The cGMP-Phosphodiesterase and Its Contribution to Sensitivity Regulation in Retinal Rods

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ABSTRACT We have used the truncated outer segment preparation to measure rod cGMP-phosphodiesterase activity, as well as its modulation by Ca^{2+} , in darkness and in light. The basal enzyme activity in darkness was $\sim 0\text{--}3\text{ s}^{-1}$, and was largely independent of Ca^{2+} concentration from 10 nM to 10 μM . The steady state activity elicited by a step of light ($\lambda = 520\text{ nm}$) was strongly enhanced by Ca^{2+} , increasing from $\sim 0.005\text{ s}^{-1}/(h\nu\text{ }\mu\text{m}^{-2}\text{ s}^{-1})$ at 10 nM Ca^{2+} to $\sim 0.16\text{ s}^{-1}/h\nu\text{ }\mu\text{m}^{-2}\text{ s}^{-1}$ at 10 μM Ca^{2+} . Based on these measurements, as well as previous measurements on the effects of Ca^{2+} on rod guanylate cyclase and the cGMP-gated channel, we have calculated the step response-intensity relation for the rod cell in steady state. This relation agrees reasonably well with the relation directly measured from intact rods. We have also evaluated the relative contributions from the three Ca^{2+} effects to rod sensitivity. At low background light intensities, the Ca^{2+} modulation of the guanylate cyclase appears to be the most important for sensitivity regulation. At higher light intensities, especially above half-saturation of the response, the Ca^{2+} modulation of the light-stimulated phosphodiesterase shows a progressively important influence on the light response; it also extends the Weber-Fechner behavior of the cell to higher intensities. The contribution of the Ca^{2+} modulation of the cGMP-gated channel is slight throughout.

INTRODUCTION

The cGMP-phosphodiesterase in rod outer segments hydrolyzes guanosine 3':5'-cyclic monophosphate (cGMP) to 5'-GMP and has a central role in the phototransduction process. This enzyme is stimulated by light through a series of reactions, starting with the activation of rhodopsin by incoming photons. Photoexcited rhodopsin activates a G protein, transducin, which in turn stimulates the activity of the cGMP-phosphodiesterase, resulting in a decrease in the free cGMP concentration in the outer segment (for recent reviews, see Lagnado and Baylor, 1992; Detwiler and Gray-Keller, 1992; Pugh and Lamb, 1993; Koutalos and Yau, 1993; Yarfitz and Hurley, 1994; Yau, 1994). In the dark, cGMP binds to and opens cation chan-

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nels on the plasma membrane of the outer segment (see Yau and Baylor, 1989 for review). These open channels sustain an inward dark current, which partially depolarizes the cell. In the light, the decrease in the cGMP level closes the cation channels to produce a membrane hyperpolarization as the light response.

The phototransduction mechanism is modulated by a negative feedback mediated by Ca^{2+} , leading to light adaptation. This Ca^{2+} feedback acts through multiple targets, including guanylate cyclase, cGMP-phosphodiesterase, and the cGMP-gated channel (see Introduction in Koutalos, Nakatani, Tamura, and Yau, 1995*a*). We are interested in the question of how well these three Ca^{2+} feedback pathways can account for the adaptation of the rod cell to background light. Moreover, we want to evaluate the relative contribution from each pathway to the overall rod sensitivity to light. To address these questions, it is necessary to characterize the Ca^{2+} -mediated feedback pathways quantitatively, preferably under conditions close to the physiological situation. We have previously measured the Ca^{2+} modulation of the cGMP-gated channel (Nakatani, Koutalos, and Yau, 1995). In our companion work (Koutalos et al. 1995*a*), we described the modulation of guanylate cyclase. Here we report measurements of phosphodiesterase activity and its dependence on Ca^{2+} . The experiments used the truncated rod outer segment preparation (Yau and Nakatani, 1985*b*; Nakatani and Yau, 1988*b*), which allows recording of membrane current from the outer segment and at the same time manipulation of the internal ionic conditions. For measuring phosphodiesterase activity, cGMP was dialyzed into a truncated outer segment, and the rate of hydrolysis was monitored by the cGMP-gated channels on the plasma membrane. The same measurements could be repeated at different Ca^{2+} concentrations.

