Platymonas subcordiformis Channelrhodopsin-2 (PsChR2) Function

II. RELATIONSHIP OF THE PHOTOCHEMICAL REACTION CYCLE TO CHANNEL CURRENTS

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Background: Channelrhodopsins (ChRs) are light-gated cation channels widely used in optogenetics. Results: The photocycle obtained from time-resolved absorption spectroscopy of PsChR2 is shown to explain photocurrent kinetics observed in HEK293 cells. Conclusion: The photochemical reactions involving two parallel photocycles result in the complex photocurrent behavior. Significance: The parallel-cycle model opens new perspectives in understanding photocurrents from channelrhodopsins.

Channelrhodopsins, such as the algal phototaxis receptor Platymonas subcordiformis channelrhodopsin-2 (PsChR2), are light-gated cation channels used as optogenetic tools for photocurrents from membrane potential in living cells. Channelrhodopsin (ChR)-mediated photocurrent responses are complex and poorly understood, exhibiting alterations in peak current amplitude, extents and kinetics of inactivation, and kinetics of the recovery of the prestimulus dark current that are sensitive to duration and frequency of photostimuli. From the analysis of time-resolved optical absorption data, presented in the accompanying article, we derived a two-cycle model that describes the photocycles of PsChR2. Here, we applied the model to evaluate the transient currents produced by PsChR2 expressed in HEK293 cells under both fast laser excitation and step-like continuous illumination. Interpretation of the photocurrents in terms of the photocycle kinetics indicates that the O states in both cycles are responsible for the channel current and fit the current transients under the different illumination regimes. The peak and plateau currents in response to a single light step, a train of light pulses, and a light step superimposed on a continuous light background observed for ChR2 proteins are explained in terms of contributions from the two parallel photocycles. The analysis shows that the peak current desensitization and recovery phenomena are inherent properties of the photocycles. The light dependence of desensitization is reproduced and explained by the time evolution of the concentration transients in response to step-like illumination. Our data show that photocycle kinetic parameters are sufficient to explain the complex dependence of photocurrent responses to photostimuli.

Channelrhodopsins (ChRs) are phototaxis receptors in unicellular algae (1) that function as light-gated ion channels when expressed in animal cells (2–5). Upon illumination, channelrhodopsins transiently increase the membrane conductance for protons and mono- and divalent cations, causing depolarization of the membrane on a fast, millisecond time scale. Their light-gated channel activity makes them a valuable optogenetic tool (4–8). To better understand the channel functions on the molecular level, the connections between the current transients and the light-induced intermediates need to be established. Kinetic intermediates are generally derived from light-induced optical absorption changes recorded in photochemical measurements. At the levels of expression obtainable for channelrhodopsins, such measurements cannot be performed with sufficient accuracy in intact cells or membrane preparations due to high light scattering in those preparations. Photoreaction kinetics for channelrhodopsins have therefore been obtained from measurements carried out on solubilized protein samples. The properties of proteins in the solubilized and membrane-embedded states may or may not be identical, and local ion concentrations at the membrane surface may influence the kinetics. However, in general, it is assumed that the kinetics of the solubilized protein are similar enough to those in the membrane that the conclusions drawn from measurements on the solubilized protein can explain the protein functions in the membrane environment. This is the ultimate goal of kinetic studies and is a testable assumption. Previous work on channelrhodopsins CrChR2 and PsChR2 indicated that laser-induced current transients roughly correlate with late photocycle intermediate(s) observed in independent photochemical studies (9, 10). The complex time dependence of the current response to step or continuous illumination, however, is difficult to reconcile with the reaction sequence derived from photochemical measurements (9, 11–14). The desire to explain the unusual

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2 The abbreviations used are: ChR, channelrhodopsin; CrChR2, Chlamydomonas reinhardtii channelrhodopsin-2; PsChR2, Platymonas subcordiformis channelrhodopsin-2.
features displayed by the current profile prompted the development of hypothetical models, called four-state models (15–17). Support for these sophisticated models from photochemical experiments is still lacking.

