Two-photon excitation of channelrhodopsin-2 at saturation

John Peter Rickgauer and David W. Tank

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We demonstrate that channelrhodopsin-2 (CR), a light-gated ion channel that is conventionally activated by using visible-light excitation, can also be activated by using IR two-photon excitation (TPE). An empirical estimate of CR's two-photon absorption cross-section at \( \lambda = 920 \text{ nm} \) is presented, with a value (260 ± 20 GM) indicating that TPE stimulation of CR photocurrents is not typically limited by intrinsic molecular excitability \( [1 \text{ GM} = 10^{-20}\text{(cm}^2\text{ s)/photon}] \). By using direct physiological measurements of CR photocurrents and a model of ground-state depletion, we evaluate how saturation of CR's current-conducting state influences the spatial resolution of focused TPE photostimulation, and how photocurrents stimulated by using low-power scanning TPE temporarily summate. We show that TPE, like visible-light excitation, can be used to stimulate action potentials in cultured CR-expressing neurons.

Channelrhodopsin-2 (CR) is a microbially derived cation channel that transiently opens in response to blue-light illumination (1). Several modified forms of the channel, each encoded by a single gene, have been expressed heterologously in excitable mammalian neurons to recapitulate this light-gated conductance. It has been shown that in CR-expressing neurons, action potentials can be triggered by single-photon excitation, using blue-light illumination, with millisecond temporal resolution (2–7). Where multiple cells are photosensitized, the optical resolution with which each cell can be independently excited determines the precision of optical neuronal interrogation.

Spatially localized excitation of individual cells is, however, difficult to achieve in thick biological tissue by using single-photon excitation. Brain tissue, for example, is an optically scattering medium that defocuses light, reducing the spatial definition and intensity of a focused beam or projected spatial pattern with increasing tissue depth (8). Additionally, excitation cannot be confined to a single z-focal plane of interest perpendicular to the axis of illumination (but see ref. 9), a geometry typically favored for in vivo studies of neural circuits.

Two-photon laser-scanning microscopy (TPLSM) using a focused IR laser beam is the method of choice by which to achieve spatially localized fluorescence excitation deep in scattering tissue, providing an intrinsic optical section around the plane of focus (10, 11). We hypothesized that an analogous method to activate CR photocurrents by using two-photon excitation (TPE) would confer a much higher degree of spatial precision for targeted neuronal photostimulation than is currently possible by using single-photon (blue-light) illumination.

Here we use whole-cell recordings of photocurrents in cultured cells to provide an initial biophysical characterization of TPE of CR. At typical light intensities used for TPLSM, we find that brief (<25 ms) CR photocurrents are described by a single-photon model incorporating depletion of the CR ground state. Our analysis is used to provide an estimate of the CR two-photon absorption cross-section, which turns out to be larger than most fluorescent molecules commonly used in TPLSM. This high sensitivity, coupled with the fact that the lifetime of the excited current-conducting state is long (>10 ms vs. <10 nsec lifetime of the typical fluorophore), leads to the condition of ground-state depletion. We refer to this condition as conducting-state saturation, in analogy to excited-state saturation of fluorophores, which occurs when high light intensity shortens the ground-state lifetime below that of the excited state, leading to an inversion of state occupancy.

Previously, it has been observed that TPE of fluorophores under saturated conditions (at the focus) can generate more fluorescence away from the plane of focus than near the plane of focus (12) (also see ref. 8); here, we find that focused TPE at typical TPLSM intensities can excite larger photocurrent amplitudes away from the plane of focus than near the plane of focus. Photostimulation of currents is better confined to the region around the focus when the laser intensity is reduced. Scanning a low-intensity TPE focus rapidly over the excitable cell membrane can recover much of the photocurrent available under whole-cell blue-light excitation, although the integrated (whole-cell) current stimulated in this way is sensitive to the scan trajectory and duration of the scan. As a practical illustration of these findings, we demonstrate that scanning TPE can stimulate action potentials in cultured neurons by using lower squared-intensity values than are commonly employed in TPLSM imaging.

