The Kinetics of Inactivation of the Rod Phototransduction Cascade with Constant Ca\(^{2+}\)

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A B S T R A C T A rich variety of mechanisms govern the inactivation of the rod phototransduction cascade. These include rhodopsin phosphorylation and subsequent binding of arrestin; modulation of rhodopsin kinase by S-modulin (recoverin); regulation of G-protein and phosphodiesterase inactivation by GTPase-activating factors; and modulation of guanylyl cyclase by a high-affinity Ca\(^{2+}\)-binding protein. The dependence of several of the inactivation mechanisms on Ca\(^{2+}\) makes it difficult to assess the contributions of these mechanisms to the recovery kinetics in situ, where Ca\(^{2+}\) is dynamically modulated during the photoresponse.

We recorded the circulating currents of salamander rods, the inner segments of which are held in suction electrodes in Ringer’s solution. We characterized the response kinetics to flashes under two conditions: when the outer segments are in Ringer’s solution, and when they are in low-Ca\(^{2+}\) choline solutions, which we show clamp Ca\(^{2+}\) very near its resting level. At T = 20–22°C, the recovery phases of responses to saturating flashes producing 10\(^{2.5}\)–10\(^{4.5}\) photoisomerizations under both conditions are characterized by a dominant time constant, \(\tau_c = 2.4 \pm 0.4\) s, the value of which is not dependent on the solution bathing the outer segment and therefore not dependent on Ca\(^{2+}\). We extended a successful model of activation by incorporating into it a first-order inactivation of R*, and a first-order, simultaneous inactivation of G-protein (G*) and phosphodiesterase (PDE*). We demonstrated that the inactivation kinetics of families of responses obtained with Ca\(^{2+}\) clamped to rest are well characterized by this model, having one of the two inactivation time constants (\(\tau_{R^*}\) or \(\tau_{PDE^*}\)) equal to \(\tau_c\), and the other time constant equal to 0.4 ± 0.06 s.

I N T R O D U C T I O N

The biochemical events underlying the activation of the phototransduction cascade in vertebrate rods and cones are now well established (Pugh and Lamb, 1993; Hofmann and Heck, 1995). Moreover, recent investigations have also shown that models of activation based on the established biochemical events of phototransduction can provide quantitatively accurate accounts of the amplification and kinetics of the electrical responses of vertebrate rods (Lamb and Pugh, 1992; Pugh and Lamb, 1993; Kraft et al., 1993), and of the α-wave of the human electroretinogram (Breton et al., 1994; Hood and Birch, 1994), which is proportional to the activation phase of the rod photocurrent (Hagins et al., 1970).

The biochemical processes governing the inactivation of the phototransduction cascade and the recovery phase of the photoresponse are less well understood than those governing activation, although a number of mechanisms have been discovered and characterized in vitro that down-regulate, or otherwise modulate each of the activation steps. The enzymatic activity of photovolved rhodopsin (R*) has been shown to be diminished by phosphorylation by rhodopsin-kinase (RK), and by the subsequent binding of the 48-kD protein arrestin (Sitaramayya and Liebman, 1983; Wilden et al., 1986; Bennett and Sitaramayya, 1988). Moreover, the activity of RK is modulated in a Ca\(^{2+}\)-dependent manner by S-modulin (alias recoverin) (Kawamura, 1993; Chen et al., 1995; Klenchin et al., 1995). The inactivation of the activated moiety of G-protein, G\(_{\alpha}\)-GTP, and of its target protein, the rod phosphodiesterase (PDE), are apparently coupled via the GTPase activating effect of PDE\(_{\alpha}\), the inhibitory subunit of the PDE (Arshavsky and Bownds, 1992). Other GTPase activating factors may
also be involved (Angleson and Wensel, 1994). In addition, the inactivation rate of the PDE-Gαs-GTP complex may depend on the occupancy of cGMP on non-catalytic binding sites on PDEα and PDEβ (Arshavsky et al., 1992, 1994). Guanylyl cyclase (GC) activity is regulated by a high-affinity, multisite calcium binding protein, GCAP (Gorczya et al., 1994; Palczweski et al., 1994), in such a way that the decline in Ca2+i that occurs during the light response increases GC activity and accelerates the recovery of the circulating current after a flash of light (Hodgkin and Nunn, 1988; Fain et al., 1989; Nakatani and Yau, 1989).

Efforts to link the recovery kinetics of the photoresponse with the biochemistry of inactivation of the photoreceptor cascade in situ must confront a number of problems. One problem is that the different biochemical inactivation processes occur concurrently in the responses of intact photoreceptors, making it difficult to analyze the contributions of individual processes to the overall kinetics. Another problem is that dynamic changes in Ca2+i accompany normal photoresponses, and, as noted above, such changes modulate several of the biochemical events hypothesized to underlie response recovery.

In an effort to confront these problems and link the kinetics of photoresponse recovery in intact amphibian rods more quantitatively to the underlying biochemistry, in the investigation presented here we used three strategies. First, we adapted the experimental approach of Fain et al. (1989) and Nakatani and Yau (1989), blocking the action of the Na+/Ca2+-K+ exchanger and eliminating Ca2+ influx, thus freezing Ca2+i; this approach allows the recovery kinetics to be characterized without the complexity of dynamically changing Ca2+i. Second, we extended a previous representation of the activation of the phototransduction cascade (Lamb and Pugh, 1992) by incorporating first-order inactivation of the two enzymatic amplifiers, R* and PDE*, and we have characterized the recovery kinetics in constant Ca2+i in terms of two corresponding theoretical time constants, τR and τPDE. Third, we adopted and extended the analysis of David Pepperberg and colleagues, who have provided evidence that a characteristic time parameter, τc, derived from the photoresponse recovery kinetics, corresponds to a time constant, τR, of first-order decay of the enzymatic activity of R* (Pepperberg et al., 1988, 1992; Kahlert et al., 1990). Our results allow us to examine the dependence of τc on Ca2+i.

The paper is organized as follows. First, in the Theory section we develop a phototransduction cascade model that incorporates inactivation steps and that is appropriate for describing responses in the choline solution. Second, we present the Methods. Third, we present data (in Figs. 3–7) characterizing the experimental protocol used to clamp Ca2+i to its resting value and evaluate its success in doing so. Fourth, we measure τc, the dominant time constant of photoresponse recovery in normal Ringer’s solution and in calcium-clamping conditions (Figs. 8 and 9) and analyze the results by applying the model (Figs. 10 and 11, and Table 1). Finally, we discuss the implications for the understanding of the mechanisms of inactivation of the transduction cascade.

**THEORY**

The theoretical description of the phototransduction cascade of salamander rods applied here has for the most part been presented in previous papers (Cobbs and Pugh, 1987; Hodgkin and Nunn, 1988; Lamb and Pugh, 1992; Pugh and Lamb, 1993). However, to incorporate a biochemical description of the recovery phase of the photoresponses, we have extended our previous formulation, which dealt almost exclusively with the activation phase (Lamb and Pugh, 1992). Moreover, to characterize the inverted photoresponses obtained in choline solution, we have found it necessary to introduce some theoretical considerations not treated in previous papers. Thus, we present here an abbreviated description of the extended model.