The current response shown by PsChR2 to a step-like illumination (10) is typical of responses reported for other ChR proteins (12), a fast peak followed by a plateau current. However, the extent of peak current inactivation or desensitization is less and the recovery to repetitive illumination steps is faster than those of CrChR2 (10). The time course of the current induced by a laser flash lacks the ultrafast transient signals seen in rhodopsin proton pumps and CrChR1 associated with proton transfer from the retinylidene Schiff base (18, 19) and follows the time dependence of the absorption transient assigned to an unidentified red-shifted intermediate (10). These properties of PsChR2, mentioned above briefly, make this protein a desirable subject for studies aimed at connecting the channel current kinetics to light-induced intermediates. Here we report on the relationship between the published channel currents produced by PsChR2 expressed in HEK293 cells, as well as current transients reported for other ChR2 proteins, and the photocycle derived from time-dependent absorption experiments on the solubilized PsChR2 protein presented in the accompanying article (21). We suggest that similar relationships may exist for ChR proteins in general.

Materials and Methods

Transient Photocurrent—Transient photocurrent in response to a short laser pulse, courtesy of Professor J. L. Spudich, was measured in HEK293 cells expressing PsChR2 as described (19). All other current transients discussed in this work have been previously published as referenced in the text.

Model Calculations—The time dependence of intermediate concentrations was calculated based on the microscopic rate constants of the kinetic scheme corresponding to the reaction scheme involved. The rate constants were arranged in a kinetic matrix, and the eigenvalues and eigenvectors of the matrix were obtained. The eigenvectors were scaled according to the initial conditions, i.e. the relative concentrations of the intermediates of the scheme at zero time. The product of the scaled eigenvectors and the time-dependent exponential functions based on the eigenvalues yielded the time dependence of the intermediate concentrations. Responses to switching on and off the light pulses were calculated separately and merged to give continuity according to the profile of the light pulse regime involved. The calculations were performed using programs written in MATLAB (MathWorks).

Results and Discussion

The PsChR2 Photocycle—The analysis of PsChR2 kinetics reported in the accompanying article (21) revealed the simultaneous presence of two photocycles in PsChR2. Cycle 1 accounted for 30% of the reacting molecules and included the reversible decay of the K state to the M state, followed by conversion to the O state, which decayed to the P state before returning to the recovered, R, state. Cycle 2 represented 70% of the reacting molecules and showed the decay of the first intermediate state, K, forming coupled equilibria with the L and M states, followed by buildup of the O state, which decayed directly to the recovered state. The microscopic rate constants, in units of s⁻¹, of the reaction steps obtained from fitting the two-cycle model (shown below) to the experimental data are placed next to the arrows in the schemes.

Based on these rate constants, the time-dependent concentrations of the intermediate states were calculated. The states likely to be involved in channel function can be evaluated by comparing the time evolution of the intermediate states with the transient cell current produced by the channel opening of PsChR2 in response to a laser pulse.

Identifying the Conductive States—In response to a short laser pulse, HEK293 cells expressing PsChR2 exhibit a transient photocurrent that reaches its maximal value between 1 and 2 ms. Fig. 1, a and b, show the concentration profiles of Cycle 1 and Cycle 2, respectively, and Fig. 1c compares the sum of the contributions by the two cycles with the transient photocurrent (black curve) reported earlier (19). It is clear that the concentration profiles of the O states in both cycles overlap with the current transient. An approximate match in the current to red-shifted intermediate accumulation has been noted previously for CrChR2 (9) and for PsChR2 (10).

The match between the O intermediate lifetimes and the photocurrent is not exact. Although quantitative differences might be expected due to uncertainties in both the current measurements and the kinetic analysis, the qualitative agreement in times for the channel opening and O state population is striking. Nevertheless, the O state in Cycle 1 starts earlier than the current, and the two cycles do not contribute to the current shape equally. To account mathematically for the lag period between the current and the O state in Cycle 1, we split the O state into two states, a nonconductive and a conductive one, by introducing a step with a 200-μs lifetime (rate constant k’₂ in xCycle 1 below) to connect the two. Thus, the O state responsible for the channel opening would appear 30 μs later than the detection of absorbance of the O form. To distinguish it from the conductive O state, the nonconductive early state we call Oₙ. A possible explanation for the delayed current is that protonation of the Schiff base in Cycle 1 occurs rapidly because a proton is readily available at a nearby group. The pore formation, however, needs more time, although it may also require the initial protonation step. Also, the color change and the flow of ions are two different responses produced by the protein and detected by two different techniques. Because the protonation of the Schiff base in Cycle 2 is a relatively slow process, with a
1.4-ms lifetime, no delay between the current and color change is expected. We emphasize that it is entirely possible that electrical potential present across the HEK293 plasma membrane in photocurrent measurements, or other differences in the PsChR2 preparation, are responsible for the kinetic mismatch between the O lifetime and the photocurrent, and that the assumption of two physically different O states or, equivalently, one with a delayed conductance response, is consistent with, but not demonstrated by the data.

