Second Harmonic Generation in Neurons: Electro-Optic Mechanism of Membrane Potential Sensitivity

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ABSTRACT Second harmonic generation (SHG) from membrane-bound chromophores can be used to image membrane potential in neurons. We investigate the biophysical mechanism responsible for the SHG voltage sensitivity of the styryl dye FM 4-64 in pyramidal neurons from mouse neocortical slices. SHG signals are exquisitely sensitive to the polarization of the incident laser light. Using this polarization sensitivity in two complementary approaches, we estimate a ~36° tilt angle of the chromophore to the membrane normal. Changes in membrane potential do not affect the polarization of the SHG signal. The voltage response of FM 4-64 is faster than 1 ms and does not reverse sign when imaged at either side of its absorption peak. We conclude that FM 4-64 senses membrane potential through an electro-optic mechanism, without significant chromophore membrane reorientation, redistribution, or spectral shift.

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Traditionally, the membrane potential of neurons is measured with intracellular electrical recordings or patch-clamping. Although these techniques have been, and continue to be, powerful methods in neuroscience, they cannot be used to monitor membrane potential at dendritic spines, which are the predominant sites of excitatory inputs into a neuron. The reason is that spines are too small (~1 μm^3) to accommodate an electrode. A promising nonlinear optical technique, second harmonic generation (SHG) (1–3), can be used to measure the electrical potential at various interfaces, e.g., charged surfactant/water (4) and charged phospholipid liposome/water interfaces (5). SHG has been recently used to measure membrane potential of biological cells in an imaging microscopy mode, using a variety of chromophores that are adsorbed to the cell membrane (3).

We recently carried out the first membrane potential measurements of individual spines using FM 4-64 (6), a fluorophore used to study vesicle recycling (7), which is also an excellent SHG chromophore (8,9). We observed a linear dependency between SHG signals and membrane potential, with a sign reversal at zero voltage, and proposed that the voltage response of FM 4-64 SHG was likely electro-optic (6). However, other mechanisms of voltage sensing, such as chromophore redistribution, reorientation, or spectral shift (10–12), could not be ruled out.

We now explore the biophysical mechanisms of FM 4-64 SHG voltage sensitivity, characterizing its polarization, wavelength dependence and the speed of its response, to better understand how the chromophores behave in response to changes in electric field.

Most push-pull styryl dyes, such as FM 4-64, possess a long molecular axis, and their hyperpolarizability tensor is dominated by a single tensor element along that axis (10,11,13–16). With this simplification and the assumption that the orientation of the dye in the plane of the membrane is isotropic and peaked at a fixed angle, the tilt angle of the dye molecule with respect to the plasma membrane can be analytically determined. Following past work on artificial membranes (10), the intensity of SHG versus the angle ϕ between excitation light polarization and the membrane normal can be expressed as

$$SHG = SHG_0\{[\langle\cos^3\theta\rangle\cos^2\phi + \langle\cos\theta\sin^2\theta\rangle\sin^2\phi/2]^2 + \langle\cos\theta\sin^2\theta\rangle^2\sin^2\phi\cos^2\phi\}, \quad (1)$$

whereby SHG_0 is a constant, θ the angle of dye axis to membrane normal, and $\langle\ldots\rangle$ denotes ensemble average of dye molecules with different orientations (Fig. 1 *A*).

We performed SHG measurements from layer-5 pyramidal neurons in neocortical brain slices, filled with FM 4-64 through a patch pipette and held at −65 mV. Due to the dominant uniaxial hyperpolarizability of FM 4-64, various parts of the plasma membrane showed strong SHG signals depending on the relative angle of the laser polarization and the local membrane, with orthogonal segments of the membrane showing negligible SHG signals (Fig. 1 *B*). Because neurons are not perfectly spherical, we sampled a small region of the soma and varied the polarization of the excitation light. The value θ was then obtained from $\cos\theta = 0.81 \pm 0.02$ (mean ± SD; $n = 5$ neurons) by fitting the data using Eq. 1 (Fig. 2), yielding an average dye tilt angle of ~36° to the membrane normal, assuming a narrow orientational distribution.