Theory

In this section, we present a simplified model of saturation-limited CR activation that is appropriate to typical experimental conditions used in TPE. We will use this model, adapted for particular illumination and scan geometries, to analyze induced membrane currents; the analysis provides estimates of the CR absorption cross-section and a foundation for optimizing TPE-based methods for neuronal stimulation.

Several empirical models addressed the dynamics of CR photocurrents under stationary illumination, assuming that illuminated molecules undergo multiple photocycles (1, 13). The model we present here is intended to describe only the transient photocurrent arising from a population of illuminated CR molecules undergoing (at most) one photocycle (Fig. S1 in the SI Appendix). Accordingly, we restrict our analysis to the initial 25 ms of recordings, where the fraction of molecules contributing to measured current through a second photocycle should be small (1, 13).

Our model assumes that the population of molecules is initially homogenous and in the excitable ground state. We addressed this issue experimentally by allowing dark intervals of at least 60 s between measurements, longer than the reported time-constant for molecules in the dark to return to the excitable ground state (7–8 s) (14).

A CR molecule with an \( m \)-photon absorption cross-section \( \sigma_m \) \( (m = 1, 2\text{ for }1-, 2\text{-photon}) \) and illuminated with intensity \( I(t) \),

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1 To whom correspondence should be addressed. E-mail: dwtank@princeton.edu.

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should absorb light at a rate \((\sigma_m/m)I(t)\)\(^m\) (15). As molecules are excited in a single-photocycle model, the concentration \(\rho_g(t)\) of molecules in the ground state is reduced exponentially according to

\[
\rho_g(t) = \rho_0 e^{-\frac{\sigma_m}{m} I(t) t^{m-1}},
\]

where \(\rho_0\) is the initial ground-state concentration. For molecules on a two-dimensional plane (e.g., on an idealized membrane surface) illuminated with a radially symmetric profile \(I(r, t)\), the rate \(dN(t)/dt\) of recruitment of a particular excited state with a population \(N(t)\), occurring with quantum efficiency \(\eta\), can be calculated by integrating over the surface, and is given by

\[
\frac{dN(t)}{dt} = \frac{\sigma_m}{m} \int I(r, t) \rho_g(r, t) dA,
\]

where \(\rho_g(r, t)\) now has an explicit spatial dependence. \(N^*(T)\), the number of current-conducting molecules at time \(t = T\) after light onset, can be calculated by weighting the excitation rate with the channel’s impulse-response function and integrating in time, giving

\[
N^*(T) = \int_0^T \frac{dN(t)}{dt} \left[ e^{-\frac{T-t_1}{\tau_1}} - e^{-\frac{T-t_2}{\tau_2}} \right] dt,
\]

where molecules enter the current-conducting population (denoted by \(\ast\)) with a latency \(\tau_1\) after excitation, and these currents decay with a characteristic time constant \(\tau_2\). If the single-channel current is \(i^*\), then the instantaneous current at time \(T\) is \(I^*(T) = i^* N^*(T)\), and the total available current from a population of \(N_i\) molecules is \(I_{max} = i^* \eta N_i\). The instantaneous fraction of available photocurrent is thus

\[
\frac{I^*(T)}{I_{max}} = \frac{N^*(T)}{\eta N_i}.
\]

In the following, we will use solutions to Eq. 4 under different excitation profiles. For clarity, we note that \(\sigma_m\) represents the molecular absorption cross-section and not the molecular action cross-section (\(\eta\sigma_m\)); if ground state absorption yields short-lived, nonconducting intermediates that return to an excitable ground state within the period of our analysis, then our estimate of \(\sigma_m\) should be taken as a lower bound.

**Results**

As a first step toward developing a method for TPE stimulation of CR, we used the depletion model to estimate the CR absorption cross-section, which sets the scale for molecular excitability. Although our focus here is \(\sigma_2\), the cross-section for two-photon excitation using IR illumination, the single-photon cross-section \(\sigma_1\) under blue-light illumination was also measured for completeness.