By combining the measurements of the different Ca^{2+} -mediated feedback pathways, we have calculated the step response–intensity relation in steady state for the rod cell. This predicted relation can be compared with direct measurements from intact rods. Finally, we have evaluated the contribution of each pathway to light adaptation by studying the effect of omitting the other pathways on the predicted response.

METHODS

Truncated Rod Outer Segment Experiments

For measurement of the phosphodiesterase activity, the truncated salamander rod outer segment was used. The methodology was as described previously (Nakatani and Yau, 1988*b*; Koutalos et al., 1995*a*). Two solution pairs were used: (*a*) The pipette contained a modified Ringer's solution with low Ca^{2+} (110 mM NaCl, 0.5 mM MgCl_2 (0.5 mM free Mg^{2+}), 2 mM bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 1.97 mM CaCl_2 (0.001 mM free Ca^{2+}), 5 mM tetramethylammonium hydroxide-4-(2-hydroxyethyl) 1-piperazineethanesulfonic acid (TMA-HEPES), and 5 mM glucose, pH 7.55) and the bath contained a choline chloride (ChCl) solution (110 mM ChCl, 0.5 mM free Mg^{2+} , 2 mM BAPTA, 5 mM TMA-HEPES, and 5 mM glucose, pH 7.55) with 0, 1, or 3 mM cGMP (Na^+ salt) and different concentrations of free Ca^{2+} . The absence of K^+ eliminated $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchange activity, which otherwise could affect internal Ca^{2+} concentration. When added, 3-isobutyl-1-methylxanthine (IBMX), adenosine 5'-triphosphate (ATP; Na^+ salt), and guanosine 5'-triphosphate (GTP; Na^+ salt) were at concentrations of 0.5, 0.2, and 0.1 mM, respectively. (*b*) The pipette contained a ChCl solution (110 mM ChCl, 0.5 mM MgCl_2 (0.5 mM free

Mg²⁺), 2 mM BAPTA, 1.97 mM CaCl₂ (0.001 mM free Ca²⁺), 5 mM TMA-HEPES, and 5 mM glucose, pH 7.55) and the bath a K⁺-gluconate solution (110 mM K⁺-gluconate, 0.5 mM free Mg²⁺, mM 2 BAPTA, 5 mM TMA-HEPES, and 5 mM glucose, pH 7.55) again containing 0, 1, or 3 mM cGMP and different concentrations of free Ca²⁺. When present, IBMX, ATP, and GTP were also at concentrations indicated above. Since there was no Na⁺ in the pipette and only small amounts of Na⁺ in the bath, the Na⁺/Ca²⁺, K⁺ exchange activity should be negligible in this case as well.

The total concentrations of MgCl₂ and CaCl₂ to be added in order to give the desired free concentrations of Mg²⁺ and Ca²⁺ were calculated according to the formulas presented in our companion article (Koutalos et al., 1995a).

Intact Rod Experiments

Two sets of experiments were carried out on intact rods. The first was to determine the relation between the inward current through the cGMP-gated channels and the Na⁺/Ca²⁺, K⁺ exchange activity. The second was to measure the step response-intensity relation and the dependence of step sensitivity on background light. In both cases, the outer segment of an intact rod was drawn into a suction pipette for recording membrane current, according to procedures described previously (Baylor, Lamb, and Yau, 1979; Lamb, McNaughton, and Yau, 1981). The bath and pipette both contained Ringer's solution. In some experiments, to remove the Ca²⁺ feedback, the inner segment of a rod was drawn into the Ringer's solution-containing pipette instead, and the bath solution was transiently switched to a 0 Na⁺/low Ca²⁺ Ringer's solution (110 mM guanidinium [guanidine hydrochloride], 2.5 mM KCl, 1.6 mM MgCl₂, and nominal 0–5 μM free Ca²⁺, pH 7.55) (Nakatani and Yau, 1988c).

All experiments were conducted at room temperature and, except for the measurement of exchange current, were performed on salamander rods. The exchange current measurements were made on toad (*Bufo marinus*) rods. The stimulating light had a wavelength of 500 nm for toad rods and 520 nm for tiger salamander rods, and was diffuse and unpolarized. In all of the figures, inward membrane current at the outer segment is plotted as negative current. Junction currents have not been subtracted.