As an alternative approach to measure dye orientation, we analyzed the polarization of the SHG signal. By separating the SHG components parallel and perpendicular to the same

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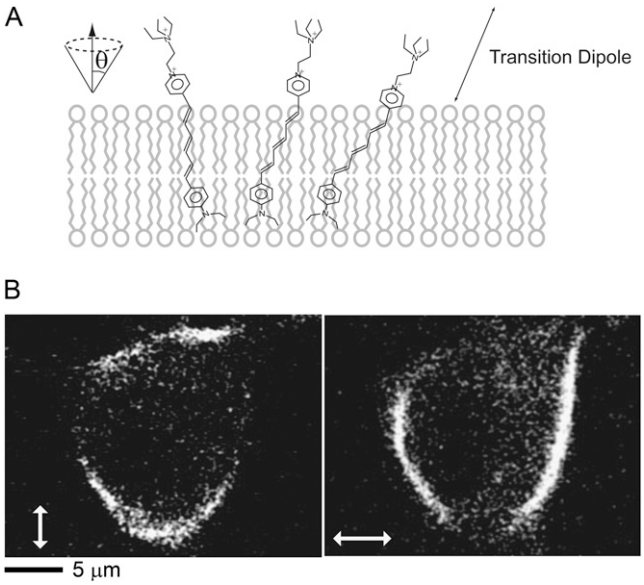

FIGURE 1 (*A*) Simplified representation of FM 4-64 geometry in membrane. Arrow shows average orientation of the uniaxial hyperpolarizability, with θ being its tilt-angle to membrane normal. (*B*) Membrane SHG signals polarization dependence; arrow indicates laser polarization.

excitation polarization, we also found an average dye tilt-angle of ~36° ($n = 2$, see Supplementary Material Fig. S1). Thus, two complementary approaches yielded the same estimation.

We then investigated whether the dye orientation could be altered by changes in the local electric field. For these experiments, we clamped the membrane potential of the neurons at voltages ranging from −95 mV to 0 mV, while SHG images of the cell were recorded at different laser polarizations (Fig. 3). No dye tilt-angle change was observed within our detection limit, even for membrane potential changes as large as 90 mV (or a change of electric field of 1.6×10^7 V/m, assuming membrane thickness of 5 nm). These results were reliably reproduced across neurons ($n = 3$). Using Eq. 1, we calculated a confidence interval for our dye tilt-angle measurement. As shown in Fig. 3, a 5° change in dye tilt-angle would result in a significant change in the shape of fitted curve.

The observed insensitivity of dye orientation to the transmembrane field is intriguing, as the change of electric field applied was on the order of tens of MV/m. One possibility is that there is a broad distribution of tilt-angles (17), which would not be sensitive to membrane potential. Another possibility is that the local electrostatic fields, e.g., dipolar, steric forces imposed by membrane phospholipids chains and other membrane biomolecules, or hydrophobic and hydrophilic forces, are the dominant orientational factors in the membranes of the neurons used in this study.

The voltage sensitivity of fluorescent probes can be due to a change in the molecular electronic transition (12), reorientation of the membrane-bound dye (18), or a voltage-dependent slow redistribution of the population of dye molecules (19). Unfortunately, studies of the mechanisms of electric field sensing by SHG chromophores are still scarce. In artificial membranes consisting of one type of phospholipid, two mechanisms of SHG electric field sensing were described: a purely electro-optical response due to a third-order polarization with one of the fields being static (10), and a combination of electro-optical and fast chromophore reorientation (11). In addition, a very slow (35–200 ms) SHG response to membrane potential changes has been reported (20), incompatible with an fast electro-optic mechanism.

To characterize the speed of the FM 4-64 response to voltage we performed fast (0.1 ms resolution) measurements of somatic membrane regions of neurons, under conditions where action potentials were generated by brief current injections (Fig. 4). In these experiments, the temporal response of FM 4-64 to a change in the electric field was instrument-limited, on the order of a microsecond timescale. As demonstrated in Fig. 4, the