**Major Steps in the Phototransduction Cascade**

The model divides the activation events of the cascade into five major steps. The first three take place at the disc membrane and can be characterized by the following chemical scheme:

\[
R \rightarrow R^* \rightarrow R_{\text{inactive}}
\]

\[
G^*_\alpha \beta \gamma \leftarrow \gamma \rightarrow G^*_\alpha^* \rightarrow G^*_{\text{inactive}}, PDE_{\text{inactive}}
\]

Step 1 is the generation of the active enzyme R* by the photoisomerization of rhodopsin and its subsequent conversion to metarhodopsin II. Step 2 is the catalytic production by R* at the rate νRG of G*, the active moiety of G-protein; the arched arrow signifies catalysis. Step 3 is the stoichiometric activation of catalytic subunits of PDE by G*. Lamb and Pugh (1992) showed that in the absence of inactivation, the numbers of the activated proteins R*, G*, and PDE* produced in an outer segment in response to a flash producing Φ photoisomerizations could be well described as these functions of time: a step (R*), a ramp (G*), and a delayed ramp (PDE*), respectively, with the amplitude of the...
TABLE I
Variables and Parameters of the Cascade Model

| Variables | Interpretation | Range/value (unit) |
|-----------|----------------|---------------------|
| \( \Phi \) | Number of photoisomerizations per flash | \( 0-3.5 \times 10^9 \) |
| \( R^* (t) \) | Number of activated rhodopsins at time \( t \) | \( 0-3.5 \times 10^9 = G_{\text{tot}} \) |
| \( G^* (t) \) | Number of free, activated G-proteins at time \( t \) | \( 0-2.6 \times 10^7 = PDE_{\text{tot}} \) |
| \( PDE^* (t) \) | Number of activated PDE catalytic subunits | \( \leq \) \( K_{i \rightarrow p} \) for channel |
| \( cG(t) \) | The concentration of free cGMP in the outer segment | \( \leq K_{d} \) for channel |
| \( F_c(t) \) | Normalized circulating current in choline at time \( t \) | \( 0-1 \) |

Parameters

| Parameters | Interpretation | Value/status |
|-----------|----------------|--------------|
| \( v_{RG} \) | Rate of production of \( G^* \) by single \( R^* \) | Estimated \( (= A/\beta_{nh} n) \) |
| \( v_{RP} \) | Rate of production of \( PDE^* \) per \( R^* \) | Estimated \( (= \beta_{nh} n) \) |
| \( \gamma_{CP} \) | Coupling efficiency factor between \( G^* \) and \( PDE^* \) | 0.8 |
| \( \tau_{RG} \) | Time constant for inactivation of \( R^* \) catalytic activity | Estimated as \( \tau \) |
| \( \tau_{RG-PDE} \) | Time constant for inactivation of \( G^*-PDE^* \) catalytic activity | Estimated by curve fitting |
| \( \alpha(t) \) | Rate of synthesis of cGMP by GC | \( \alpha_{\text{dark}} \) (see Eq. 11) |
| \( \alpha_{\text{dark}} \) | Dark rate of cGMP synthesis | \( 2.4 \mu M s^{-1} \) \( (= \beta_{\text{dark}} cG_{\text{dark}}) \) |
| \( \beta_{nh} \) | Rate constant of cGMP hydrolysis by single PDE catalytic subunit in a volume equal to that of the outer segment cytoplasm | \( 1.9 \times 10^{-5} s^{-1} \) |
| \( \beta(t) \) | Rate constant of PDE activity in outer segment | \( =PDE^*(t) \) \( \beta_{nh} + \beta_{\text{dark}} \) |
| \( \beta_{\text{dark}} \) | Rate constant of cGMP hydrolysis in the dark | \( 0.6 \) \( s^{-1} \) |
| \( cG_{\text{dark}} \) | Resting cytoplasmic concentration of cGMP | \( 4 \mu M \) |
| \( n \) | Hill coefficient of the cGMP-activated channels in situ | 2.5 |
| \( A \) | Amplification constant; equal to \( v_{RG} cGP_{nh} n \) | Estimated |
| \( \rho \) | Ratio of slope conductances: \( g_{Si}/g_{cG} \) (Eq. 11; cf. Fig. 1) | 2.3 |

The formulation and symbols are those adopted by Lamb and Pugh (1992) and Pugh and Lamb (1993); the calculations are done for an isotropic rod outer segment; whereas this is an oversimplification, it can be computationally justified under certain conditions (Lamb and Pugh, 1992). The value of \( \beta_{\text{dark}} \) in this table reflects recent advances in the characterization of the kinetic parameters of amphibian rod PDE (Dumke et al., 1994). The value of \( \beta_{\text{dark}} \) is taken from the investigation of Hodgkin and Nunn (1988); they give \( A = \beta_{\text{dark}} cG_{\text{dark}} \). For such intensities \( PDE^*(t) \), the number of active phosphodiesterase catalytic subunits in the outer segment at time \( t \) after a flash can be predicted to obey approximately the following equation:

\[
PDE^*(t) = \Phi v_{rg} C \left( e^{-t/\tau_{RG}} - e^{-t/\tau_{PDE}} \right).
\]

Here \( \Phi \) is the number of photoisomerizations produced by the flash \( v_{rg} \), the rate of production of \( PDE^* \)'s per fully active \( R^* \), \( \tau_{RG} \), and \( \tau_{PDE} \) are time constants for the inactivation of \( R^* \) and \( PDE^* \) as seen in the scheme 1–3, and \( C = (\tau_{RG}/\tau_{PDE})/ (\tau_{RG} - \tau_{PDE}) \) is a constant. The rate \( v_{rg} \) of production of \( PDE^* \)'s is related to the rate of production of \( G^* \)'s in step 2 by \( v_{ rg} = v_{ RG} \ cGP \), where \( cGP \) is a coupling coefficient that is expected to be close to unity (Lamb and Pugh, 1992; Lamb, 1994). At early times when the exponentials in Eq. 4 could be approximated by their Taylor series, the equation PDE*(t) could be approximated by the first terms in their Taylor series. For flashes producing up to 10 or more \( R^* \)'s per disc face, or \( \approx 20,000 \) total photoisomerizations (Lamb and Pugh, 1992; Lamb, 1994). For such intensities \( PDE^*(t) \), the number of active phosphodiesterase catalytic subunits in the outer segment at time \( t \) after a flash can be predicted to obey approximately the following equation:
lor series, Eq. 4 reduced to \( PDE^*(t) = \Phi \psi_{Rg} t \), which is the ramping result of Lamb and Pugh (1992). Eq. 4 neglects the brief delay in activation that has been examined previously (Lamb and Pugh, 1992); this delay is of little consequence on the relatively long time scales examined here. The delay can be introduced into Eq. 4 by replacing \( t \) with \( t' = t - t_{eg} \), with \( t_{eg} \approx 15-20 \) ms, and by setting \( PDE^*(t') = 0, t' \leq 0 \). This was done in the computational implementation.

Whereas the local depletions of the unactivated forms of G-protein and PDE that occur at the disc surface during activation are implicitly incorporated into the activation parameters \( \psi_{Rg} \) and \( \psi_{pr} \), the global depletions of these unactivated forms that are expected to occur at sufficiently high intensities are neglected. This neglect is because the intensities used in these experiments should not cause such depletions (see below, Computational Implementation of the Model).

The fourth major step of the cascade is the regulation of the concentration of free cGMP, \( cG \), by the two enzymes, PDE and GC. In an isotropic rod outer segment this rate equation governs \( cG \), the concentration of free cGMP:

\[
d\frac{dcG}{dt} = \alpha - \beta cG . \tag{5}
\]

In Eq. 5, \( \alpha \) is the rate of synthesis of cGMP by GC and depends on a number of factors, including (and especially) \( Ca^{2+}; \beta \) is the rate constant of cGMP hydrolysis by phosphodiesterase, which is generally time- and light-dependent, and dependent on the buffering power of cGMP.

The final major step in the cascade is the relationship between \( cG \) and the recorded circulating current. In previous formulations, this relationship has been expressed succinctly in terms of \( F \), the fraction of circulating current present at any instant:

\[
F(t) = \frac{J(t)}{J_{dark}} = \left( \frac{cG(t)}{cG_{dark}} \right)^n. \tag{6}
\]

In Eq. 6, \( cG_{dark} \) is the concentration of cGMP in darkness (at rest), \( J(t) \) is the (light-sensitive) circulating current measured at any instant in time in a particular solution, \( J_{dark} \) is the magnitude of \( J \) in darkness, and \( n \) is the Hill coefficient of the cGMP-activated conductance.

Five established principles underlie the use of Eq. 6 to characterize the responses of non-voltage-clamped rods in Ringer’s solution. First, the current/voltage relation of the cGMP-activated current in normal Ringer’s solution has a very shallow slope in the normal operating voltage range of the rod (Baylor and Nunn, 1986). Second, the cGMP-activated current equilibrates within milliseconds with free cGMP (Cobbs and Pugh, 1987; Karpen et al., 1988). Third, the voltage dependence and cGMP dependence of the cGMP-activated channels are assumed to be independent of one another, i.e., to act as separable functions (Baylor and Nunn, 1986). Fourth, capacity currents and electrogenic exchange currents are generally negligible with respect to the circulating current (see Cobbs and Pugh, 1987; Lamb and Pugh, 1992). Fifth, \( cG \ll K_{1/2} \), the half-saturation point of the Hill relation for the current through the channels (Yau and Baylor, 1989). Although all five principles involve approximations, in normal Ringer’s solution the approximations are very good, and Eq. 6 provides a faithful representation of the cGMP-activated current over the normal voltage range of the rod.