Under spatially uniform illumination, the solution to Eq. 4 for the time-dependent photocurrent \(I^*(T)\), expressed as a fraction of the total available photocurrent \(I_{max}\), is given by

\[
\frac{I^*(T)}{I_{max}} = \sum_{k=1,2} (-1)^k \frac{\tau_2 \eta}{\tau_k - \tau_2} \left( e^{\frac{\tau_k - T}{\tau_2}} - e^{\frac{T - \tau_k}{\tau_2}} \right),
\]

where \(\tau_k = m(\sigma_m/m)^{-1}\) is the ground-state lifetime for one-photon \((m = 1)\) and two-photon \((m = 2)\) illumination, and \(\tau_1, \tau_2\), for \(k = 1, 2\) refer to the latency and current decay time constants respectively, as in Eq. 3. Our general strategy was to analyze the shape of initial transient whole-cell membrane current under voltage-clamp \((-50\,\text{mV})\) in CR-expressing HEK293T cells under a set of spatially uniform illumination conditions of increasing intensity. As intensity increases, \(\tau_p\) is shortened and depletion becomes more rapid, which changes the initial transient shape in a systematic way that depends upon the specific value of \(\sigma_m\) (Fig. 1B); fitting the set of traces to the model for known illumination intensities allows estimation of \(\sigma_m\).

Wide-field illumination of CR-expressing cells by using blue light from an LED \((\lambda = 470 \pm 13\,\text{nm})\) stimulated fast-rising inward currents that reached a transient peak amplitude, and subsequently decayed toward a steady-state value with reduced amplitude (Fig. 1A). Transient currents (here, \(t = 0\) to \(10\,\text{ms}\)) stimulated by four or more different intensities were then fit simultaneously (i.e., as families) to Eq. 5: one representative family with fits overlaid is shown in Figure 1C. Fits

![Fig. 1. Estimating \(\sigma_2\) with measurements of saturation-limited excitation. (A) CR photocurrents stimulated experimentally by using wide-field blue-light illumination (\(\lambda = 470 \pm 13\,\text{nm}\); blue traces, above) or IR TPE focused to a large-diameter spot (\(\lambda = 920 \pm 6\,\text{nm}\); red traces, below); experimental illumination geometries are depicted at right (\(\sigma_m = 35\,\mu\text{m})\); overline indicates illumination epoch (500 ms); trace shading denotes incident intensities (\(I^2\)); see SI Appendix. Each trace is the average of 5–10 repeat trials. (Inset, Lower) Photocurrents excited by using mode-locked (ML) and non-mode-locked (no ML) pulses at constant average power; scale bars are 40 \(\mu\text{A} , 32\,\text{ms}\). Traces are five-trial averages. (B) Numerically simulated transient photocurrents, generated by using Eq. 5 for each indicated value of \(\sigma_1\) (\(\sigma_2\), lower traces); I values from A (\(I^2\), lower), and \(t = 0\)–10 ms (0–25 ms, lower). Amplitudes in each frame are scaled to maximum values reached during the interval (maximum values are indicated below). (C) Experimental currents from A with overlaid fits to Eq. 5. Boxed values of \(\sigma_1\) (\(\sigma_2\), lower) were obtained by fitting the family of traces simultaneously to Eq. 5, and then used to generate the overlaid fits.
to 35 of these families (175 recordings from 8 cells) yielded an estimate for $\sigma_1$ of $5 \pm 1 \times 10^{-17} \text{cm}^2$ (median ± SEM; range $1 - 7 \times 10^{-17} \text{cm}^2$), which is comparable to $\sigma_1$ measured for purified Vohax channelrhodopsin $[1.7 \times 10^{-16} (16)]$.

