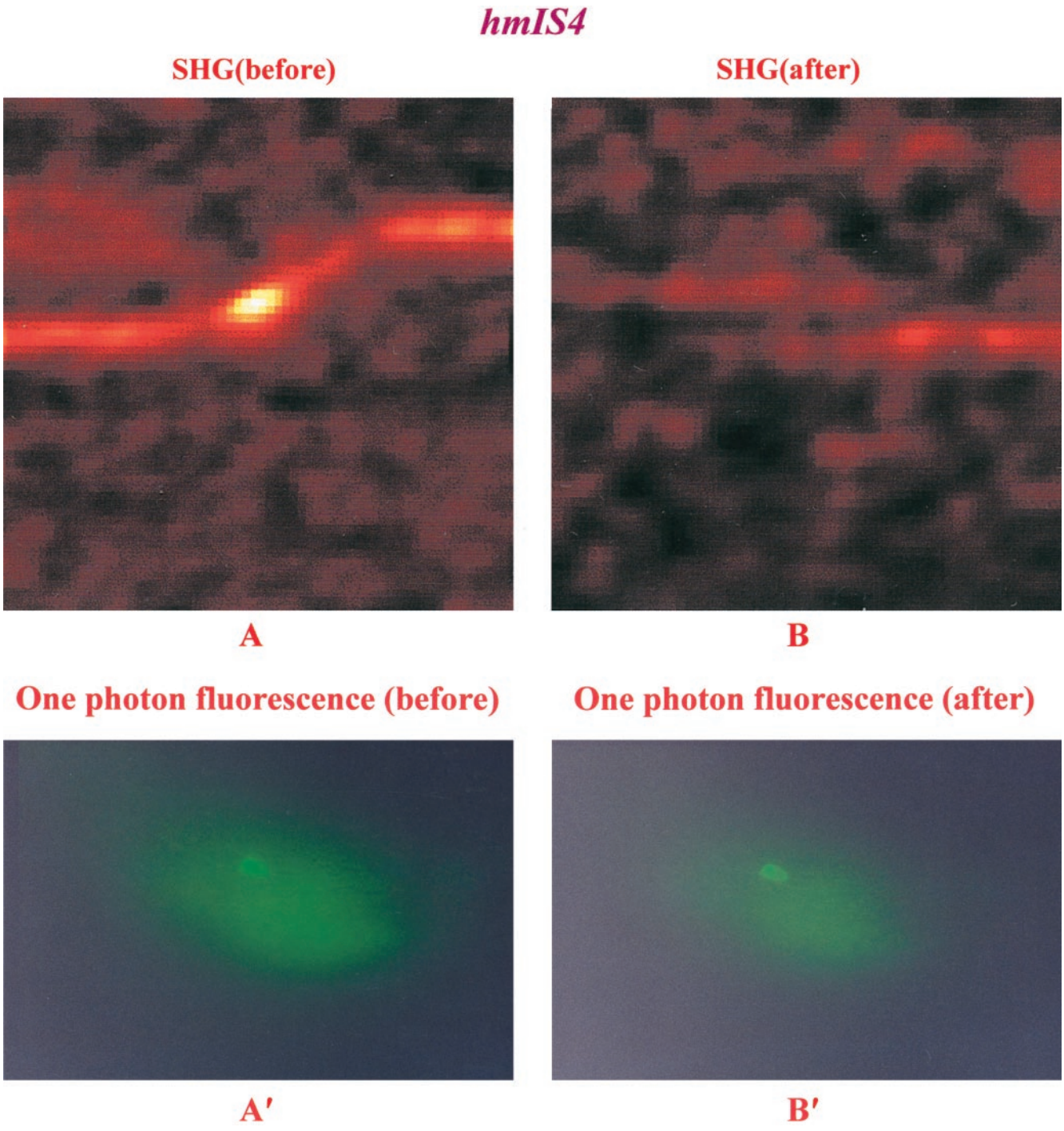


FIGURE 1 Second harmonic (*A*, *B*, *C* and *D*) and one photon fluorescence images (*A′*, *B′*, *C′* and *D′*) of PVD cells in *C. elegans* before and after the addition of choline in non-mutant transgenic animals, *hmIS4* (*A*, *A′* and *B*, *B′*, respectively) and in a DEG-3 mutant transgenic nematode, *hmIS4*; 4773, which lacks choline sensitivity (*C*, *C′* and *D*, *D′* respectively). All the second harmonic images have been reproduced with the same scale.