Eq. 6 will not apply under conditions in which the relevant region of the current/voltage relation of cGMP-activated current has a significant slope, as it does in a choline solution containing almost no permeant cations. Fig. 1 serves to illustrate why Eq. 6 is inappropriate and also helps to explain the alternative relationship we have adopted. Unlike the situation in normal Ringer’s solution (Fig. 1 A), in a choline solution lacking permeant cations the cGMP-activated current is outward (Fig. 1 B) and has a significant slope conductance, violating one of the principles underlying Eq. 6. To develop an appropriate alternative to Eq. 6, we begin with the assumption that under the recording conditions

\[
J_{is} = -J_{CG}. \tag{7}
\]

Here, \( J_{is} \) is the inner segment component of the circulating current, and \( J_{CG} \) is the outer segment, cGMP-activated component (Fig. 1 B). Eq. 7, like Eq. 6, assumes electrogenic exchange and capacity currents to be negligible. The problem with using Eq. 7 to derive an alternative to Eq. 6 is that in choline solution, both sides of Eq. 7 depend on the membrane potential, as illustrated in Fig. 1 B. To deal with this complication, we approximated the current/voltage relations of the inner segment current and of the cGMP-activated current by linear relations over the range of voltage excursion that the membrane potential undergoes during a saturating light response in choline solution:

\[
J_{CG} = g_{CG} \left( \frac{cG}{cG_{dark}} \right)^n (V - V_{CG}), \quad V_{CG} < V \leq V_{is}; \tag{8}
\]

\[
= 0, \quad V \leq V_{CG};
\]

\[
J_{is} = (V - V_{is}), \quad V_{is} \leq V \leq V_{CG}. \tag{9}
\]

In Eqs. 8 and 9, \( V \) is the membrane potential, \( V_{CG} \) the reversal potential of the cGMP-activated conductance in choline solution, \( V_{is} \) the reversal potential of the in-
which is either in Ringer’s solution, or in 0-Ca²⁺ choline solution.


\(\text{membrane current (µA)}\)

\(\text{membrane potential (mV)}\)

\(\text{A} \quad 60 \quad 50 \quad 40 \quad 30 \quad 20 \quad 0 \quad -20 \quad -30 \quad -40 \quad -50 \quad -60 \quad -70 \quad -80 \quad -90 \quad -100 \)

\(\text{B} \quad 60 \quad 50 \quad 40 \quad 30 \quad 20 \quad 0 \quad -20 \quad -30 \quad -40 \quad -50 \quad -60 \quad -70 \quad -80 \quad -90 \quad -100 \)

**Figure 1.** Current/voltage curves for the inner segment and outer segment components of the circulating current of a model salamander rod, the inner segment of which is held in an ideal suction pipette in normal Ringer’s solution, and the outer segment of which is either in Ringer’s solution, or in 0-Ca²⁺ choline solution (see inset, Fig. 3 for the actual configuration of a rod in the suction pipette). (A) Outer segment in normal Ringer’s solution. \(J_{cG}\) identifies the cGMP-activated current of the outer segment in Ringer’s solution, and follows the current/voltage relation of Baylor and Nunn (1986). The asymptotic magnitude of \(J_{cG}\) at hyperpolarized voltages is assumed to be 73 pA, based on the salamander rod whole-cell data of Cobbs and Pagh (1987) and Miller and Korenbrot (1994). \(J_{s}\) is the steady state inner segment current/voltage curve of Bader et al. (1979); the filled circles plot their actual measurements, through which a smooth curve has been interpolated. The resting membrane potential of the model rod in Ringer’s solution is \(V_{m} = -35 \text{ mV}\); this is the potential when \(J_{cG}\) and \(J_{s}\) are in balance, i.e., equal in magnitude and opposite in sign. \(V_{cG}\) and \(V_{s}\) identify the membrane potentials at which the curves \(J_{cG}\) and \(J_{s}\) intersect the 0-current axis. During a light response the membrane potential is driven from \(V_{m}\) toward \(V_{cG}\) as the cGMP-activated current declines. (B) Outer segment in 0-Ca²⁺ choline solution. When the outer segment is jumped into a choline solution containing no permeant cations, \(J_{cG}\) (unbroken curve) shifts to the left of the inner segment current curve \(J_{s}\). The inner segment component of the circulating current \(J_{c}\) nonetheless follows the same current/voltage relation as in A, since the inner segment remains in Ringer’s solution inside the suction pipette. Thus, the potential \(V_{cG}\) at which \(J_{cG}\) and \(J_{s}\) are in balance is now hyperpolarized with respect to \(V_{cG}\), and the circulating current reverses its direction of flow (e.g., as seen in the data of Fig. 3). The values of \(V_{cG}\) and \(V_{s}\) were estimated as follows. The two open symbols with error bars represent circulating current magnitudes measured in 0-Ca²⁺-choline under two conditions: at rest (C) and when \(cG\) is raised to a high level (D). These two data points are placed on the graph so that they lie on the \(J_{c}\) curve, which has been extrapolated with a polynomial approximation (dotted curve). At rest, \(J_{cG}\) (unbroken curve) must pass through a value equal in magnitude and opposite in sign to the point C; this condition determines the value \(V_{cG}\). When \(cG\) is raised to a high level, \(J_{cG}\) (dashed curve) becomes much steeper than at rest, the overall circulating current is limited by \(J_{s}\) and \(V_{cG}\) driven arbitrarily close to \(V_{cG}\). Thus, \(V_{cG}\) is identified as the potential corresponding to the lower data point (D). During a light response in choline, as cGMP declines from its resting value the membrane potential will depolarize from \(V_{cG}\) toward \(V_{cG}\), and thus the maximal voltage excursion is expected to be \(\sim 10 \text{ mV}\). The magnitudes of the data points (C, D) were obtained from suction electrode measurements made in 0-Ca²⁺-choline and are reported in connection with Fig. 5; they assume a suction electrode collection efficiency of two thirds (the error bars are \pm 1 \text{ SD}). The curves \(J_{cG}\) in B were generated with the same form of current/voltage relation as used by Baylor and Nunn (1986), but are completely outwardly rectified, reflecting the absence of permeant cations in the choline solution. In the model developed in the text, \(J_{cG}\) and \(J_{s}\) are approximated by straight lines (Eqs. 8 and 9, respectively) over the potential range between \(V_{cG}\) and \(V_{s}\), and for \(cG\) at and below its resting value \(cG_{\text{dark}}\).
current is nearly zero (Baylor and Nunn, 1986). It also bears mention that Eq. 10 is not dependent on the specific values of \(V_{\text{rest}}\) and \(V_i\).

In sum, the assumptions underlying Eq. 10 are reasonable and consistent with established facts. Moreover, whereas Eq. 10 is more complex than Eq. 6, it differs principally in having a saturating relation between \(cG\) and \(F_c\), even when \(cG \ll K_{i/2}\). Finally, as we will show, when combined with Eq. 4, Eq. 10 allows extension of the cascade theory to the full time course of families of responses of rods whose outer segments are in a choline solution with negligible amounts of permeant cations.

**Computational Implementation of the Model**

For intensities that do not lead to global depletion of the unactivated forms of G-protein and PDE, Eq. 4 is expected to be an accurate representation of \(PDE^\bullet(t)\), the number of photoactivated PDE catalytic subunits generated by the chemical scheme 1-3. For the values of the parameters involved (Tables I and II) and the intensities used in the experiments reported here, such depletion can be shown to be negligible to modest. For example, for \(\lambda = 0.06\ s^{-1}, n = 2.5, \beta_{\text{sub}} = 1.9 \times 10^{-3}\), one can derive from Table 1 the estimates of \(\nu_{\text{RF}} = 1,260\ s^{-1}\), and \(\nu_{\text{RG}} = 1,580\ s^{-1}\). For \(\tau_{\text{RF}} = 2.4\ s\) and \(\tau_{\text{PDE}} = 0.4\ s\), according to Eq. 4 the peak production of \(PDE^\bullet\) will occur at \(< 2\ s\), and so for \(\Phi = 2 \times 10^4, PDE^\bullet(t) \leq \Phi \nu_{\text{RF}} \times 2 = 2.5 \times 10^7\), a value less than the total complement of PDE catalytic subunits. If \(\tau_{\text{RF}}\) is lower, significant depletion of the inactive form of PDE will occur at still higher intensities. Since there is more than 10 times as much G-protein as PDE, significant depletion of unactivated G-protein will not occur until much higher intensities.