The concentration profiles of the nonconductive $O_n$, the conductive O state of Cycle 1, and the open channel O state of Cycle 2 are shown in Fig. 2 (blue, green, and red curves, respectively). When the concentrations of the conductive O form of Cycle 1 and the O form of Cycle 2 are summed in a 1:1 ratio, the resulting curve has its peak value almost a millisecond later than the current peak (Fig. 2, cyan and black curves, respectively). An acceptable match can be found only when the concentrations of the O states of Cycle 1 and Cycle 2 are summed in a 3.6:1 ratio (Fig. 2, pink curve). Thus, the channel created by the $O_{cy1}$ in Cycle 1 appears to be 3.6 times more conductive than the channel produced by the O state in Cycle 2. This is an unexpected observation based on the almost identical absorption spectra of the O states in the two cycles. However, it is consistent with the decoupling between pore and O state formation in Cycle 1 suggested above. The absorption spectra report only the structure around the retinal chromophore and may not be sensitive to distant changes in the protein conformation. The differences in structure of the O states in Cycle 1 and Cycle 2, which lead to the differences in conductivity, may also be responsible for the different recovery routes observed in the two cycles. The more open channel structure of the O state in Cycle 1 recovers in two steps, whereas the less open O state in Cycle 2 recovers in a fast single transition.

The laser-induced transient current is of primary importance in identifying the states responsible for channel opening. However, the laser technique is not the most frequently used technique in electrophysiological studies aimed at possible application of channelrhodopsins in optogenetics. In practical applications, the light source is likely to be less intense than a laser pulse, and thus the delivery of the needed number of photons would take a longer time. Recording the current in response to step-like light pulses of several seconds duration is the standard method of channelrhodopsin studies. The time dependences of photocurrent responses for channelrhodopsins, mainly for CrChR2, are described in detail in a number of publications (for review see Ref. 12), which allows us to compare the predictions by the two-cycle model with the experimental observations and to test whether the model fits the basic properties not only of PsChR2 but of ChR2 proteins in general.

Modeling the Channel Current Induced by Long Light Pulses—Channel currents evoked by light pulses of moderate intensity and a few seconds duration show a number of interesting kinetic features that are quite different from the transient current seen following a short and intense laser pulse. The current response to a light step starts with a fast rise, reaching an
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initial peak value, called the peak current, and then the current decays to a lower, but steady level and stays at this plateau until the light pulse ends. This current profile is characteristic not only of PsChR2 (10) but also ChR proteins in general (12). The decay of the peak current is called desensitization or inactivation, and this unusual phenomenon described in earlier studies on ChR proteins initiated a number of hypothetical models, the most complex being called the four-state model, having two light-induced and interconnected open-channel states, which decay to two closed-channel dark states (15). The rate of conversion between the two open states was later proposed to depend on the intensity of the light step applied (16). These models, aimed at explaining the current transients under continuous illumination, or long-lasting light pulses, have little connection to the actual photocycle intermediates deduced from time-resolved spectroscopy measurements (12, 13). The photocycle kinetics, interpreted in terms of a sequential mechanism, show relatively simple time dependences of intermediate concentrations (13), which are difficult to reconcile with the far more complex time course of the current response to a light step. It has been suggested that the protein, responding to a longer but less intense continuous illumination, may have a cycle that is more complex than the photocycle derived from laser experiments that use very intense pulses (12).

We deduced the two-cycle model from laser-induced absorption changes. Thus, if the protein has different cycles when it responds to an intense laser or moderate continuous illumination, the model we deduced should fail when attempting to reproduce the experimental current response observed in light step experiments. To test the model, we modified Cycle 1 and Cycle 2 by adding a light-driven step connecting the recovered state, R, to the K state in each of the cycles, and thus turning the schemes into true cycles. Also, a step connecting O_s to O is included in xCycle 1.