receptor is eliminated, whether the SHG from GFP was sensitive to membrane potential. The effect of choline on PVD cells in *hmIS4*, non-mutant transgenic nematodes, is shown in Fig. 1, *A* and *B*. In this figure the SH image of GFP in these cells is reproduced before and after the addition of 0.1 M choline. Fig. 1 *A* shows a field of view of 25 × 25 μm. Such a large field of view was chosen in order to see the soma of the PVD cell (that is clearly seen in the middle of the image) with two processes on either side. It is worth mentioning that the DES-2 GFP chimera is expressed throughout the cell with a larger concentration in the region of the soma. As can be seen in Fig. 1 *B*, upon the addition

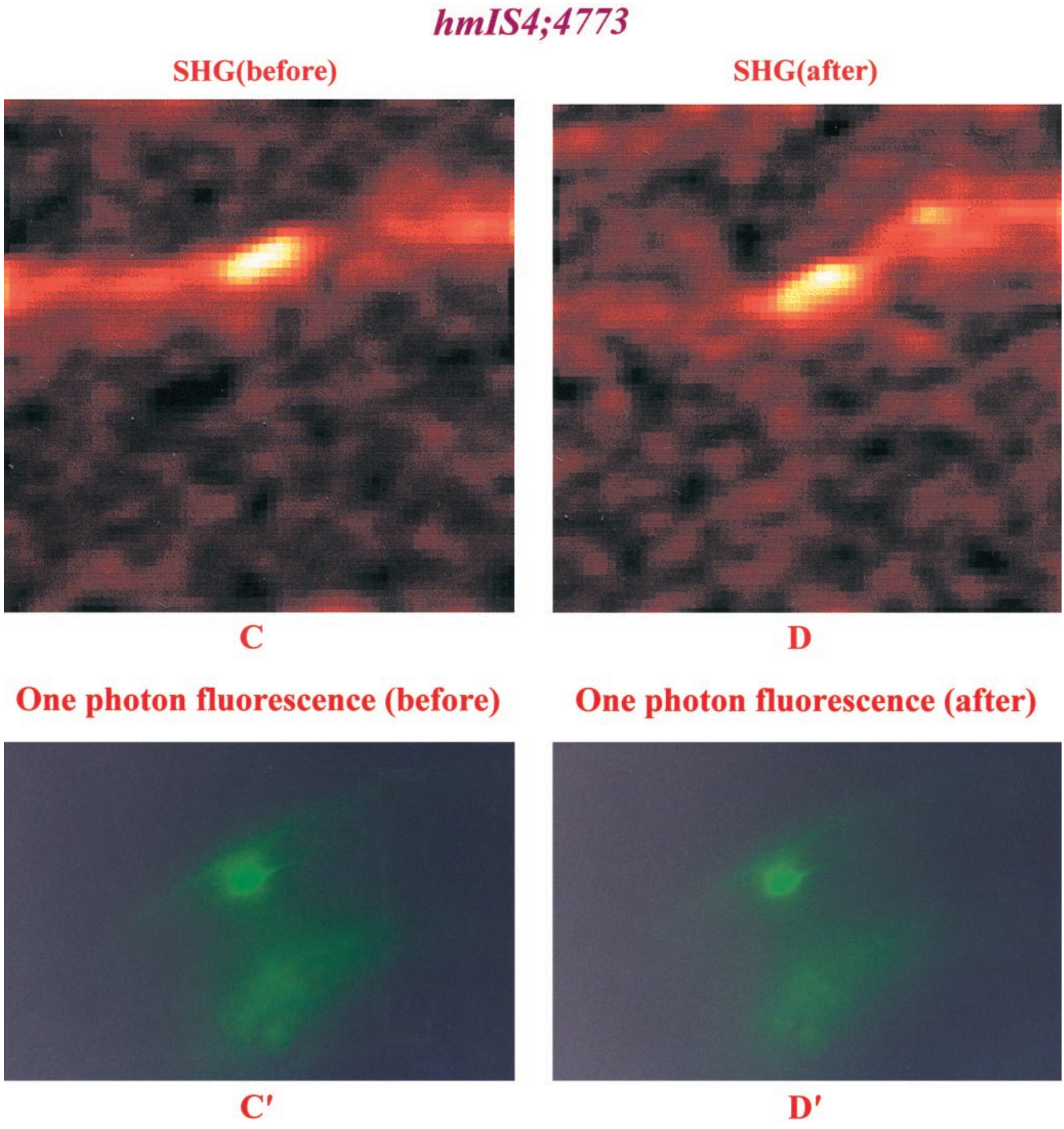
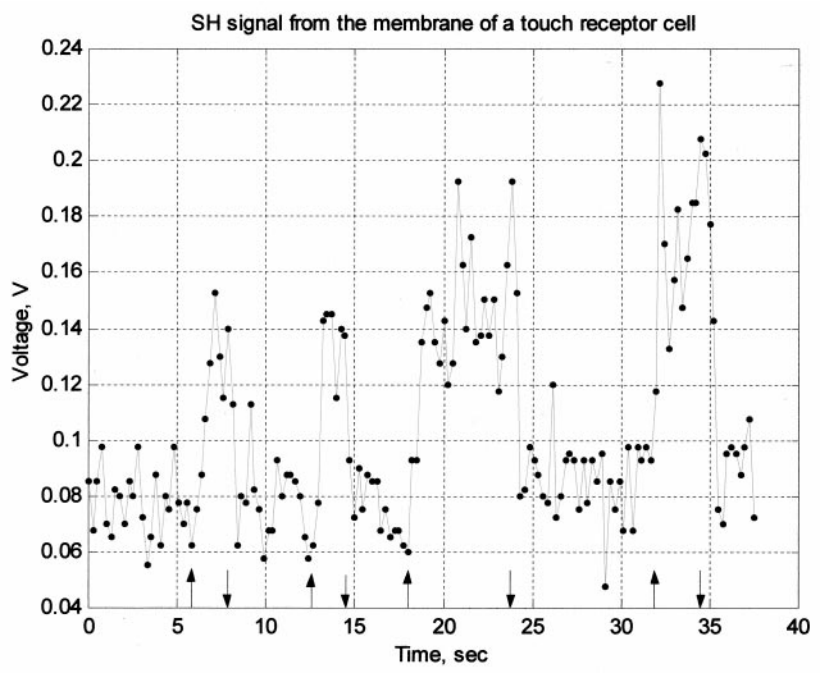