THEORY

Symbols.

- I_s = intensity of light step in $h\nu \mu\text{m}^{-2} \text{s}^{-1}$
- G = cGMP concentration at intensity I_s
- C = Ca²⁺ concentration at intensity I_s
- G_D = cGMP concentration in darkness
- C_D = Ca²⁺ concentration in darkness
- J = cGMP-gated current at intensity I_s
- J_D = cGMP-gated current in darkness
- j = fractional cGMP-gated current at intensity I_s
- R_s = fractional step response at intensity I_s
- S_s = step sensitivity at background light intensity I_s
- n = Hill coefficient for the activation of the channels by cGMP
- $\alpha(C)$ = guanylate cyclase activity at Ca²⁺ concentration C
- K_g = half-point for Ca²⁺ inhibition of guanylate cyclase
- m = Hill coefficient for Ca²⁺ inhibition of guanylate cyclase
- β_D = basal phosphodiesterase activity in darkness
- $\beta^*(C)$ = light-stimulated phosphodiesterase activity (per unit light intensity) at Ca²⁺ concentration C

- K_p = effective half-point for Ca^{2+} stimulation of phosphodiesterase
 w = Hill coefficient for Ca^{2+} stimulation of phosphodiesterase
 β^*_{max} = effective maximum activity of light-stimulated phosphodiesterase per unit light intensity
 $\gamma(C)$ = multiplication factor accounting for the effect of a Ca^{2+} concentration C on the cGMP-gated channel
 γ_∞ = maximum fractional Ca^{2+} inhibition of the cGMP-gated channel
 K_c = half-point for the Ca^{2+} inhibition of the cGMP-gated channel
 ℓ = Hill coefficient for Ca^{2+} inhibition of the cGMP-gated channel

Calculating the Step Response and Sensitivity of the Intact Rod

The approach is similar to that previously described (Tamura, Nakatani, and Yau, 1991), except here we incorporate the multiple Ca^{2+} feedback pathways and ignore Ca^{2+} buffering by considering only the steady state situation.

(A) *In the presence of Ca^{2+} feedback.* The negative feedback mediated by Ca^{2+} on phototransduction has three targets: the guanylate cyclase, the light-stimulated phosphodiesterase activity, and the cGMP-gated channel.

The effect on the cyclase can be expressed as

$$\alpha(C) = \frac{\alpha(0)}{1 + \left(\frac{C}{K_g}\right)^m}, \quad (1)$$

where $\alpha(C)$ is the cyclase activity at Ca^{2+} concentration C , $K_g = 87 \text{ nM } \text{Ca}^{2+}$, $m = 2.1$, and $\alpha(0) = 13 \mu\text{M cGMP s}^{-1}$ (see Koutalos et al., 1995). These values were obtained in the presence of 2 mM GTP as substrate. Depending on experimental conditions, slightly different values have been obtained for $\alpha(0)$. This parameter, however, affects only the computed cGMP concentration in the dark, G_D , but not any of the calculations on light sensitivity described here.

The Ca^{2+} effect on the light-stimulated phosphodiesterase activity is described in the Results section and can be expressed as

$$\beta^*(C) = \frac{\beta^*_{\text{max}}}{1 + \left(\frac{K_p}{C}\right)^w}, \quad (2)$$

where $\beta^*(C)$ is the light-stimulated phosphodiesterase activity (per unit light intensity) at Ca^{2+} concentration C , $K_p = 400 \text{ nM } \text{Ca}^{2+}$, $w = 1.0$, and $\beta^*_{\text{max}} = 0.16 \text{ s}^{-1}/(h\nu \mu\text{m}^{-2} \text{ s}^{-1})$.

Finally, the effect of Ca^{2+} on the cGMP-gated channel can be expressed as

$$\gamma(C) = \gamma_\infty + \frac{1 - \gamma_\infty}{1 + \left(\frac{C}{K_c}\right)^\ell}, \quad (3)$$

where $\gamma(C)$ is a multiplication factor for the cGMP-gated current at Ca^{2+} concentration C , $K_c = 48 \text{ nM } \text{Ca}^{2+}$, $\ell = 1.6$, and $\gamma_\infty = 0.4$ is the corresponding factor at high Ca^{2+} concentrations (see Nakatani et al., 1995).