The shapes of the current responses to an intense laser pulse, shown in Fig. 2, and to a light pulse of moderate intensity, as modeled and shown in Fig. 3, are readily explained by their origins. The current transient seen in a laser experiment reflects molecular events, the rise and decay of the channel-forming intermediates that follow the synchronous and instant excitation of molecules in their ground state. Because of the asynchronous and prolonged excitation of the molecules in the ground state, the current profile being produced by a light step reflects the onset of a steady state. As discussed later in more detail, the intermediates in a reaction sequence display a transient response to a light step before relaxing to a steady state level. The relative steady state concentration, c, of an intermediate is proportional to the fraction its lifetime represents in the total lifetime of the cycle, c ~ τ/Στ. In xCycle 1, the decay of the P state, with 0.71 s⁻¹ rate constant, is much slower than the decay of all other states, including the R state, with a 20 s⁻¹ decay rate constant used in the modeling. Thus, the P state builds up at the expense of all the other states. The O state, which is responsible for the open channel, starts with a higher initial concentration as the light-induced flow is the highest at the beginning of the light pulse, and later relaxes to a relatively low steady state level as shown in Fig. 3. The details of the concentration profiles for

![Figure 3. The concentration transients of the conductive O states of xCycle 1 and xCycle 2 (blue and green curves, respectively) in response to a light pulse of 1-s duration having a light rate of 20 s⁻¹. The red and cyan curves represent the combined transients of O_cy1 and O_cy2 in a 1:1 and 3.6:1 ratio, respectively, where cy1 and cy2 represent Cycle 1 and Cycle 2.](image)
all of the intermediate states will be discussed later. In xCycle 2, the O state has the lowest decay constant in the chain leading to the recovered R state. It thus will have a relatively high steady state level. Even so, it does not accumulate to the same extent as the P state in xCycle 1 because in xCycle 2, the decay of the O state is not the slowest step; rather the light-induced decay of the R state is the slowest one for low and moderate light intensities. In the case of the 20 s⁻¹ light rate constant used in the modeling, the O state accounts for roughly 10% of the total concentration in the steady state. Because the O state corresponds to the open channel protein state, it is responsible for most of the plateau current seen in the step response in Fig. 3. Note that the concentration of the O state in xCycle 2 and that of the P state in xCycle 1, which precede the recovered state in the chains, do not display transient peaks; rather they build up steadily.

The characteristic current response, a peak followed by a plateau, to a light step stimulus is a general feature of all two-cycle models that have the pattern of open and closed channel states shown in Cycle 1 and Cycle 2. The combination of an open state followed by a closed state with a slower decay rate, -O-P-R in Cycle 1, produces a peak current, and an open state recovering directly and having the slowest decay rate in the cycle, -O-R in Cycle 2, produces a plateau current regardless of the actual values of the individual reaction rate constants. Thus, variations in rate constants caused by differences in the environment that do not change the open/closed state pattern shown above, as may occur between membrane bound and solubilized proteins, have little influence on the shape of the current response and do not alter our conclusions.

The Recovery of the Peak Current in Multistep Experiments—Characterization of ion channels often involves measuring the current response to a train of alternating light and dark steps. It is frequently observed that the peak current produced by a second light step is lower in amplitude than the peak invoked by the first light pulse, whereas the plateau regions are reproduced reasonably well (12, 15). This phenomenon is called the peak current recovery and observed also for PsChR2 (10).

In the two-cycle extended model, the peak current is the response of xCycle 1. To produce a full size peak current, xCycle 1 has to recover completely. At the end of the light pulse, most of the molecules are in the nonconductive P state and must convert into the R state before responding to the second light pulse. The smaller peak amplitude during the second light pulse means that xCycle 1 has not fully completed its course in the dark period between the two pulses because the decay of the P state is slow. Only that fraction of the molecules that had already recovered into the R state during the dark period is ready to respond. The peak current recovery phenomenon in our two-cycle model is the result of the open-closed state sequence, -O-P-R, in Cycle 1, which produces a peak current due to the transient O state and delays the recovery due to the nonconductive long-lived P state. The -O-R sequence in Cycle 2, which produces most of the plateau current, recovers directly and having the slowest decay rate in the cycle, -O-R in Cycle 2, produces a plateau current regardless of the actual values of the individual reaction rate constants. Thus, variations in rate constants caused by differences in the environment that do not change the open/closed state pattern shown above, as may occur between membrane bound and solubilized proteins, have little influence on the shape of the current response and do not alter our conclusions.