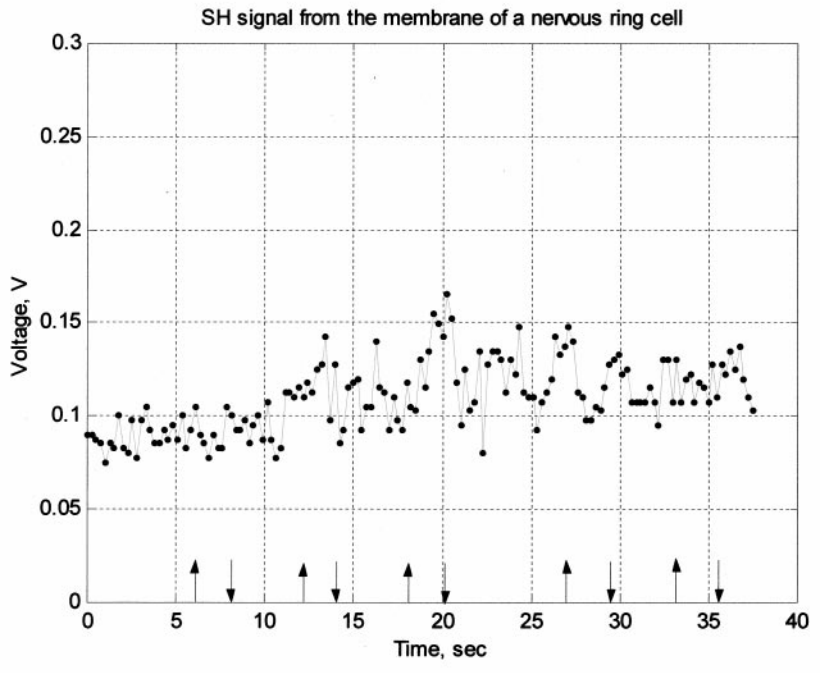
FIGURE 1 Continued

of choline there is significant reduction in the SH signal from the cell in comparison to the background.

The one photon fluorescence images obtained during these experiments are shown in Fig. 1, *A′* and *B′*. These images clearly show that the cell did not move out of focus in going from the case before the addition of choline to after the choline was added even though for technical reasons the preparation had to be moved for the choline addition. To achieve the same focus the PVD cells, which could be readily recognized, were returned to the same field of view and first the one photon image was used to bring the focus to the same point. Then, the focus was finely adjusted to detect the 4 highest intensity pixels of SH generation in the field.



A



B

FIGURE 2 The effect of imposed pressure on the SHG of mechanosensory ALM touch receptor cells (*A*) and cells near the nervous ring (*B*) when a needle controlled by a micromanipulator is brought into contact with the coverslip that is placed on top of the animal. Up and down arrows on the graphs in *A* and *B* show when the pressure was imposed and removed respectively from the coverslip. The experiments on the ALM cells were performed as described in Fig. 1 except for the use of the *uIS9* strain that carries an integrated array expressing a MEC-2 GFP chimera (Huang et al., 1995).