We want to obtain the relation in steady state between the fractional light-sensitive current in the intact rod and the light step intensity. Assuming constant GTP concentration and that the rod outer segment is a homogeneous, well-stirred compartment, we have at steady state

$$\frac{dG}{dt} = \alpha(C) - [\beta_D + \beta^*(C) I_s] G = 0 \quad (4)$$

$$\frac{dC}{dt} = (\text{Ca}^{2+} \text{ Influx}) - (\text{Ca}^{2+} \text{ Efflux}) = 0. \quad (5)$$

In Eq. 4, the total phosphodiesterase activity is expressed as the sum of the dark basal rate, β_D , and the light-stimulated rate; the latter is taken to be proportional to the light step intensity I_s (see Results and Discussion). The K_m of the $\text{Na}^+/\text{Ca}^{2+}, \text{K}^+$ exchanger for Ca^{2+} is 0.9–1.6 μM (Schnetkamp, 1991; Lagnado, Cervetto, and McNaughton, 1992; Gray-Keller and Detwiler, 1994), much higher than the intracellular free Ca^{2+} concentration in the rod outer segment (see Results and Discussion). At the same time, the fraction of cGMP-gated current carried by Ca^{2+} is not affected by light (see Fig. 6 C). Thus,

$$(\text{Ca}^{2+} \text{ Efflux}) \propto C \quad (6)$$

$$(\text{Ca}^{2+} \text{ Influx}) \propto J, \quad (7)$$

where J is the cGMP-gated current. Eq. 6 implies that the $\text{Na}^+/\text{Ca}^{2+}, \text{K}^+$ exchanger can in principle decrease free Ca^{2+} to near zero, which may not be strictly correct (see Discussion). Nonetheless, the linearity between the exchange current and the cGMP-gated current observed up to $\sim 70\%$ saturation of the light response (Fig. 6 C) suggests that Eq. 6 should be valid at least up to several hundred $\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$ (see Fig. 9 A), and possibly at higher intensities as well. From Eqs. 6 and 7, we have $C \propto J$, or

$$\frac{C}{C_D} = \frac{J}{J_D} = j \quad (8)$$

where C_D and J_D are the Ca^{2+} concentration and the cGMP-gated current in darkness, and j is the normalized cGMP-gated current. Thus,

$$C = jC_D \quad (9)$$

The physiological cGMP concentration range in the outer segment is considerably lower than the half-activation constant, $K_{1/2}$, for the channel (Nakatani and Yau, 1988b), so we can write $J \propto G^n$. Incorporating the Ca^{2+} modulation, we get

$$J \propto \gamma(C) G^n$$

or

$$j = \frac{J}{J_D} = \frac{\gamma(C)}{\gamma(C_D)} \left(\frac{G}{G_D} \right)^n. \quad (10)$$

Rearranging terms,

$$G = \left[j \frac{\gamma(C_D)}{\gamma(C)} \right]^{1/n} G_D. \quad (11)$$

From Eq. 4, we have

$$\alpha(C) = [\beta_D + \beta^*(C) I_s] G. \quad (12)$$

Thus, in the dark:

$$G_D = \frac{\alpha(C_D)}{\beta_D}, \quad (13)$$

where G_D is the cGMP concentration in darkness.

Substituting Eqs. 9, 11, and 13 into Eq. 12, we obtain

$$j = \left[\frac{\alpha(jC_D)}{\alpha(C_D)} \right]^n \left[1 + \frac{\beta^*(jC_D)}{\beta_D} I_s \right]^{-n} \left[\frac{\gamma(jC_D)}{\gamma(C_D)} \right]. \quad (14)$$

Given Eqs. 1–3 and $\beta_D = 0.3 \text{ s}^{-1}$ (see Results), j can be evaluated for different I_s values by solving Eq. 14 numerically. For calculations, we adopt $C_D = 200$ or 500 nM .