To estimate $\sigma_2$, we used a long focal-length lens to focus pulsed IR excitation ($\lambda = 920 \pm 6 \text{ nm}$) to a large-diameter spot in the sample plane, generating an approximately uniform squared-intensity profile across the full diameter of targeted cells (Fig. 1.4, Inset; $\omega_{np} = 35 \mu\text{m}$). In this optical configuration, TPE stimulation of CR-expressing cells stimulated inward photocurrents that reached a transient peak amplitude, followed by decay toward a reduced stationary amplitude during sustained illumination (Fig. 1.A). Trials at comparable average power but using non-mode-locked pulses did not stimulate photocurrents (Fig. 1.A, Inset). Maximum rise-rates of photocurrents showed a nearly power-squared dependence that was not observed in single-photon excitation trials (Fig. S2 in the SI Appendix). Over this range of squared-intensities ($0.3 - 1.8 \times 10^{18} \text{W/cm}^2 \text{s}$), currents stimulated by TPE reached peak amplitudes that were 10–40% of the peak amplitude stimulated by high-intensity blue-light illumination of the same cells.

Families of transient photocurrents (here, $t = 0 - 25 \text{ ms}$) were fit simultaneously to Eq. 5, computing $\tau_p$ for the case of two-photon excitation ($m = 2$); a representative family with fits overlaid is shown in Fig. 1C. Fits to 5 families of curves, each including 6 or more intensities (286 recordings total, averaged to 42 across 5 cells) yielded an estimate of $\sigma_2 = 2.6 \pm 0.2 \times 10^{-18} \text{cm}^2/\gamma$ (range $2.5 - 3 \times 10^{-18} \text{cm}^2/\gamma$) or $260 \pm 20 \text{ GM}$ [1 GM $= 10^{-50} \text{cm}^4/\gamma$/photon]. This value is similar to $\sigma_2$ reported for bacteriorhodopsin, a microbial proton pump homologous to CR, near its spectral two-photon absorption peak (290 GM) (17).

This value is also higher than most other values reported for fluorophores common to TPLSM [e.g., EGFP, DsRed2; 40–100 GM (18)], implying that TPE stimulation of CR photocurrents should not be excitation-limited at intensities compatible with TPLSM imaging.

**Photostimulation with a Diffraction-Limited TPE Focus.** Whole-cell stimulation with a large beam waist (as shown in Fig. 1) represents one approach to TPE photostimulation, but spatially precise photostimulation requires a smaller excitation volume. Typically, high-resolution TPLSM imaging is achieved by focusing excitation pulses through a high numerical aperture objective, generating fluorescence mostly within a diffraction-limited volume near the focus (11). However, when the integrated rate of excitation away from the focus exceeds the rate near the focus (8), as can occur under saturated conditions (12), this resolution can be significantly degraded.

We tested how this condition would affect spatially precise TPE stimulation of CR photocurrents by using a high-N.A. objective lens to generate a small TPE focus ($\approx 1 \mu\text{m}^2$ in the plane of focus; $40\times/0.8$ N.A., Olympus LUMPlanFL/IR). The geometric center of the focus was held stationary on the upper surface of a CR-expressing cell for brief exposures (32 ms), and photostimulated currents were recorded. Because transmembrane CR currents require excitation of membrane-bound CR molecules, the amplitude of photocurrents excited at the focus should scale (approximately) with the area of the illuminated membrane. However, although the area illuminated in the plane of focus accounted for less than 0.1% of the total cell surface area, currents stimulated in this configuration sometimes exceeded 10% of the amplitude stimulated by whole-cell illumination at high intensities (N=4 stimulated locations from three cells; $9 \times 10^{15} \leq I \leq 1 \times 10^{16} \text{W/cm}^2 \text{s}^2$). This excess suggested that these photocurrents were not arising exclusively from excitation of the in-focus membrane.

To determine whether these currents included a component excited away from the plane of focus (here, from the opposing cell membrane), we stepped the plane of focus away from the cell (+z in Fig. 2) and repeated these measurements. Out-of-focus excitation contributed significantly to the measured current; recordings from one cell, shown in Fig. 2B, illustrate that photocurrents could be stimulated at moderate sample plane powers (40 mW) even when the plane of focus was several tens of microns above the cell.