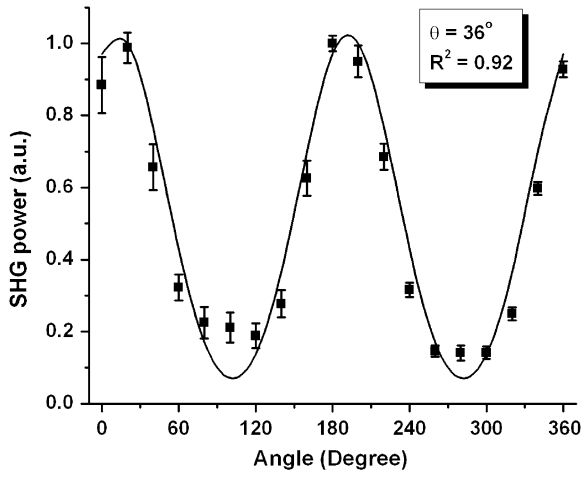

FIGURE 2 SHG power versus angle of laser polarization (mean ± SE, $n = 5$ neurons) . Line is a nonlinear fit, based on Eq. 1.

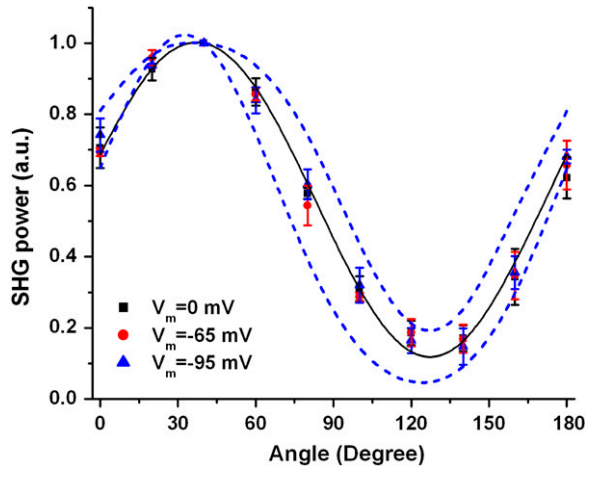

FIGURE 3 Polarization dependence of SHG signal at different membrane potentials. Solid curve is the best fit with $\theta = 38°$; dotted lines are fitted curve with $\theta = 43°$ and $\theta = 33°$, respectively.

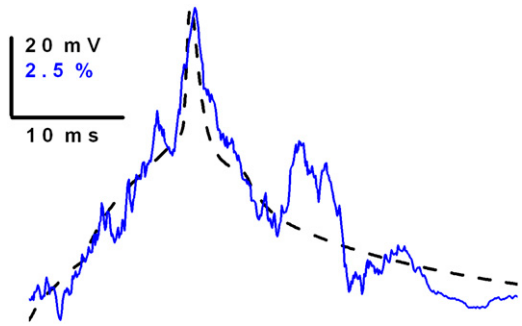

FIGURE 4 Fast kinetics of action potential SHG measurements. Relative change in SHG (*blue line*) shows similar kinetics as the electrical signal, recorded with a whole-cell electrode (*dashed black line*). Note the linearity of the optical response to voltage and how the peaks coincide.

SHG signal from FM 4-64 tracked the fast time course of the action potential, without any appreciable delay.

A fast SHG response to the electric field could be due to a Stark shift in the spectrum, and a telltale sign of this would be that the SHG relative change would switch signs as the SHG frequency moves through the absorption peak. In agreement with other styryl dyes measurements (20), we did not observed a change in sign when imaging FM 4-64 SHG on either side of its absorption peak (~500 nm). Specifically, the change in SHG in response to a 100-mV depolarization was 10.3 ± 0.8% at 850 nm ($n = 15$), 10.6 ± 1.1% at 900 nm ($n = 13$), and 14.8 ± 1.2% at 1064 nm ($n = 19$).

We conclude that the potential sensing mechanism of FM 4-64 in neurons is predominantly electro-optical. No physical reorientation of the dye was detected upon changing membrane potential and the response was as fast as our time resolution and had no wavelength dependency in its sign. Finally, the similar linearity of the response at slow (6) and fast (Fig. 4) temporal resolutions indicates that the mechanisms of voltage sensitivity are likely to be the same at these two different timescales. Although the exact mechanism of SHG potential sensing is not critical as long as the response is fast, linear, and calibrated before any quantitative interpretation, its understanding is important for improving probe design, a crucial issue given that SHG measurements of fast voltage transients in spines and dendrites are currently limited by poor signal/noise ratio (6,21). Chromophores that combine strong electro-optic and molecular orientation response (11) would be ideal for optimal SHG potential imaging with high sensitivity.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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