It is also important to emphasize at this point that the laser that was used for these experiments was a Nd:YAG with a pulse width of 100 ps. Unlike femtosecond lasers that are usually employed in nonlinear optical imaging this laser has a much larger depth of field and small changes in focus have little effect on the intensity of the second harmonic

generation. Thus, these simultaneous one photon fluorescence images, together with the additional optical fine tuning described above, confirm that the results obtained in Fig. 1, *A* and *B* were a result of the addition of choline and not due to alterations in the microscopic imaging.

The above data were collected on five *hmIS4* transgenic animals. The reduction of the SH signal upon choline addition was 64.5 ± 3.33% for the 4–5 brightest pixels in the PVD cells of each animal. An appropriate control for this experiment would be the response of a mutant lacking the DES-2/DEG-3 receptor. In Fig. 1, *C* and *D*, the SH image of a PVD cell in such a mutant DEG-3 transgenic worm, *hmIS4;4773* in which the same GFP construct is expressed. As can be seen in Fig. 1, *C* and *D*, the SH image before and after the addition of choline to this mutant shows little change in the signal relative to the background. Five DEG-3 transgenic mutant animals were investigated and in each case every pixel in the image had a change of less than 5% in the signal upon the addition of choline.

GFP expressed in mechanosensory cells is sensitive to mechanical stimulation

To check the generality of the membrane potential sensitivity of the GFP SHG we used wtGFP fused to MEC-2 which is a membrane protein expressed in the touch receptor cells (Huang et al., 1995). As in the previous construct, GFP is located close to the membrane on the intracellular side. A fine needle was brought into contact using a micromanipulator with the region of the coverslip under which the nematode of interest was positioned. The GFP SH signal from one of the ALM touch receptor neurons was then recorded as a function of time as this fine tip exerted pressure on the coverslip above the nematode. The results are seen in Fig. 2, *A* and *B*. In Fig. 2 *A* it can be seen that there is a direct correlation between an increase in the SH signal and the times at which the needle point touched the coverslip above the nematode (4 animals studied). As a control, cells close to the nervous ring from 5 DES-2-GFP transgenic animals were examined in the same fashion. These cells should not exhibit a response to touch and as can be seen in Fig. 2 *B* there is essentially no alteration in the SHG as a response to the pressure exerted by the needle.

DISCUSSION

The results indicate that SHG of GFP in selected cells of *C. elegans* is a sensitive monitor of membrane potential. In the case of the mechanoreceptor experiments, the SH signal increased between 1.63 and 2.02 with the imposition of pressure. These alterations were opposite in direction to those that were observed in the choline sensitive PVD cell experiments. Previous measurements have shown that there is an approximately 10% change in the SH intensity for every 10 mV change in membrane potential (Ben-Oren et al., 1996). Such sensitivity occurs when the chromophore dipole is oriented perpendicular to the plane of the membrane across which the membrane potential alterations are occurring.

Based on previous results, (Bouevitch et al., 1993; Ben-Oren et al., 1996; Peleg et al., 1999) the large alterations of the SH signal upon depolarization of the PVD cells with choline indicate that the axis of the GFP chromophore along which the induced dipole is generated by light is close to being perpendicular to the membrane plane across which the voltage alterations are occurring. From the GFP crystal structure, the GFP protein is composed of essentially an 11 β barrel picket fence architecture surrounding a chromophore that is positioned (Fig. 3) in an orientation that is close to perpendicular to the axis around which the β barrels are aligned (Ormo et al., 1996; Yang et al., 1996). It is known that GFP has regions of the β barrel surface that are more hydrophobic and other regions that have a propensity for specific charges. Such regions of GFP could play a role in orienting the protein embedded chromophore with the