The total light-sensitive current is the sum of the cGMP-gated current and the $\text{Na}^+/\text{Ca}^{2+}, \text{K}^+$ exchange current. Since the latter is proportional to the former (Fig. 6 C), the fractional light-sensitive current is the same as the fractional cGMP-gated current, j . The normalized light response amplitude, R_s , is then given by

$$R_s = 1 - j \quad (15)$$

and the step sensitivity, S_s , by

$$S_s = \frac{dR_s}{dI_s} = -\frac{dj}{dI_s}. \quad (16)$$

(B) *In the absence of Ca^{2+} feedback.* In this case, $C (= C_D) = C_D$, and Eq. 14 reduces to

$$j = \left[1 + \frac{\beta^* (C_D)}{\beta_D} I_s \right]^{-n}. \quad (17)$$

The step response and sensitivity are then given by

$$R_s = 1 - \left[1 + \frac{\beta^* (C_D)}{\beta_D} I_s \right]^{-n} \quad (18)$$

and

$$S_s = n \frac{\beta^* (C_D)}{\beta_D} \left[1 + \frac{\beta^* (C_D)}{\beta_D} I_s \right]^{-(n+1)}. \quad (19)$$

An equation identical to Eq. 18 has also been derived by Forti, Menini, Rispoli, and Torre (1989), Matthews, Fain, Murphy, and Lamb (1990), and Tamura et al. (1991).

Evaluation of the Contribution of Each Ca^{2+} Feedback Pathway

To evaluate the contribution of a particular Ca^{2+} feedback pathway to rod sensitivity, we simply make the other targets invariant with respect to Ca^{2+} concentration.

Thus, with Ca^{2+} modulation of the guanylate cyclase only, Eq. 14 becomes

$$j = \left[\frac{\alpha (jC_D)}{\alpha (C_D)} \right]^n \left[1 + \frac{\beta^* (C_D)}{\beta_D} I_s \right]^{-n}. \quad (20)$$

With Ca^{2+} modulation of the phosphodiesterase only, we have

$$j = \left[1 + \frac{\beta^* (jC_D)}{\beta_D} I_s \right]^{-n}. \quad (21)$$

With Ca^{2+} modulation of the cGMP-gated channel only, we have

$$j = \left[1 + \frac{\beta^* (C_D)}{\beta_D} I_s \right]^{-n} \left[\frac{\gamma (jC_D)}{\gamma (C_D)} \right]. \quad (22)$$

Finally, for a rod with Ca^{2+} modulation of both guanylate cyclase and phosphodiesterase but not the cGMP-gated channel, we have

$$j = \left[\frac{\alpha (jC_D)}{\alpha (C_D)} \right]^n \left[1 + \frac{\beta^* (jC_D)}{\beta_D} I_s \right]^{-n}. \quad (23)$$

Eqs. 20–23 can be solved numerically as before to evaluate j as a function of I_s , from which R_s and S_s can be obtained from Eqs. 15 and 16.

Reciprocal Sensitivity

Another way to examine the relative contributions from different Ca^{2+} modulations to rod adaptation is by using increment threshold, T_s , defined as $1/S_s$, as the parameter of study. Consider an increase in the background light intensity from I_s to $I_s + dI_s$. The concentrations of cGMP and Ca^{2+} ,

as well as the fractional current, will change by dG , dC , and dj , respectively. In steady state, we have, from Eq. 12,

$$d\alpha(C) = [d\beta^*(C)] I_s G + \beta^*(C) [dI_s] G + [\beta_D + \beta^*(C) I_s] dG \quad (24)$$

or

$$\frac{d\alpha}{dC} dC - \left[\frac{d\beta^*}{dC} I_s G \right] dC - [\beta_D + \beta^*(C) I_s] dG = [\beta^*(C) G] dI_s. \quad (25)$$

The first and second terms on the left side of Eq. 25 represent the influence of the Ca^{2+} feedback on the cyclase and light-stimulated phosphodiesterase, respectively. The third term represents the hydrolysis of cGMP. Viewed in terms of the change in current, dj , this last term also contains the contribution from the Ca^{2+} modulation of the channels. From Eq. 11, we have

$$dG = \frac{1}{nj} \left[j \frac{\gamma(C_D)}{\gamma(C)} \right]^{1/n} G_D dj - \frac{1}{n\gamma(C)} \left[j \frac{\gamma(C_D)}{\gamma(C)} \right]^{1/n} G_D \frac{d\gamma}{dC} dC. \quad (26)$$