Over a range of z-focal plane positions (relative to the cell equator), the amplitude of photostimulated currents (measured at $t = 15 \text{ ms}$) initially increased with increasing distance from the equator, generating a minimum amplitude near the equator, an intermediate amplitude near the cell membrane, and a maximum amplitude where the plane of focus was above the cell (+z in Fig. 2; N = 4/4 cells; 20–40 mW). This trend was also observed in 3/3 cells where the plane of focus was stepped below the cell equator (−z in Fig. 2). In trials using 20–40 mW of power, currents with the largest total charge, $Q_T$ (membrane current integrated from $t = 0$ to $t = 15 \text{ ms}$) occurred when the plane of focus was separated from the cell equator by 15–25 μm.

These trends could be reproduced in numerical simulations predicting the total current generated near and away from the plane...
of focus. Cell-membrane geometry was approximated by two parallel planes (top and bottom membrane surfaces) separated by a distance $h = 10 \, \mu m$ along the optical axis (Fig. 2A), with an equatorial plane at a distance $z$ from the plane of focus. Under illumination with a Gaussian squared-intensity profile, the solution to Eq. 4 for a specified value of $z$ is given by

$$I(r, T) = \sum_{k=1}^{\infty} \left(-1\right)^{k+1} \frac{1}{k} \int_0^T \frac{1}{r} \left(1 - e^{-\frac{r^2}{4kT}}\right) e^{-\frac{T}{2k}} \, dt,$$  

where $\tau_g = 2(\sigma^2 I_0(z))^{-1}$ is the ground-state lifetime under two-photon excitation. In this formulation, increasing the separation between the plane of focus and an excitable surface not only decreases the incident peak squared intensity, $I_0^2$, but also increases $I_{\text{max}}(z)$ because the area illuminated is larger (see SI Appendix).

In simulations using 40 mW of power, positioning the plane of focus at one surface (e.g., green trace at $z = +5 \, \mu m$, Fig. 2A, Inset) concentrated incident TPE on that surface, generating a current that rapidly attained a peak, with an amplitude limited by the small illuminated area near the focus. Simultaneously, diffuse excitation on the opposing surface (blue trace, Fig. 2A, Inset) stimulated a current that rose less rapidly, but which reached a larger amplitude at peak. The summed two-surface current, approximating the whole-cell current (black trace), thus had a larger amplitude than predicted by the area excited at the focus.

At moderate power (40 mW), the maximum integrated current amplitude occurred in both simulations and experiments when the whole cell was below the plane of focus ($z = 30–35 \, \mu m$ in simulations, $z = 20–25 \, \mu m$ in experiments), at a distance that was reduced as the total sample-plane power was reduced (Fig. 2B). In simulations using very low power ($P = 0.1 \, mW$), where the effects of saturation should be reduced, integrated current amplitudes were highest when the plane of focus coincided with the membrane (Fig. 2B, Inset), as expected of TPE under nonsaturated conditions.

**Stimulation with a Moving Focal Spot.** For some types of measurements, out-of-focus excitation manipulated to maximize whole-cell current activation (as shown in Fig. 2) could represent a second approach to TPE photostimulation. A third approach, which could make better use of the nonlinear properties of TPE, would be to use low-power TPE to stimulate currents primarily at the focus, and to scan the focus, in time, across the photosensitized membrane.

![Fig. 3](image)