The Light-dependent Rate of Desensitization—Desensitization or inactivation, the decay of the peak current to the steady state level in light step experiments, is an important and interesting channel property, the origin of which has been obscure (12). It has also been found that the rate of the transition responsible for the inactivation of the channel is light-dependent. Exponential fits to the falling part of the peak current revealed that the channel desensitizes faster at higher light pulse intensities. No such light-dependent rates have been reported in absorption studies. In the first four-state channel model (15), two conductive states, connected via a reversible

![Figure 4](image)

**FIGURE 4. a, reproduction by the two-cycle model of the current transient produced by a train of two 1-s light pulses with a 1-s dark period between them. The concentrations of OCy1 and OCy2 states are combined in a 3.6:1 ratio to mimic the current. (Cy1 and Cy2 represent Cycle 1 and Cycle 2.) b shows the current contribution by the O state (blue curve) and the partial recovery of the produced R state of Cycle 1 in the dark period (green curve).**
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FIGURE 5. The contributions of the two cycles to the reproduced current transients in response to 1-s light pulses of different intensities. The light rates are 10, 20, 40, and 80 s⁻¹ (blue, green, red, and cyan curves, respectively), as shown in b. a and b show the contributions by Cycle 1 and Cycle 2, respectively. c shows the total current reproduced by the two cycles after normalization of the transient to the plateau current level for each light intensity.

The Origin of the Light-dependent Desensitization Rate—Using the two-cycle model derived from time-dependent absorption data, we were able to reproduce all the basic observations regarding the experimental channel currents and, more importantly, the light-dependent desensitization of the current. What is the origin of the light dependence? Analyzing the time evolution of intermediates and their concentration transients in multistep photoreactions following short laser and step-like long pulse excitations provides the key to answer this question.

Analysis of the Concentration Transients—To understand the relationship between the rate constants in xCycle 1 and the kinetics of desensitization, the concentration transient displayed by the O conductive state, we have to solve the different-step, were proposed to account for the peak current deactivation. However, for better reproduction of the light-dependent deactivation rates, the idea of a light-assisted transition between the conductive states was introduced and added to the improved four-state model (16). Note that light-dependent kinetics and complications arising from secondary photoreactions (9), which are more prominent at high light intensities, are not part of our discussion. A comparison of the reported action spectra of channel currents generated in HEK293 cells (10) with the absorption spectra of solubilized PsChR2 shows no noticeable contributions from secondary photoreactions at normal light intensities. This is also likely to apply to CChR2.

In our two-cycle model, there is no connection between the two cycles and no light-assisted transition between the conductive O states to facilitate the peak current decay in a light-sensitive manner. To test the light dependence of desensitization, we modeled the current response to light steps of different intensity by varying the value of the light rate constant in the extended cycles from 10 s⁻¹ to 80 s⁻¹. The results, the concentrations of the conductive O states, are shown in Fig. 5. Fig. 5, a and b, display the current response produced by xCycle 1 and xCycle 2, respectively, for 10, 20, 40, and 80 s⁻¹ light rate constants. As expected, the reproduced channel currents increase with light intensity as more molecules of the R state are driven into the cycles. The rise in current level deviates from linearity at higher light intensities as the pool of molecules in the R state becomes exhausted. For a better comparison with the current signals measured in experiments, Fig. 5c shows the sum of currents reproduced by the two extended cycles (colors as in panel a), normalized to the plateau level. The desensitization rate produced by the two-cycle model is clearly light-dependent. The similarity between these curves and the ones published earlier (15, 16) on ChR2 proteins is striking.

The light dependence of the desensitization can also be tested by applying a second light step on top of a weaker first one. Experiments on ChR2 reported on this type of light dependence (15). We modeled the experiment by applying a first pulse with 4 s⁻¹ light rate and a second pulse of higher intensity, with 20 s⁻¹ light rate, at 1 s after the start of the first pulse. Because xCycle 1 is responsible for the peak current, the results for this cycle only are shown in Fig. 6. The blue curve in Fig. 6a is the concentration transient for the O state, and the green curve is the response observed when the second pulse is applied to a relaxed dark state. Because of depletion of the R state by the first pulse, the amplitude of the O concentration transient produced by the second pulse is very small, roughly the size produced by the much weaker first pulse. In Fig. 6b, the concentration time dependences for the P state (broken curve) and for the recovered R state (solid curve) are plotted for the two-pulse sequence (blue curves) and for the single pulse applied to the dark state (green curves) to show the accumulation of the molecules in the nonconductive P state and the simultaneous depletion of the recovered R state. The first pulse of lower intensity drives a large fraction of molecules from the R state into the cycle, and little is left for the larger second pulse. In Fig. 6c, the transient of the superimposed second pulse (blue curve) is compared with the transient of the pulse applied to the relaxed state (green curve) after normalizing their amplitudes. Before normalization, the steady state levels were subtracted. The comparison shows that the rate of desensitization becomes higher when a pulse is superimposed on a weaker background light. Thus, the channel senses the total light intensity and displays a rate of desensitization as if the second pulse had a higher intensity.
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This simple xCycle 1 scheme, sxCycle 1, is a sequential scheme without back reactions, one that has a relatively easy analytical solution. We will recite some of the equations, or their approximations, to describe the time dependence of intermediate concentrations in laser studies as well as in light-step experiments. In the description, we refer to the “rise” and “fall” of the transient concentrations and restrict the term “decay” to the rate constants of transitions between molecular states in the schemes.