After substituting Eqs. 9, 11, and 26 into Eq. 25, and using $dC = C_D dj$ and $dR_s = -dj$, we obtain

$$dI_s = T_1 dR_s + T_2 dR_s + T_3 dR_s + T_4 dR_s, \quad (27)$$

where

$$T_1 = \frac{-\frac{d\alpha}{dj}}{\left[j \frac{\gamma(C_D)}{\gamma(jC_D)} \right]^{1/n} \beta^*(jC_D) G_D} \quad (28)$$

$$T_2 = \frac{\frac{d\beta^*}{dj} I_s}{\beta^*(jC_D)} \quad (29)$$

$$T_3 = -\frac{[\beta_D + \beta^*(jC_D) I_s] \frac{d\gamma}{dj}}{n\gamma(jC_D) \beta^*(jC_D)} \quad (30)$$

$$T_4 = \frac{\beta_D + \beta^*(jC_D) I_s}{nj\beta^*(jC_D)}. \quad (31)$$

The derivatives $d\alpha/dj$, $d\beta^*/dj$, and $d\gamma/dj$ can be obtained from Eqs. 1–3 using $C = jC_D$. The values of T_1 through T_4 are all positive after the substitutions. The physiological interpretation of Eq. 27 is as follows. An increase in intensity, dI_s , that produces an increase in response, dR_s , can be expressed as the sum of four terms. One term, $T_4 dR_s$, stands for the requirement of a decrement in the cGMP concentration, whereas the other three stand for the requirement of overcoming the Ca^{2+} feedback. Eq. 27 can be rewritten in terms of the reciprocal sensitivity, or threshold:

$$T_s = \frac{1}{S_s} = \frac{dI_s}{dR_s} = T_1 + T_2 + T_3 + T_4. \quad (32)$$

In Fig. 12, the threshold, T_s , is normalized to unity, and the fractional contributions of T_1 through T_4 are shown.

RESULTS

Phosphodiesterase Activity and Ca^{2+} Dependence

We first describe the light-activated phosphodiesterase activity and its dependence on Ca^{2+} . Fig. 1 A shows currents elicited by 3 mM cGMP at different Ca^{2+} concen-

trations from a truncated salamander rod outer segment. The arrow indicates the time of truncation. The pipette contained a ChCl solution, whereas a K^+ -gluconate solution containing 0.2 mM ATP and 0.1 mM GTP was used for intracellular dialysis (solution pair B in Methods). ATP was supplied to support normal rhodopsin kinase activity, which phosphorylates photoisomerized rhodopsin to trigger its deactivation; GTP, on the other hand, is required for the activation of the G protein transducin, which in turn activates phosphodiesterases. The K_m of rhodopsin kinase for ATP is 2 μ M (Palczewski, McDowell, and Hargrave, 1988), and the activation of transducin requires GTP with an apparent affinity between 0.1 and 20 μ M (Bennett and Dupont, 1985); thus, the ATP and GTP concentrations in our experiments were nonlimiting. The GTP would also serve as substrate for the guanylate cyclase in the rod outer segment, being converted into cGMP. However, at 0.1 mM

FIGURE 1. Measurement of light-stimulated phosphodiesterase activity as a function of Ca^{2+} concentration. (A) Membrane current recorded from a truncated salamander rod outer segment in the presence of a steady 520-nm background of $27.8 \text{ h}\nu \mu\text{m}^{-2} \text{ s}^{-1}$ in intensity. The pipette contained a ChCl solution; a K^+ -gluconate solution in the bath containing 0.2 mM ATP and 0.1 mM GTP was used for intracellular dialysis. The arrow indicates time of truncation. The current rise soon after the truncation was due to a junction current resulting from a bath solution change (see text). The break in the recording is to remove an artifact caused by valve switching. The reason for the relaxations on the plateaus of the cGMP-induced currents is unknown. Bandwidth DC-10 Hz. (B) Expanded records of the decays of the cGMP-elicited currents from A. The traces have been synchronized at the time of switching bath solution to 0 cGMP (time 0). Ca^{2+} concentrations were as follows: 1 μ M—first exposure (trace 1), 300 nM (trace 2), 100 nM (trace 3), 1 μ M—second exposure (trace 4), and 10 nM (trace 5).