**Fig. 3.** Photocurrents stimulated by focused TPE. (A) Strip scan. (i) TPLSM fluorescence image of a CR-expressing cell (gray = volume-filling Alexa 594, projected in $z$; yellow = CR-EYFP, single $z$-focal plane). Highlighted region indicates the boundary of a strip-scan stimulation trajectory (schematically represented in red), in which the TPE focus was oscillated at 1 kHz ($\gamma$) while scanning across the cell ($\omega$). (ii) TPE-stimulated photocurrent recorded during a strip-scan (gray = 5 repeats overlaid; black = average), and trial-averaged TPE fluorescence recording during strip scans (yellow = CR-EYFP; dye-filled region indicates thresholded intracellular Alexa 594 fluorescence). Horizontal axis denotes scan time (0–1024 ms; scan distance is 57 $\mu m$). (B) Temporal summation of photocurrents stimulated by a moving TPE focus: maximum photocurrents (scaled) predicted for CR molecules excited in a temporal sequence, occurring over a total time of $T_s$ approximating progressive excitation by a moving focal spot (Eq. 7). (Inset) Schematic representation of fast and slow scans over a fixed number of molecules. (C) Photocurrents stimulated by focused TPE scans [N.A. = 0.2; $I_0^2 = 3.8 \times 10^{14} \mu A^2 / \mu m^2$, (Left); 2.5 $\times 10^{14} \mu A^2 / \mu m^2$, (Right)], illustrating the effect of scan consolidation (i.e., reducing total scan time, $T_s$). Peak currents are indicated and connected by dotted lines. Raster scan times (grayscale; shorter = faster = left) were varied by changing the number of lines in a fixed-area raster. Currents stimulated using spiral-scan trajectories, scanning inward from the cell boundary to the center, are shown in red ($T_s = 27$ ms, (Left); $T_s = 16$ ms, (Right)). Traces are registered in time by the measured onset of fluorescence during each scan, and represent average response from three or more trials.
inward photocurrents were excited primarily when the focus was within 1 μm of the inferred membrane edge, where fluorescence associated with the cell boundary was also excited (78% of deflections > 2σ above the mean, using 1 ms bins; 30 scans across 3 cells, 1–7 mW).

Under conditions where currents are generated primarily at the focus, the focus must be scanned across a large population of molecules to generate large-amplitude currents. However, if scanning photostimulation takes much longer than CR’s current decay-constant (τc), currents stimulated early in the scan should not summate with currents activated later in the scan (e.g., Fig. 3A). To estimate how the total scan time, Ts, could influence the amplitude of scan-stimulated photocurrents, we reasoned that scanning across a uniform concentration of molecules (Nt; molecules total) at a constant velocity should excite molecules at a constant rate (i.e., dN/dt = K in Eq. 2). In the typical condition in which Ts > τc, the solution to Eq. 4 is closely approximated by

\[
\frac{I^*}{I_{\text{max}}} = \frac{\tau_c}{Ts} (1 - e^{-\frac{Ts}{\tau_c}}).
\]

In this formulation, a scan sequence lasting n times longer than τc (Ts = nτc) would limit the peak photocurrent to \((1 - e^{-n})/n\) of the available current (Fig. 3B). For a typical TPLSM whole-cell raster scan time \(Ts = 250 \text{ ms}\) and \(τc = 20 \text{ ms}\, Eq. 7\) predicts that the scan-stimulated current amplitude would be < 10% of the whole-cell stimulated current amplitude. It also predicts that the peak current amplitude, which scales inversely with \(Ts\), could increase significantly by scanning over the same area in less time, which we here refer to as “consolidation.”

To test whether consolidation would allow larger-amplitude currents to be generated with low-power excitation, we measured currents stimulated by scans of varying duration, Ts, over the cell surface. To stimulate currents from all membrane areas (top, bottom, and side of a cell) from a single (x, y) location in these scans, we extended the TPE volume along the optical axis (in z) by adjusting the effective N.A. of the objective with an iris (N.A. = 0.2–0.5) (see Fig. S1 in the SI Appendix).

Fig. 3C demonstrates the effect of consolidation over a ten-fold range of Ts in two cells; the fastest scans (shown in red) followed spiral-like trajectories patterned after cell geometries (see SI Appendix), and the other scans (grayscale) were produced by changing the number of lines in a raster. In general, photocurrent deflections arising from intersections of the scan trajectory and excitable cell membrane were observable in both slow scans and fast scans, and whole-cell current amplitudes increased as these deflections were consolidated by reducing Ts. Compared with slow-scanning currents (Ts > 100 ms), faster-activated currents that reduced the time-to-peak to 15–30 ms increased the peak amplitude by a factor of 3.4. Amplitudes increased more rapidly at low Ts, in agreement with the dependence predicted by Eq. 7.