Eq. 5 was solved with the fourth–fifth order Runge-Kutta routine ODE45 of MatLab. The time-varying forcing factor \(\beta(t)\) was computed as \(\beta(t) = \beta_{\text{dark}} + PDE^\bullet(t)\beta_{\text{sub}}\), where \(\beta_{\text{dark}}\) and \(\beta_{\text{sub}}\) are given in Table I. The term \(\alpha\) was also allowed to be time dependent, as expressed in Eq. 12; the rate parameter \(k_a\) was estimated from application of Eq. 5 to the DC-LC trace (e.g., lowermost trace in Fig. 7).

The normalized circulating current in choline, \(F_c(t)\), was computed with Eq. 10, with \(\rho = 2.5\), the value estimated from the results reported in the context of Fig. 5. For computational convenience it was assumed that \(cG = cG_{\text{dark}}\) at \(t = 5\ s\) after the jump to choline.

An equation for capacity current was also added to provide a good account of the activation phase of responses to bright flashes. This addition can be made without knowledge of the absolute values of \(V_i\) providing a membrane time constant can be assumed, and the cell impedance does not change during the activation phase of the response. Based on the work of a number of laboratories on salamander rods, we expect \(\tau_m\) to be \(\sim 20\ ms\). Cobbs and Pugh (1987) showed how \(\tau_m\) can be estimated from the responses to bright flashes.

**METHODS**

Some of the methods used in this investigation have been reported previously (Cobbs and Pugh, 1987). The description presented here emphasizes those experimental procedures and analyses that are novel or especially important for this report.

**Animals and Tissue Preparation**

Larval tiger salamanders (Ambystoma tigrinum) were obtained from Charles Sullivan (Nashville, TN) and maintained in aerated, charcoal-filtered tanks on a diet of minnows. Retinas were prepared and rods isolated, as described previously.

**Solutions**

All solutions were made with tissue-culture-grade water (W3500, Sigma Chemical Co., St. Louis, MO) and reagent-grade chemicals. The contents of the normal amphibian Ringer's solution in millimolars were: NaCl, 111; KCl, 2; CaCl2, 1.0; Na2HPO4, 0.5; MgCl2, 1; MgSO4, 0.5; EDTA, 0.01; HEPES, 3. The pH was adjusted with NaOH to 7.4. We also added 2 mg/ml fatty-acid-free bovine serum albumin (A6003, Sigma Chemical Co.) to the Ringer's solution; this seemed particularly helpful in keeping cells viable immediately after the chopping, when debris from ruptured cells is present.

In preliminary experiments aimed at maintaining constant Ca\(^{2+}\); in rods, we used several different 0-Na\(^+/\)low-Ca\(^{2+}\) external solutions, including solutions containing either guanidinium (Nakatani and Yau, 1989; Fain et al., 1989) or K\(^+/\) or Mg\(^{2+}\) as the only cation; each of these conditions provided some useful data. However, we found that the 0-Na\(^+\) solution that yielded stable recordings for the longest periods was a choline solution (Hodgkin et al., 1984; Matthews, 1995), the constituents of which were (in mM): choline–chloride, 115; HEPES, 3; glucose, 10; and small amounts of Ca\(^{2+}\), buffered with 2 mM EGTA (L657-5, Baker); we emphasize that no Mg\(^{2+}\) or any other permeant cation was added. The pH of choline solutions was adjusted by adding of small amounts of tetramethylammonium. The osmolarity of the choline solutions was measured with a vapor pressure osmometer (model 5500, Westcor Inc., Logan, UT), and matched empirically to that of the Ringer's solution with small adjustments of the choline–chloride concentration; this adjustment helped maintain long-term stability of rods.

**Estimation of Free Calcium in the Choline Solutions**

Methods for calculating free Ca\(^{2+}\) in physiologic solutions buffered with EGTA have been examined by Durham (1983) and Tsien (1983). In the absence of Mg\(^{2+}\), the four stability constants involved are those for the binding of the first and second protons to EGTA\(^{-}\), that for the binding of Ca\(^{2+}\) to EGTA\(^{-}\), and that for the binding of Ca\(^{2+}\) to EGTA-H\(^{+}\), respectively. Based on
Durham's (1983) survey and an analysis of the literature, we adopted the following values for these constants at 20°C: pKm = 9.5, pKmG = 8.94, pKmG = 11.38, and pKmGn = 5.7. A program was written in MatLab that iteratively solved the set of simultaneous binding equations involved and that checked for conservation of the EGTA and calcium. Experimentally, we used only five levels of Ca\(^{2+}\), with choline. Specified in terms of (free Ca\(^{2+}\) / total Ca\(^{2+}\)) the first four levels were (7.4 nM / 900 µM); (6.1 nM / 800 µM); (2.3 nM / 400 µM); and (1.0 nM / 200 µM). The fifth level, 0-Ca\(^{2+}\) choline, refers to solutions with 2 (or, rarely, 10) mM EGTA and no added Ca\(^{2+}\). We emphasize that other than the very small amounts of Ca\(^{2+}\) no permeant cations were added to the choline solutions.

**Recording Chamber and Solution Exchange**

All experiments were performed at room temperature (20–22°C) under continuous infrared illumination (λ > 850 nm) in a light-tight Faraday cage built over an inverted microscope (IM-35, Carl Zeiss, Inc., Thornwood, NJ) on a vibration-isolation table. Fig. 2 illustrates the experimental chamber and superfusion system, which was modeled on previously reported superfusion chambers for photoreceptors (Hodgkin et al., 1985, 1987; Lamb and Matthews, 1988). The top (1) and bottom (2) of the chamber are 1-mm-thick glass slides, mounted on Plexiglas blocks (3, 4). The sides of the chamber are open to permit entry of electrodes from both sides. The chamber dimensions are 8 × 3 × 1.4 mm, and its volume is 33 µl.

In the experiments, two solutions continually flowed into the chamber under gravity feed; the main flow was through the body of the block (inlet 1) and usually carried Ringer's solution; the secondary flow, which usually carried choline test solutions, was through a central polycarbonate tube (5, inlet 2), having an outside diameter of 600 µm, and an inside diameter of 300 µm. A six-way tap allowed selection of additional solutions for the secondary flow. The solution exit was through a channel in the body of block 4 and was connected to a small open bath in which fluid was maintained at a constant level by continuous suction through a small tube. A Ag/AgCl reference electrode was connected to the bath by means of a short salt bridge with a wick; the electrode was connected to the signal ground of the recording amplifier. Gravitational gradients and flow resistance were adjusted to give flow rates of 0.5–1.0 ml min⁻¹ for the main flow, and 0.05–0.1 ml min⁻¹ for the test flow. Control experiments with dye in the central axis showed that with these rates the flows are laminar, with sharp boundaries extending from the tube orifice up to 1 mm beyond the end of the tube. The flow rates were chosen to be as low as possible to prevent bending of the rods, yet able to produce good laminar boundaries.

For electrical recording, the inner segment of a salamander red rod was drawn into a tapered suction pipette filled with normal Ringer's solution. The resistance of the suction electrode without a rod inserted was 0.5–0.6 MΩ and with the rod in place increased to 2–3 MΩ. Each rod was carefully positioned in the pipette so that the inner segment/outter segment junction was just outside the point of maximal pipette constriction (see Fig. 2 B and Fig. 3, left inset); this was done to ensure that the entire outer segment was exposed to the choline superfusion solution. Previous experiments combining suction electrode recordings and whole-cell recordings showed that when a rod is optimally placed in the tapered type of suction electrode that we construct, approximately two thirds of the circulating current is recovered by the suction electrode (Cobbs and Pugh, 1987). Because of the positioning of the rods, the suction electrode collection should be between one half and two thirds.