In the description, we will analyze cycles with four intermediates. Therefore, we start with the general formulas for this type of reaction sequence. The concentration time dependence of intermediates A, B, C, and D in a linear sequential scheme without back reactions is described by the following reaction and equations (20).

$$\begin{align*}
A & \quad \longrightarrow \quad B \quad \longrightarrow \quad C \quad \longrightarrow \quad D \\
\text{Reaction 1} & \\
A &= A_0 \times \exp(-k_At) \\
B &= A_0 \left( -k_A/(k_A - k_B) \times \exp(-k_Bt) + k_A/(k_A - k_B) \times \exp(-k_At) \right) \\
C &= A_0 \left( [k_Ak_B/(k_A - k_B)(k_A - k_C)] \times \exp(-k_Bt) - [k_Ak_B/(k_A - k_B)k_B - k_C] \times \exp(-k.Ct) \right) \\
D &= A_0 \left( 1 - [k_Ak_B/(k_A - k_B)(k_A - k_C)] \times \exp(-k_Bt) - [k_Ak_B/(k_A - k_C)] \times \exp(-k.Ct) \right)
\end{align*}$$

Laser Experiments—First we apply these equations to describe the concentration time dependence of intermediates in the sxCycle 1 scheme as it occurs in laser experiments. Because of the short duration and high light intensity in a laser experiment, the first transition, R to KM, is too fast for the common absorption studies. It is observed only in ultrafast laser photochemistry. In nanosecond spectroscopy, as used in this study, the kinetics starts with the KM intermediate, and the scheme can be shortened by omitting the first step.

$$\begin{align*}
K_M \quad \longrightarrow \quad O \quad \longrightarrow \quad P \quad \longrightarrow \quad R \\
\text{Reaction 2} & \\
K_M &= K_{M0} \exp(-k_At) \\
O &= K_{M0} \left( \exp(-k_At) - \exp(-k_Bt) \right)
\end{align*}$$

The rate constants in this scheme follow a descending order, $k_2 \gg k_3 \gg k_4$. Replacing $k_A$, $k_B$, and $k_C$ by $k_2$, $k_3$, and $k_4$, respectively, the integrated forms of the intermediate concentrations, to a good approximation, are as follows.

$$\begin{align*}
K_M &= K_{M0} \exp(-k_At) \\
O &= K_{M0} \left( \exp(-k_At) - \exp(-k_Bt) \right)
\end{align*}$$

Partial equations that describe the concentration changes in the cycle. The cycle, however, is too complex to solve analytically; therefore, we are going to use a simplified version of xCycle 1 that serves our purpose equally well. We skip the equilibration between K and M states and replace it with a single intermediate, KM, and combine the O, and O states into one. For simplicity, we call them KM, O, P, and R intermediates. The simplified sequential scheme is arranged in a linear chain for convenience.

$$\begin{align*}
R \quad \rightarrow \quad \text{KM} \quad \rightarrow \quad \text{O} \quad \rightarrow \quad \text{P} \quad \rightarrow \quad \text{R} \\
sxCycle 1
\end{align*}$$
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\[ P \sim \text{KM}_0 \{e^{-k_4 t} - e^{-k_5 t}\} \quad \text{(Eq. 7)} \]

\[ R \sim \text{KM}_0 \{1 - e^{-k_6 t}\} \quad \text{(Eq. 8)} \]