Compared with raster scanning across cells using the full objective N.A. (=0.8) and TPLSM image acquisition scan times (Ts > 100 ms), which typically stimulated peak currents representing <10% of the whole-cell illuminated current (6 ± 3%; N = 5 cells), consolidating the scan (i.e., reducing Ts) to allow whole-cell stimulation in < 100 ms allowed the peak photocurrent to increase by a factor of 5.8 (20 averaged recordings, each with ≥3 sweeps, across 7 cells). In four cells where membrane geometries were compatible with a full scan of the membrane in <16 ms, currents could attain peak amplitudes that were over half (52–84%) of the peak amplitudes stimulated by whole-cell blue-light illumination of the same cells.

Two-Photon Spike Stimulation in Cultured Neurons. A common application of CR is blue-light stimulation of action potentials in excitable neurons. As a practical demonstration of our findings, then, we showed that consolidated TPE spiral scans can be used to stimulate action potentials in cultured neurons.

Dissociated superior cervical ganglion (SCG) neurons transduced to express CR (see Materials and Methods) were targeted for whole-cell recordings, filled, and imaged to define trajectories of soma-targeted spiral scans; the z-dimension of the TPE focal volume was adjusted to approximately match the z-dimension of cells (N.A. = 0.2–0.5). In six neurons where wide-field blue-light illumination depolarized membrane voltages above spike threshold in 5/5 trials, TPE stimulation of the same cells depolarized cells above threshold in most trials, generating spikes (50 spikes out of 60 trials, 10 trials per neuron; min 5/10, max 10/10) (Fig. 4).

Discussion

Our results demonstrate that the kinetics of transient CR currents stimulated by TPE can be quantitatively captured in a one-photon model that incorporates ground-state depletion. This framework was used to estimate the CR absorption cross-section and to explore the effects of stimulation geometry and duration. Here, we review these results from the perspective of experimental design for single-cell stimulation.

CR has a high two-photon absorption cross-section (≈260 GM at 920 nm). When CR containing membrane is continuously illuminated by using the focus of a standard 40 × 0.8 N.A. objective and 1 mW of power, >99% of the CR molecules should be excited within 10 μs (see Eq. 6). Because of the long lifetime of the excited conducting state, continuing illumination or higher intensity at the same position does not produce more current as the ground state is substantially depleted, with further excitation yielding a saturated response. When viewed on the msec time scale, a small but rapidly rising pulse of current is produced that represents most channels open from that patch of membrane and which decays away on the tens-to-msec time scale (τ2). This basic finding has two important
implications for localized TPE photostimulation using CR: (i) at high light intensities that rapidly saturate the excited photocurrents from an in-focus patch, excitation away from the focus can produce photocurrents in other membranes from the same cell (or in other cells) that can easily exceed focally excited currents; and (ii) at lower intensities that still excite most available current from an in-focus patch but which also reduce out-of-focus excitation, scanning the focused spot rapidly across the entire excitatory membrane area of a cell on a time scale less than $\tau_2$ will produce the largest current for stimulation.

Both of these effects are captured quantitatively and can be studied more generally as a function of light intensity and geometry in the framework described by Eqs. 1–4, where photocurrents represent the rate of excitation as integrated, in the mathematical sense, over the duration and membrane-excitable area of illumination in a single-photon model. In our experiments and simulations, transient photocurrents at high light intensity (much higher than focused spot saturation) are largest when the focal spot is off the membrane (Fig. 2). Photocurrent stimulation could be better confined to the small region near the focus by using lower-intensity excitation (Figs. 2–3), although such currents were (naturally) much smaller in amplitude because the membrane area contributing to the current is very small. This finding is consistent with studies that use focused single-photon excitation of CR (19, 20), wherein the spatial precision attainable with similar parked-beam protocols was also improved by reducing excitation intensity.