Once the rod was properly seated in the suction pipette, the chamber and pipette were arranged so that the rod occupied a standard position 180–200 µm from the center of the superfusion tube in an x-y plane containing the tube’s central axis (Fig. 2 B). Recording then commenced. Changes in the solution superfusing the rod were effected by a computer-controlled motor-mike (Oriel Corporation, America, Stamford, CT) which was mounted rigidly on the stage of the microscope and which translated the chamber along the x coordinate. The forward movement of the chamber situated the rod on the superfusion tube’s central axis, < 100 µm from the orifice; the reverse movement caused the chamber to return to its original position, so that the rod was again in the main flow. The speed of the solution change was estimated from the kinetics of the junction current recorded with a rod exposed to a bright light; 90% of the junction current occurred in 200–300 ms (see Fig. 3).

**Light Stimulation and Intensity Calibrations**

Rods were stimulated with circularly polarized 500-nm light from a multibeam optical system constructed on the vibration table and situated outside the Faraday cage (Cobbs and Pugh, 1987). One channel of the stimulator could deliver flashes (usually 10 ms in duration) or steps of any duration. The other channel delivered 20-µs flashes. Both stimulus channels contained optical wedges that were under the control of a microcomputer, allowing 0.02 log unit steps in intensity over 3 log units of intensity. Additional neutral density filters were available in each of the channels. A dedicated microcomputer having customized software controlled the timing of the light stimuli and their synchronization with the stage movements.

Light intensities were calibrated regularly at the microscope stage with a Pin-10 photodiode (United Detector Technology) that had been calibrated absolutely by the National Research Council of Canada. Intensities were expressed in flux density units (quanta µm⁻² s⁻¹ or quanta µm⁻² flash⁻¹) at the image plane of the microscope. The optical wedges and neutral filters were recalibrated for these experiments. For simplicity in reporting the results, we assume that a salamander red rod has a collecting area of 18 µm² for the circularly polarized, 500-nm stimuli used here (see Lamb and Pugh, 1992). This collecting area reflects the use of a 500-nm rather than a 520-nm stimulus (the λmax of salamander porphyropsin), and a 20-degree tilt of the rod with respect to the horizontal plane. In the text below, stimuli are expressed in photoisomerizations, Φ or Φ s⁻¹, obtained by multiplying the quantal flux density at the stage by the assumed collecting area. No corrections have been applied for loss of light caused by scattering in the chamber.

**Electrical Recording and Data Analysis**

A patch-clamp amplifier (model 8900, Dagan Corp., Minneapolis, MN) with a 1-GΩ head stage having 10 kHz bandwidth served as the current-to-voltage transducer for measuring the membrane current of the rod inner segment. The output of the Dagan amplifier was fed to a four-pole analogue Bessel filter (model
Fig. 3 illustrates the basic experimental protocol used in the dark, resting state. Shown are suction electrode malization, curve fitting, and other routine analysis procedures. Analogue filter was set at a value appropriate to avoid aliasing, with a microcomputer (486/33, Gateway) that used software that patterned on that of Fain et al. (1989; Fig. 4) and was number of features of the method. The experiment was allowed for averaging records, subtracting baselines, response normomized software constructed on the MatLab platform. The software for off-line processing (Axotape, Axon Instruments, Foster City, e.g., at 200 Hz for 500-Hz sampling. Stimulus markers were recorded on one of several parallel data channels.

Continuous digitized records were divided into appropriate time blocks based on the stimulus markers and analyzed with customized software constructed on the MatLab platform. The software allowed for averaging records, subtracting baselines, response normalization, curve fitting, and other routine analysis procedures.

RESULTS

Fig. 3 illustrates the basic experimental protocol used throughout this paper and serves further to illustrate a number of features of the method. The experiment was patterned on that of Fain et al. (1989; Fig. 4) and was designed to maintain Ca\textsuperscript{2+}, constant at the level it has in the dark, resting state. Shown are suction electrode traces obtained from a rod whose outer segment was exposed repeatedly to 0-Ca\textsuperscript{2+} choline solution. For 10 of the traces the rod was stimulated with each of a series of increasingly intense flashes in Ringer’s solution, and then again after 5 s in choline solution, with the same flash; a saturating flash was then given to determine the residual circulating current in choline solution, and the rod was returned to the Ringer’s solution. For the two traces marked LC (light control), the rod was stimulated with a saturating flash before being moved to choline; for the trace marked DC (dark control), only a single flash was given in choline, at the time when the second flash was normally delivered.

Of critical importance for the kinetic analyses that we undertake here is that the rod remain stable for a long recording period. For the rod of Fig. 3, the superfusion cycle Ringer’s → choline → Ringer’s was executed more than 90 times, allowing repeated responses to each flash to be obtained. During the nearly 3 h of recording, the circulating current and light response kinetics in Ringer’s solution were maintained with only modest change. In general we found that rods having initial photocurrents under these recording conditions of 35–50 pA (Table II) were stable for dozens of solution changes and for recording periods of 2 h or more.

**Experiments Examining the Stability of Ca\textsuperscript{2+} in Low-Ca\textsuperscript{2+} Choline Solution**

Since most previous experiments in which investigators have attempted to maintain Ca\textsuperscript{2+}, constant in rods have employed guanidinium or lithium as the charge carrier of cGMP-activated current, it is important to examine the evidence for stability of Ca\textsuperscript{2+} in choline solution. Some pertinent evidence is presented in Fig. 3 in the inset showing the difference trace, DC–LC. This difference trace reveals that the light-sensitive current increased steadily at a rate of 0.5 pA s\textsuperscript{-1} during the 15-s exposure to the low-Ca\textsuperscript{2+} choline solution. Assuming that \( \beta_{dash} \), the rate constant of cGMP hydrolysis in darkness, remains constant during the choline exposure, the steady increase in circulating current necessitates a steady increase in \( \alpha \), the rate of cGMP synthesis (Eq. 5). Because the most likely cause of the increase in \( \alpha \) is a steady decline of Ca\textsuperscript{2+}, a careful examination is required of the suitability of the 0-Ca\textsuperscript{2+} choline condition for maintaining Ca\textsuperscript{2+}, near its resting level.

To test the hypothesis that a decline of Ca\textsuperscript{2+} underlies the steady increase in circulating current and to assess the rate and magnitude of changes in Ca\textsuperscript{2+}, we performed experiments designed to vary Ca\textsuperscript{2+} over its natural range in normal Ringer’s solution, and to establish the maximum circulating current achievable in 0-Ca\textsuperscript{2+} choline solution.

Fig. 4 A shows data obtained from a rod repeatedly jumped into 0-Ca\textsuperscript{2+} choline solution in darkness. In this experiment, to clearly delineate any changes in circulating current, a saturating flash was delivered at a series of different times after the jump. These data confirm the inference drawn from the DC–LC trace in Fig.
3, viz., the circulating current steadily increases in 0-Ca2+ choline. They also yield the additional observation that the rate of increase declines over 30 s. The finding that the rate of increase declines was replicated on several rods (see Fig. 6 C). In the experiments reported here, we never kept a rod in choline longer than 30 s.

Fig. 4 B shows results from the same rod, but in each case exposed before the switch to choline to a step of light that suppressed about two thirds of the circulating current in Ringer’s solution. An increase in circulating current in choline over the level seen in Fig. 4 A was expected, because the light step in Ringer’s solution lowers Ca2+, thereby increasing GC activity and leading to elevated cG once PDE activity has declined to its dark level (see Eq. 5). The maximum circulating current in choline in Fig. 4 B was 53 pA, 2.4-fold greater than the circulating current recorded at the same point in time without prior light exposure (Fig. 4 A). The maximum magnitude of the circulating current seen in Fig. 4 B provides a quantitative perspective on the rate of increase of current in the dark shown in Fig. 4 A, for it shows the magnitude of the rate, 0.5 pA s−1, to be relatively small. Moreover, 53 pA represents a lower bound on the maximum possible current in 0-Ca2+ choline solution, since partial suppression of the circulating current in Ringer’s solution does not cause Ca2+; to decline to its lowest possible value, nor does it cause GC to achieve its maximum rate (Hodgkin and Nunn, 1989). We now describe additional experiments designed specifically to estimate the maximal possible current achievable in 0-Ca2+ choline.