The concentration of KM falls with the \( k_2 \) rate constant to zero, and the concentration of O rises with the same \( k_3 \) and falls with the \( k_4 \) rate constant. The concentration of P rises with the \( k_3 \) and falls with the \( k_4 \) rate constants. The R form recovers, approximately, with a rising exponential function, \( 1 - e^{-k_6 t}\). When the experimental data produced by the shortened scheme are fit to a sum of exponentials, it is found that the apparent rates in the fit are the same as the \( k_2 \), \( k_3 \), and \( k_4 \) microscopic rates. The transient concentrations of the intermediates rise and fall with the rate constants as seen in the shortened scheme above. If the intermediates in the scheme are all single molecular states, as in Cycle 1 and Cycle 2, the experimental apparent rates or decay constants can be regarded as molecular characteristics associated with the probabilities of converting one molecular state into another one. This seemingly trivial argument is the key to understanding and interpreting the peculiarities of the kinetics under continuous illumination.

**Light Step Experiments**—Under constant light illumination of moderate intensities, the R to KM transition is no longer the fastest one; in fact it is often one of the slowest rates in the cycle. At moderate light intensities, such as the 20 s \(^{-1}\) light rate constant used in the modeling, among the rate constants in the xCycle 1 scheme, only \( k_3 \), having a 0.7 s \(^{-1}\) value, is smaller than \( k_5 \). Because the P to R transition is so slow, 0.7 s \(^{-1}\), there is a negligible recovery of the R state from the P state in the time window where all the other transitions occur. Thus, to describe the main characteristics of the kinetics under continuous illumination, the simplified xCycle 1 can be shortened by omitting the last step.

\[ k_5 \quad k_2 \quad k_3 \]

\[ R \rightarrow \text{KM} \rightarrow \text{O} \rightarrow \text{P} \]

**Reaction 3**

As was done above for the laser experiment, the rate constants \( k_{2x}, k_{3y}, k_{5y} \), and \( k_{2c} \) in the general equations are replaced by \( k_{5y}, k_{2y}, \) and \( k_{3y} \), respectively. Because \( k_2 \gg k_y, k_3, \) the concentration time dependence of the intermediates in this scheme, is described, with good approximation, by the following equations:

\[ R \sim R_0 \exp(-k_y t) \quad \text{(Eq. 9)} \]

\[ \text{KM} \sim R_0 \left(k_3/k_5\right) \{e^{-k_5 t} - e^{-k_5 t}\} \quad \text{(Eq. 10)} \]

\[ \text{O} \sim R_0 \left[k_3/(k_3 - k_5)\right] \{e^{-k_3 t} - e^{-k_3 t}\} \quad \text{(Eq. 11)} \]

\[ P \sim R_0 \left[1 - \left[k_3/(k_3 - k_5)\right]e^{-k_3 t} + \left[k_3/(k_3 - k_5)\right]e^{-k_3 t}\right] \quad \text{(Eq. 12)} \]

The KM concentration transient does not rise with \( k_{5y} \), the rate constant of the step pointing to KM in the scheme. Instead it rises with \( k_3 \), which belongs to the decay step of KM in the scheme. The light rate constant, \( k_{5y} \), describes the decrease of the KM concentration transient. The concentration transient for the O intermediate also falls with the \( k_y \) rate constant, and rises with \( k_5 \), which belongs to its decay step in the cycle. Note that the concentration transient has very small amplitude for KM because \( k_3 \) is small when compared with \( k_5 \), whose value is \( 5 \times 10^3 \) s \(^{-1}\). The transient is much larger, and therefore detectable, for the O intermediate because the ratio of \( k_3 \) to \( k_5 \) is around seven at the light intensity used in the modeling. For moderate light intensities, the concentration transient for the P intermediate rises with \( k_5 \), and this is true even for the scheme that has the P to R step. The analysis above shows that the transient concentrations of most of the intermediates do not rise and fall with the rate constants as seen in the scheme. The “odd” behavior of the concentration transients, when compared with the “normal” behavior observed in the laser experiment, reflects the order of rates of the consecutive steps in the mechanism. If the supply rate constant for an intermediate is larger than its decay rate constant, the transition follows the normal, fast-to-slow step order and the concentration transient for the intermediate rises with the supply rate constant and falls with the decay rate constant, as in the laser experiment. If the supply is slower than the decay, the transition follows the odd slow-to-fast step order and the concentration transient for the intermediate rises with the decay rate constant and falls with the supply rate constant, as in the light pulse experiment. For light pulses of high intensity, the light rate constant, \( k_{3y} \), becomes larger than \( k_y \). In that case, the equation for the O concentration shows that the O intermediate displays a normal behavior; its concentration transient rises and falls the way it is observed in laser experiments. The desensitization rate constant thus increases with the light intensity until it reaches the value of the true decay rate constant of the O state. The increase is linear at low light intensities and shows saturation behavior at high intensities as it approaches the true decay rate limit, in accord with experimental observations (16). Having these guidelines, we can examine the concentration transients reproduced by xCycle 1 under continuous illumination.