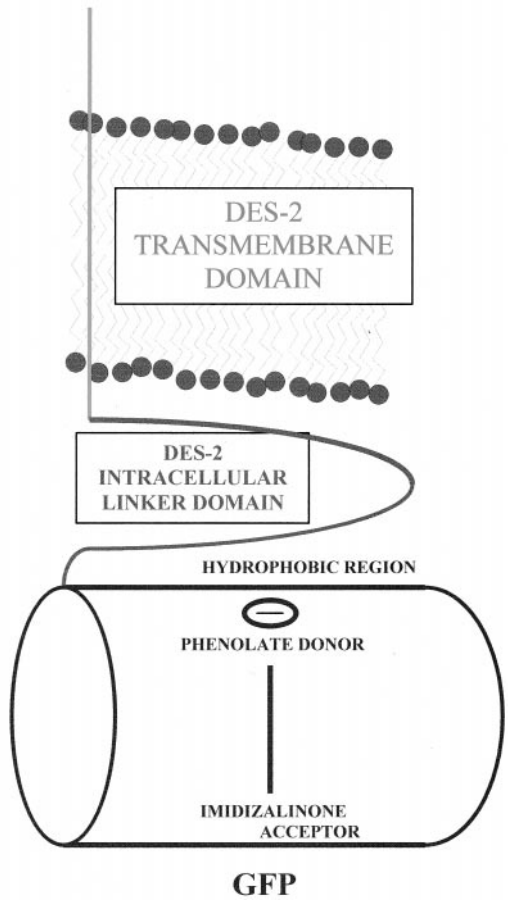


FIGURE 3 Diagrammatic representation of a hypothetical orientation of the GFP molecule attached to the DES-2 transmembrane domain by a DES-2 intracellular linker domain in choline sensitive PVD cells.

negatively charged phenolate either at or away from the membrane. Upon light excitation the electron redistribution in the chromophore is known to occur in the highly polarizable excited state from the phenol (wild type) or phenolate (S65C mutant) to the imidazolinonone as demonstrated by the excited state proton transfer from the phenolate in wild type GFP. In spite of the traditional view that a membrane field only affects components fully embedded in the membrane and GFP is certainly not embedded in the membrane, the presence of such a field in close proximity to a redistribution of highly polarizable electrons under the action of the electromagnetic field of light could seriously perturb the chromophore electron redistribution. Such a perturbation would alter the induced dipole and this would alter the observed SH signal which is extremely sensitive to such perturbations. This could be the origin of the affect that we have observed.

Based on the above mechanism, knowing that GFP is associated with the intracellular side of the membrane envelope and knowing that the effect of choline is most likely a depolarization of the PVD cell membrane, we can suggest based on the observed results that, in PVD cells, the GFP chromophore is aligned with the electron donating phenolate closer to the membrane leaflet than the imidazolinone group of the chromophore. Thus, upon excitation the electron movement will occur in the direction of the field which points toward the inside of the cell. As a result of depolarization with choline, the extent of excited state electron redistribution will be reduced, reducing the induced dipole and thus reducing the SH signal. Further work will be required to understand the structural motifs in the β barrel that lead to the chromophore orientation that is indicated by our results and the associated SH signals and to understand why in the case of mechanosensory cells, where the situation in the literature is less defined as to change in membrane potential, there is an opposite signal to what has been observed in the PVD depolarization by choline.

Even with this caveat, our work does however indicate, that, the phenomenon of membrane potential sensitivity of GFP SHG in *C. elegans* seems quite general since three cell types (cells near the nervous ring (Lewis et al., 1999), PVD and touch cells), two different chimeras (DES-2/DEG-3 and MEC-2), and wild type GFP together with one mutant GFP, S65C, all showed SHG and alterations in SHG as a response to functional stimuli. It has previously been shown that the direction of the change of the SHG is associated with either depolarization or hyperpolarization of the membrane potential (Bouevitch et al., 1993) and thus it appears that SHG of GFP constructs in *C. elegans* offers a general tool to couple the electrophysiological response of the animal to important functional responses.

Future experiments will be able to couple these measurements with a complex of sensory responses. For example, if, instead of a needle, a force sensing micropipette (Shalom et al., 1992) with appropriate control of pressure, as in an atomic force microscope that is integrated into the nonlinear optical microscope (Lieberman et al., 1996), is used directly on the animal, then protocols of pressure and odor could be designed to probe for learned responses in the animal. Also SHG has the potential to be combined with the nonlinear optical phenomenon of two photon fluorescence of GFP constructs that report on intracellular calcium (Miyawaki et al., 1997). Thus, it may be possible to relate membrane potential alterations in selected cells with specific chemical changes in these cells. Furthermore, if a titanium:sapphire laser with wavelength tunability and femtosecond pulses was available to us in our present experiments we could tune the wavelength of the laser in order to relate the membrane potential alterations to the concentration of membrane attached GFP as detected by two photon fluorescence. This would allow for absolute membrane potential measurements. Finally, such titanium:sapphire lasers can emit femtosecond pulse durations instead of the 100-ps pulse durations available in our present experimental set-up. With this laser a SH signal could be detected with a resident time per pixel of 1 μs. Such measurements have the potential of providing dynamic information on membrane potential alterations. In summary, this confluence of physics with molecular biology, optically detected electrophysiological changes, sensory processes and behavior could have a significant impact in addressing important questions in neurobiology.

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