To estimate the maximum circulating current achievable in 0-Ca2+ choline, we performed experiments similar to that of Fig. 4 B but varied the intensity of the light steps in Ringer’s solution; such experiments are similar to the experiments performed by Fain et al. (1989) to evaluate calcium clamping in solutions having guanidinium as the charge carrier through the cGMP-activated channels. We also performed experiments in which we jumped rods into 0-Ca2+ choline containing 50–500 μM IBMX, with and without prior light adaptation. These maneuvers are expected to increase cG, both by further increasing GC activity before the jump into choline and by decreasing PDE activity after the jump (Hodgkin and Nunn, 1988). Fig. 5 presents results from three such experiments. In each panel the trace labeled D is the circulating current observed when the jump into 0-Ca2+ choline solution was made in darkness and without IBMX; the other traces are circulating currents obtained when the cell was exposed before the jump to a step of light that suppressed at least 50% of its current (L) or obtained when IBMX was added to the choline solution (I). For seven rods exposed to various combinations of light steps and IBMX, the maximum circulating current in 0-Ca2+ choline solution was 71 ± 15 pA (mean ± SD). The value 71 pA provides an estimate of the circulating current achieved when the cGMP conductance is very strongly activated in 0-Ca2+ choline, and the membrane potential approaches VCG (see Fig. 1 B); let us call this value Jmax. For these same seven rods, the circulating current measured in darkness at 5 s after the jump was 22 ± 5 pA; call this value Jdark (In Fig. 1 B, Jmax and Jdark, corrected from suction electrode collection efficiency, are plotted as the two open symbols.) The ratio Jmax/Jdark is

![Figure 3](image-url)
71:22 = 3.2. Computing the ratio \( j_{\text{max}}/j_{\text{dark}} \) for each cell and averaging, we obtained the mean ratio (±SD) 3.3 (± 0.8). By instantiating Eqs. 8 and 9 twice, first for the rest (dark) condition in choline \( (V = V_{\text{rest}}, j = j_{\text{dark}}, cG = cG_{\text{dark}}) \), and second for the maximally activated condition \( (V = V_{\text{G}}, j = j_{\text{max}}) \), the following expression can be derived:

\[
\rho = \frac{g'_{\text{is}}}{g'_{\text{cG}}} = \frac{j_{\text{max}}}{j_{\text{dark}}} - 1. \tag{11}
\]

Thus, we obtained the estimate \( \rho = g'_{\text{is}}/g'_{\text{cG}} = 2.3 \) needed for application of the model (Eq. 10). We emphasize that this estimate of \( \rho \) does not depend on the specific values of \( V_{\text{G}}, V_{\text{C}}, \) or \( V_{\text{rest}} \) (in choline).

Aside from the reversed direction of circulating current flow, the most striking difference between the results we have obtained in choline solution containing negligible permeant cations (except for Ca\(^{2+}\)) and those previously reported for which guanidinium and lithium were used to carry the circulating current and maintain Ca\(^{2+}\), constant, is that in choline very much lower concentrations of Ca\(^{2+}\) yield approximately steady circulating currents. Fig. 6 presents data obtained from 11 rods, each exposed repeatedly to one or more choline solutions containing 0, 2.3 nM, or 7.4 nM Ca\(^{2+}\). Here two kinds of data are presented: symbols represent photocurrent magnitudes, measured (as in Fig. 4) with saturating flashes given at the times when the points are plotted; the continuous traces are difference traces, DC-LC, as in the inset of Fig. 3. The current magnitudes are most accurately estimated with the saturating flashes (symbols); however, the difference traces give an additional, useful estimate of the overall time course.

The data in Fig. 6 C establish the generality of the steady increase in circulating current in 0-Ca\(^{2+}\) choline, and the data in Fig. 6, A and B, demonstrate that this steady increase in current can be reversed by Ca\(^{2+}\) in the nanomolar range. In particular, the data in Fig. 6 B show that for Ca\(^{2+}\) = 2.3 nM, the circulating current is steady for 30 s or more. These results support the hypothesis that the increase in current in 0-Ca\(^{2+}\) choline (Figs. 4 A and 6 C) is the result of a decline in Ca\(^{2+}\) in the outer segment.

Further evidence that Ca\(^{2+}\) declines in the outer segment in 0-Ca\(^{2+}\) choline but not in choline containing 2.3 nM Ca\(^{2+}\), is presented in Fig. 7. The upper panel plots four responses of a rod jumped into 2.3 nM Ca\(^{2+}\); the responses, which were obtained at times ranging from 1.2 s to 29 s after the jump into choline, are nearly identical, as are the photocurrent magnitudes. The lower panel plots the responses of the same rod to flashes delivered at the same times, but now jumped into 0-Ca\(^{2+}\) choline. In contrast to those in the upper panel, the responses in the lower panel show a progressive increase in circulating current and a speeding of recovery, both of which are characteristic of a gradual lowering of Ca\(^{2+}\); (Fain et al., 1989).

**Conclusions Regarding Ca\(^{2+}\) Stability in Low-calcium Choline**

Our initial experimental goal was to find an experimental condition in which Ca\(^{2+}\), in a rod could be maintained stably at its resting level, so that the light-response kinetics could be examined without the complexity of dynamically changing Ca\(^{2+}\). Two relevant conclusions emerge from consideration of the results of Figs. 4–7. The first is that while Ca\(^{2+}\) declines in 0-Ca\(^{2+}\) choline, the rate of decline is relatively slow, in the sense that the change in GC activity is apparently quite small over 20 s. For example, from Fig. 6 C, one finds that the normalized rate of increase of circulating current is \( \sim 0.02 \) s\(^{-1}\) over the initial 20 s. With a rate of 0.02 s\(^{-1}\), a 40% total increase in circulating current would occur in 20 s. For a Hill coefficient of 3 (in Eq. 10) and \( \rho = 2.3 \), this 40% increase in current corresponds to a 19% increase in cG. Assuming the PDE rate constant, \( \beta \) in Eq. 4, is unchanged, the 19% increase in cG corresponds to a 19% increase in guanylyl cyclase ac-
FIGURE 5. Circulating currents of three rods jumped into 0-Ca²⁺ choline in the darkness (traces labeled D) or after exposure in Ringer’s solution to a step of light, as in Fig. 4 (traces labeled L). Traces labeled I indicate that the 0-Ca²⁺ choline contained 500 μM IBMX. The fraction of the circulating current suppressed by the light steps in Ringer’s and the intensities in Op s⁻¹ were B (0.48, 470) and C (0.75, 1900). B and C show the variety of behavior exhibited when light-steps in Ringer’s solution are used to decrease Ca²⁺ before the jump to choline.

FIGURE 6. (A–C) Circulating currents of 11 rods jumped into choline solutions containing Ca²⁺ as indicated on the graphs. Symbols show the photocurrent amplitudes, measured with saturating flashes (as in Fig. 4), and noisy traces show continuous DC-LC measurements (as in the inset of Fig. 3). Filled symbols represent data of rods exposed to two or three levels of Ca²⁺; thus, when a filled symbol is repeated in A–C, it refers to data of the same rod. Open symbols in different panels represent data from different rods. Error bars give ±1 SD. For display clarity, the continuous traces were digitally filtered with a Gaussian filter having f = 0.6 Hz (Colquhoun and Sigworth, 1983).