Consistent with the high CR cross-section and Eq. 7, we found that low-intensity, focused TPE stimulation at moderate N.A. values could generate currents with amplitudes exceeding 50% of the whole-cell stimulated photocurrent by rapidly scanning the focal volume over the excitable cell membrane. Fast-scanning stimulation should be compatible with most TPLSM systems, although photocurrents stimulated in this way reached amplitudes that decreased sharply as whole-cell scan times exceeded $\tau_2$. Alternate approaches to achieving large-amplitude currents with spatial precision might use molecules with longer $\tau_2$ values (e.g., C128 mutants (3)) (see Eq. 7), although long $\tau_2$ values should also render such molecules more sensitive to collateral excitation away from the plane of focus (see Eq. 6). These properties could be complemented or mitigated with spatially targeted expression strategies, e.g., by using molecular trafficking signals (21).

Flexible methods for TPE photostimulation of CR or other opsins might take advantage of optical techniques recently developed for TPLSM imaging, including focal-array scanning (22), multiple z-focus plane excitation (23), or beam-shaping with a spatial light modulator (24). Our framework may provide some guidance in the development of these techniques.

Materials and Methods

Cell Culture and Gene Expression. CR expression used the plenti-EF1a-hChR2-EYFP-WPRE plasmid a gift from K. Deisseroth (Stanford University, Stanford, CA). Transient CR expression in low-passage HEK293T cells (Invitrogen; cultured in DMEM +10% FBS) was attained by using calcium phosphate precipitation (0.5–1 μg DNA/35 mm plate), and physiology was performed within 24–96 h of transfection. Stable expression of CR in dissociated neurons from E17 rat SCG (provided by K. McCarthy and L. Enquist (Princeton University, Princeton, NJ); cultured in supplemented Neurobasal medium) was attained by lentiviral transduction 4–7 days in vitro; vectors were grown as described (2). Physiology was performed within 2–4 weeks of transduction.

Electrophysiology. Whole-cell recordings under voltage clamp (−50 mV) and current-clamp (Fig. 4) were made at room temperature under low ambient-light conditions, using an N = 0.1 headstage and a BVC700A bridgevoltage clamp amplifier (Dagan), and then digitized and recorded at 20-40 kHz using the pClamp 10 recording platform (Digidata 1322a, Clamplex 10, Axon/Molecular Devices). See SI Appendix for solutions and other recording parameters.

Data Analysis. Data were analyzed using scripts written in Matlab (v7.6.0, Mathworks). Photocurrent amplitudes in voltage-clamp recordings were determined relative to the mean holding current during a 5–10 ms window preceding the synchronously recorded light-on trigger signal, and low-pass filtered by using least-squares polynomial smoothing (Matlab’s sgolayfilt function; order 1, frame size 5). Averaged traces, where shown, represent the mean of the time-registered recordings. Groups of recorded currents were fit simultaneously and dynamically given in the text by using nonlinear least-squares optimization (see SI Appendix).

Optical Stimulation Sources. Blue light for single-photon photostimulation trials was supplied by a Luxeon V LED (470 ± 13 nm; Star Her, lamp pattern, #LXHL-LB5C, Philips Lumileds) powered by a variable voltage supply (#72-6615, Tenma) with the current switched on and off by using a metal oxide semiconductor field effect transistor. Pulsed IR excitation for photostimulation and fluorescence image acquisition was supplied by a mode-locked Ti:Sapphire laser tuned to 920 ± 6 nm (Mira 900, Verdi V10-pumped; Coherent), packaged into a home-built apparatus based on an Olympus BX51 microscope (see Fig. S1 in the SI Appendix). Laser-scanning excitation for photostimulation and fluorescence image acquisition was controlled by using software written in LabView (National Instruments) to issue command signals driving deflections of galvanometer-driven scan mirrors (8210 series, Cambridge Technology). Actual galvanometer positions during these scans were monitored by digitizing and recording the position feedback signals from the galvo amplifiers (Micromax 677, Cambridge Technology) synchronously with the electrophysiology data.

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