**The Concentration Transients in Light Step Responses Produced by xCycle 1**—The current response to a light pulse as predicted by the two extended cycles was shown in Fig. 3 for a 20 s \(^{-1}\) light rate constant. There we depicted only the time-dependent concentrations of the conductive O states. In Fig. 7, we show the concentration transients to a light step for all of the states involved in the extended Cycle 1. The blue, green, red, cyan, pink, and orange lines represent the concentration transients for the K, M, O, and R states, respectively. Because the concentration transients are very different in amplitude for the different molecular states, we normalized the K, M, O, and O states, according to their steady state level, bringing their plateau regions to the steady state level of the R state. All the characteristics of the rise and fall of the intermediate concentrations, briefly outlined above, can be seen in this figure.

The concentration transient of the K state (blue curve) rises with the true, microscopic decay rate constants obtained in the laser experiment. In xCycle 1, the K state decays in two steps: first to the M state, forming an equilibrium mixture between the two, which then decays to the O state. The decay constant of the latter transition controls the rise of the M concentration transient. The rise of the O state transient occurs with
because the pool of molecules in the R state is nearly depleted of the channel. It is the rate constant of the R state depletion, does not belong to the molecular state responsible for opening connected to any channel property, or to any adjustment in the rate constant of desensitization in our model is not connected to any experimental kinetic transient. The light dependence of other experimental kinetic transients do not necessarily represent the true microscopic formation and decay rates of the particu-
lar states to which the transients belong. This statement applies not only to the light step experiments discussed here, but to any other experimental kinetic transient. The light dependence of the rate constant of desensitization in our model is not connected to any channel property, or to any adjustment in the open state configuration via modification of the O state, which is responsible for the open channel. The rate constant itself does not belong to the molecular state responsible for opening of the channel. It is the rate constant of the R state depletion, driven by the continuous light. Thus, it not only is, but must be, light-dependent according to our model.

Having found the source of light dependence, it is now easy to understand the current response, shown in Fig. 6, caused by a second light pulse of 20 s\(^{-1}\) rate constant superimposed on the first one having a 4 s\(^{-1}\) rate constant. The signal is small because the pool of molecules in the R state is nearly depleted with the first light pulse of lower intensity. The transient produced by the second light pulse falls with a light rate constant that is the sum of the light rate constants of the two light pulses present simultaneously, 24 s\(^{-1}\) in the model calculations. It is this moderate increase in the rate that is seen in Fig. 6b.

**Conclusion**—We have shown that the photoreactions and the channel currents of PsChR2, a member of the ChR2 group, can be adequately described on the molecular level by two independent parallel photocycles. Cycle 1 contains the K, M, O, and P states and is responsible for 30% of the converted molecules. The transitions in Cycle 2 involve the K, L, M, and O states and account for 70% of the reacting molecules. The O states in the cycles are responsible for the open channel. The O state in Cycle 1 appears 30 \(\mu\)s earlier than the current response and shows a 3.6 times higher conductivity than that of the O state in Cycle 2. The two cycles reproduce the current transients under different illumination regimes, including single and multiple light pulses of different intensities. The desensitization phenomenon is linked to the transient current prior to the establishment of steady state kinetics in Cycle 1. The recovery of the peak current is explained by the presence of the nonconductive P state with a slow decay rate in Cycle 1. The analysis of the light-induced concentration transients revealed the source of the light dependence of the desensitization rate constant reported for ChR2 proteins. It was found that the desensitization rate is a characteristic of the transients leading to the steady state and is identical to the rate of the light-induced depletion of the recovered state. Our results show that a single molecular mechanism with a single set of microscopic rate constants describes both the kinetics of the photochemistry and the light-induced currents of PsChR2. Differences in photocycle kinetics may exist between the solubilized and membrane embedded protein. However, they do not seem to be critical in the evaluation of channel current properties.

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