Photocurrent Kinetic Analysis

Fig. 8 shows averaged responses of the rod of Fig. 3 to a series of flashes. The saturating responses in Ringer’s solution (Fig. 8 A) and those in 0-Ca²⁺ choline (Fig. 8 B) follow two laws first noted by Baylor et al. (1974) in their investigation of turtle cones and by many investigators since: first, the recovery phase of the responses to saturating flashes is approximately invariant in shape when translated on the time axis; second, geometric increases in light intensity produce linear increments in the recovery time. This latter result is more clearly illustrated in Fig. 8 C, where we plot the time to 50% recovery as a function of the flash intensity on a semilogarithmic plot. Although confirming previous findings obtained from rods in normal Ringer’s solution (Peperberg et al., 1992), Fig. 7 also presents an entirely novel result: the slopes of the lines best fitting the recovery half-times are nearly identical: τ_r = 2.56 s and τ_r = 2.46 s per e-fold change in intensity for recoveries in Ringer’s solution and choline, respectively. Fig. 9 shows that the parallelism of the lines characterizing the recovery half-times in Ringer’s solution and in low-Ca²⁺ choline is quite general. Here we plot the estimates of τ_r obtained from saturating responses in low-Ca²⁺ choline against τ_r determined from responses in Ringer’s for 11 rods from which we obtained response families in both conditions, as in Fig. 8. In Fig. 9, open symbols represent data of rods exposed to 0-Ca²⁺ choline, whereas filled symbols represent data of rods exposed to choline containing 2.3 nM Ca²⁺. The hypothesis of equality of τ_r’s is represented in Fig. 9 by the positive diagonal, i.e., the unit slope line through the origin. 10 of the 12 data sets are consistent with the hypothesis, while two deviate significantly by having τ_r determined in choline reliably higher than τ_r determined in Ringer’s. It is noteworthy that different rods that do not deviate from the hypothesis of equality of
Figure 7. Circulating currents and photoresponses of a rod jumped into choline containing 2.3 nM Ca\textsuperscript{2+} (upper panel), or 0-Ca\textsuperscript{2+} choline (lower panel). The flash (Φ = 7,100) was delivered to the rod after (a) 29 s, (b) 11 s, (c) 4.1 s, and (d) 1.25 s in choline. Traces are single records, Gaussian filtered (γ = 7 Hz). Note that the scale of the ordinate of the upper panel is twice that of the lower panel. The full time course of the circulating current changes in choline for this rod are shown in Band C of Fig. 6 (filled diamonds).

τ\textsubscript{c}'s do differ in a highly reliable manner from one another in their value of τ\textsubscript{c} (Fig. 9). It is highly unlikely that these differences among rods in τ\textsubscript{c} were caused by differences in temperature, which was always 20–22°C.

The parallelism of the recovery half-time semilog plots captured in the equality τ\textsubscript{c}'s in Fig. 9 renders the vertical shift, ΔT, between the curves interesting, for it provides a gauge of the shift of sensitivity caused by the decline in Ca\textsuperscript{2+} from its resting level (in choline) to the very low level it reaches after saturating flashes in Ringer's solution. For example, for the data of Fig. 8 C, the vertical shift is ΔT = 6.5 s. By virtue of the parallelism, one can compute that in Ringer's solution a flash must be exp(ΔT/τ\textsubscript{c}) = 13-fold more intense to produce a response having the same recovery half-time as it would have for Ca\textsuperscript{2+} near its resting level. Table II (last column) gives sensitivity shift factors computed in this manner for all the rods studied.

Following Pepperberg et al. (1992), and interpreting τ\textsubscript{c} as τ\textsubscript{R}, we implemented the model described in the Theory section to describe the photoresponses in low-Ca\textsuperscript{2+} choline. Fig. 10 illustrates the application of the model to the responses of the rod of Fig. 8. To satisfactorily account for the data we found it necessary to augment the model with one equation in addition to those given in the theory section:

\[
α(t) = α_{\text{dark}} (1 + k_0 t).
\]  

This equation expresses the observation reported in Figs. 4 A and 6 C that there appears to be a steady increase in guanylyl cyclase activity in 0-Ca\textsuperscript{2+} choline. Eq. 12 may be evaluated by the degree to which the theory provides a good description of the dark control (DC–LC) trace in Fig. 10. To fit the responses at times longer...
than 10 s, when the rate of increase of current in 0-Ca\(^{2+}\) choline declines (Figs. 4 A and 6 C), \(k_0\) in Eq. 12 can be replaced by \(k_0 \exp(-t/\tau_\alpha)\), where \(\tau_\alpha\) ranges from 15 to 25 s for different rods.

Fig. 11 A presents averaged photocurrents of a rod in choline having 2.3 nM Ca\(^{2+}\), and theoretical curves. In Fig. 11 B the recovery half-time data for the saturating responses are presented, along with the best-fitting straight lines. We note a minor problem with the theoretical account of these responses. The dark-control trace (DC–LC) is virtually flat for 30 s (see Fig. 6, solid diamonds), and thus reflects balanced dark cyclase and PDE activity; thus, in terms of Eq. 12, \(k_0 = 0\). However, in order to fit the recoveries to the more intense flashes well, \(k_0\) must be set at a nonzero value. As a compromise, we have fixed \(k_0\) at the value 0.002 s\(^{-1}\) for the entire family of theoretical traces.

Table II summarizes the application of the theoretical analysis to a population of rods from which we obtained response families including at least four saturating responses from which \(\tau_\alpha\) was estimated. In every case we found that the entire family of responses could be well described with the theory, as in Figs. 10 and 11. In two cases, we found that good reproduction of the responses to one or more intense flashes required \(k_0\) to be set to a higher value than that required to fit the dark-control trace and the dimmer flashes. In Table II, these cases (rods 5 and 10) are indicated by showing two values in the column listing \(k_0\); the unbracketed value gives that which was used to fit most of the traces, whereas the bracketed value gives the value required to fit the responses to the most intense flashes.

### DISCUSSION

**Ca\(^{2+}\) Invariance of the Dominant Recovery Time Constant**

When an amphibian rod is stimulated with a saturating flash, Ca\(^{2+}\) declines within a few seconds from a resting value near 400 nM to a much lower value, probably 50 nM or lower (Ratto et al., 1988; Lagnado et al., 1992; Gray-Keller and Detwiler, 1994). As summarized in Fig. 9, a novel and robust finding of this investigation is that \(\tau_\alpha\), the dominant time constant of circulating current

### TABLE II

| Rod | Figs. | \(J_{\text{max}}\) | \(\tau_\alpha\) | \(\text{Ca}^{2+}\) | \(J_{\text{max}}\) | \(A\) | \(\tau_\beta\) | \(\tau_{\text{PDE}}\) | \(K_0\) | \(\Delta T\) | \(\exp(\Delta T/\tau_\alpha)\) |
|-----|------|-----------------|---------------|----------------|-----------------|-----|---------------|----------------|------|---------|----------------|
| 1   | 9    | 43 ± 0.5        | 1.6           | 0              | 37 ± 2          | 0.11| 1.9           | 0.40           | 0.015| 4.7     | 5.8           |
| 2   | 9    | 38 ± 4          | 2.7           | 0              | 33 ± 4          | 0.04| 2.7           | 0.50           | 0.052| 6.1     | 9.6           |
| 3   | 5, 8, 9, 10 | 50 ± 2        | 2.6           | 0              | 21 ± 2          | 0.045| 2.5           | 0.47           | 0.016| 6.5     | 12.8          |
| 4   | 6 C, 9| 47 ± 3          | 1.8           | 0              | 23 ± 1          | 0.048| 1.7           | 0.40           | 0.052| 5.5     | 23.2          |
| 5   | 9    | 48 ± 2          | 2.7           | 0              | 30 ± 2          | 0.044| 2.5           | 0.30           | 0.005| 5.5     | 9.3           |
| 6   | 9    | 30 ± 0.5        | 2.3           | 0              | 19 ± 2          | 0.065| 2.4           | 0.50           | 0.026| 6.8     | 14.4          |
| 7   | 9    | 41 ± 0.5        | 2.4           | 0              | 18 ± 2          | 0.060| 2.5           | 0.50           | 0.030| 5.6     | 9.8           |
| 8a  | 6 C, 9| 38 ± 1          | 2.2           | 0              | 16 ± 2          | 0.085| 2.3           | 0.40           | 0.028| 5.0     | 9.2           |
| 9   | 6 B, 9| 24 ± 2*         | 2.8           | 2.3            | 12 ± 1          | 0.060| 2.9           | 0.45           | 0.067| 8.5     | 19.7          |
| 10  | 6 B, 9| 37 ± 1          | 2.3           | 2.3            | 13 ± 2          | 0.055| 2.8           | 0.45           | 0.001| 7.9     | 22.1          |
| 8b  | 6 B, 9| 43 ± 2          | 2.2           | 2.3            | 12 ± 2          | 0.08  | 2.0           | 0.40           | 0.006| 5.6     | 14.4          |
| 11  | 6 B, 6 C, 7, 9, 11 | 31 ± 2*       | 2.0           | 2.3            | 12 ± 0.4        | 0.047| 2.2           | 0.35           | 0.002| 6.5     | 18.0          |
| Mean ± SD |        | 41 ± 7          | 2.30 ± 0.37   | —              | 0.06 ± 0.02     | 2.36 ± 0.37 | 0.43 ± 0.06 | —               | 0.024| 6.2 ± 1.1 | 14.0 ± 5.6   |

Column 1 identifies the rod; rod number 8 was recorded from under three conditions, Ringer's solution and two different levels of Ca\(^{2+}\). Column 2 gives figures in which data from the rod appear. Column 3 gives the maximum circulating current in Ringer's solution; these values are somewhat diminished from the values that would be obtained were the outer segment not positioned completely outside the suction electrode (see Methods). The values denoted with an asterisk * were obtained in experiments in which the outer segment was placed more than 5 µm outside the pipette constriction in an effort to eliminate any influence of the Ringer's solution inside the suction pipette. Column 4 gives the value of \(\tau_\alpha\), estimated from families of saturating flashes in Ringer's solution, as in Figs. 8 C and 11 B. Column 5 gives the value of \(\text{Ca}^{2+}\), for the choline test solution. Column 6 gives the maximum circulating current recorded in choline, measured 1 s after the jump for rods in 0-Ca\(^{2+}\) choline, and 5 or 12 s after the jump for Ca\(^{2+}\), = 2.3 nM. Column 7 gives the estimate of the amplification constant (see Table I). Column 8 gives \(\tau_\beta\), estimated from families of saturating flashes in choline, which is identified in the model as a first-order time constant for inactivation of R\(^*\). Column 9 gives the estimate of the time constant for simultaneous inactivation of G\(^*\) and PDE\(^*\). Column 10 gives the apparent rate of activation of GC in choline (Eq. 11), obtained from fitting a response family, including and especially, the dark control trace; the two bracketed entries indicate instances in which a higher value of the parameter had to be used to fit one or more of the responses to more intense flashes. Column 11 gives the vertical shift in seconds between parallel lines fit to the recovery half-time plots (e.g., Figs. 8 C, 11 B); column 12 converts the vertical shift into an effective change in sensitivity, as described in the text.
was varied to find the best fit of the model to the traces. FIGURE 10. Application of the theoretical model to photocurrent traces are the normalized records from Fig. 8 B; the dashed lines give calculations done with the model. The magnitude of the maximal photocurrent 5 s after the jump into choline was $20.9 \pm 2.1 \text{pA}$ (26 responses). The values of the model parameters are provided in Table II. To optimize the fitting, $\tau_{\text{c}}$ was set equal to $\tau_{\text{R}}$ obtained from regression analysis of the choline data in Fig. 8 C; $\tau_{\text{PDE}}$ was varied to find the best fit of the model to the traces.

recovery after a saturating flash, has essentially the same value in Ringer’s solution as in low-Ca$^{2+}$ choline solution. This finding implies that the biochemistry underlying $\tau_{\text{c}}$ has little or no sensitivity to changes in Ca$^{2+}$ in the physiologic range. In particular, if, as hypothesized by Pepperberg et al. (1992), $\tau_{\text{c}}$ can be ascribed to the inactivation of $R^*$, then the hypothesis that the lifetime of $R^*$ is regulated by changes in Ca$^{2+}$ is falsified. Others, investigating the role of Ca$^{2+}$ in regulating response sensivity, have proposed that the lifetime of $R^*$ might be calcium dependent (Torre et al., 1986; Kamura, 1993; Lagnado and Baylor, 1994).

Kinetic Sufficiency of Two First-order Inactivations in the First Three Cascade Steps

Our application of the model of the G-protein cascade (Figs. 10 and 11, and Table II) demonstrates that when Ca$^{2+}$ is maintained near its resting level, the recovery phase of the first three steps of the cascade can be adequately characterized by two first-order decay steps: a dominant decay step with time constant of 1.6–2.8 s, and a faster decay with a time constant of about 0.3–0.5 s. By incorporating these time constants into the successful activation model of Lamb and Pugh (1992), we have shown that these decay steps can be formally ascribed to the inactivation of the two enzymatic amplifiers, $R^*$ and PDE*. In previous investigations, it has been proposed that three different time constants might be needed to characterize the inactivation of the first three steps of the cascade (Tamura et al., 1991).

A strong caution needs to be voiced regarding the identity of the mechanisms underlying the two first-order inactivations in the model. In keeping with the hypothesis of Pepperberg et al. (1992), we identified the dominant time constant, $\tau_{\text{c}}$ with a hypothetical first-order inactivation of the enzymatic activity of $R^*$. Unfortunately, the ability of the model to provide a good account of the responses in clamped Ca$^{2+}$ (Figs. 10 and 11) provides no support for the identification. This follows, because over the intensity range of our experiments, Eq. 4 is linear in $\Phi$ and the two time constants, $\tau_{\text{R}}$ and $\tau_{\text{PDE}}$, are formally interchangeable, as is necessary in any cascaded linear system. Identification of the mechanism underlying $\tau_{\text{c}}$ will require kinetic and pharmacologic analysis of recoveries to flashes of higher intensities than used here (Pepperberg et al., 1992).

Estimate of the Magnitude of the Sensitivity Changes Caused by Decline in Ca$^{2+}$

Whereas the dominant time constant of recovery, $\tau_{\text{c}}$, remains largely unaffected by the decline in Ca$^{2+}$, that occurs during the response to saturating flashes in Ringer's solution, the overall time course of recovery clearly is sped up. Because of the parallelism of the recovery half-time semilog plots (Figs. 8 B, 9, and 11 B), $\exp(\Delta T/\tau_{\text{c}})$ provides a measure of the overall effect on apparent sensitivity of the biochemical mechanisms that respond to the decline in Ca$^{2+}$. The average overall effect was 14-fold, but in many cases it was 20-fold or more (Table II, last column). A 14- to 20-fold sensitivity shift is equivalent to all but the compressive gain con-
trol in salamander rod light adaptation (Matthews et al., 1988; Nakatani and Yau, 1988). For example, Matthews et al. (1988) found that steady lights that suppress 90% of the circulating current produce a 160-fold decline in dim-flash sensitivity, when sensitivity is defined in the absolute units of pA/\( \Phi \) for dim flashes. Since 10-fold of this overall sensitivity reduction is the result of the compressive effect of the 90% decrease in current, the residual factor of sensitivity change not accounted for is 16. This is essentially the same factor we found to decrease the effectiveness of saturating flashes in Ringer’s solution.

**Calcium Balance in Low-Na⁺ Solutions**

In previous investigations in which the maintenance of constant Ca²⁺ was effected by removing external Na⁺ and most Ca²⁺, guanidinium or Li⁺ was substituted for Na⁺ as the current carrier through the cGMP-activated conductance, and concentrations of Ca²⁺ of 1–3 μM were used to maintain Ca²⁺ near rest (Fain et al., 1989). Assuming that Ca²⁺ at rest is ~400 nM (see above), and that rods in guanidinium have \( V_{\text{rest}} \approx 30 \text{ mV} \), the Ca²⁺ required for circulating current stability in guanidinium is ~25- to 75-fold higher than the concentration predicted by the Nernst equation, were Ca²⁺ in equilibrium. In contrast to the previous investigations, our evidence shows Ca²⁺ to be stable in choline solutions containing no other permeant cations except Ca²⁺ estimated to be 2.3 nM (Fig. 6), a concentration ~1,000-fold lower than those of the earlier experiments. If \( V_{\text{rest}} \approx -67 \text{ mV} \) (\( \lambda_{G} = 12 \text{ pA} \); Table II; cf. Fig. 1 B), then the Ca²⁺ required for circulating current stability, 2.3 nM, is very near the predicted equilibrium concentration, 2.0 nM. These calculations suggest that there is considerable residual Ca²⁺ pumping in the dark steady state in the earlier guanidinium/lithium experiments, and little in the choline experiments presented here.

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