GTP, the rate of cGMP production would only be $\sim 1 \mu\text{M cGMP s}^{-1}$ (Koutalos et al., 1995a), which is small compared with the rates of cGMP hydrolysis encountered in the present experiments (these rates are several micromolar cGMP s^{-1} or much higher, calculated from the product of the phosphodiesterase activity and the cGMP concentration during the measurement). A light step at 520 nm and $27.8 \text{ h}\nu \mu\text{m}^{-2} \text{ s}^{-1}$ in intensity was present throughout the experiment shown in Fig. 1. Owing to the high hydrolytic activity of the light-stimulated phosphodiesterase, the high concentration of cGMP, 3 mM, had to be used to generate a sufficiently large current for study. Junction currents have not been subtracted in the figure, and the outward current appearing $\sim 10 \text{ s}$ after truncation was due to a switch of the bath solution from ChCl, in which the truncation was carried out, to the K^+ dialysis solution. The decays of the cGMP-elicited currents upon removing bath cGMP are shown in Fig. 1 B, synchronized at the time of switching the bath solution to 0 cGMP (time 0). The rate of current decay increased with increasing Ca^{2+} concentration, indicating increased phosphodiesterase activity. In each trace, the latency for the current decline is not necessarily correlated with the decline rate; this is because the cGMP concentration inside the outer segment had not necessarily reached steady state before the bath cGMP was switched off.

The activity of the phosphodiesterase can be calculated from the current decay, as follows. The cGMP concentration inside the outer segment decays as a result of both diffusion out of the outer segment and hydrolysis by the phosphodiesterase. The rate of cGMP loss through hydrolysis is a first-order process with rate constant β , where $\beta = V_{\text{max}}/K_m$ is the hydrolytic activity of the phosphodiesterase. This holds when the enzyme operates in its linear range, valid for cGMP concentrations significantly below the enzyme K_m value of 70–95 μM (Wensel and Stryer, 1986; Dumke, Arshavsky, Calvert, Bownds, and Pugh, 1994). The rate of cGMP loss through diffusion is also a first-order process with rate constant r , where r is a parameter related to the cGMP diffusion coefficient (Koutalos et al., 1995a). The total rate of cGMP loss is therefore also a first-order process with rate constant $(\beta + r)$. Thus, the cGMP concentration should decay exponentially with rate $(\beta + r)$. At low cGMP concentrations, the current, $J(t)$, will also decay exponentially with rate constant $n(\beta + r)$, where n is the Hill coefficient for the activation of the cGMP-gated channels by cGMP; that is,

$$J(t) \propto e^{-n(\beta + r)t} \quad (33)$$

The rate r can be independently measured in darkness and the presence of IBMX, under which conditions $\beta = 0$. Adopting $n = 2$ (Koutalos et al., 1995a), β can then be calculated. A more rigorous derivation of Eq. 33 from diffusion theory is presented in the Appendix.

The decline time courses of the currents shown in Fig. 1 B, with the initial values normalized, are plotted in semilog scales in Fig. 2 A. The late decline of each current trace is exponential in time course, as expected from Eq. 33, with respective rates of 8.55, 4.11, 2.49, 6.80, and 0.77 s^{-1} at 1 μM , 300 nM, 100 nM, repeated 1 μM , and 10 nM Ca^{2+} . The error in the measurement of these rates was $\sim 0.1 \text{ s}^{-1}$, arising from the uncertainty in fitting the linear part of the semilog plot. The Ca^{2+} modulation of the cGMP-gated channels (Hsu and Molday, 1993; Gordon and Zim-

merman, 1994; Chen, Illing, Molday, Hsu, Yau, and Molday, 1994; Nakatani et al., 1995) should not interfere with the measurements; this is because the Hill coefficient, n , for the channel activation by cGMP is approximately the same at high and low Ca^{2+} (Nakatani et al., 1995), and the channel half-activation constant, $K_{1/2}$, while Ca^{2+} dependent, does not enter into Eq. 33. With the same outer segment, the experiment was subsequently repeated in darkness and with 0.5 mM IBMX

FIGURE 2. (A) Semilog plot of the current decays shown in Fig. 1 B (bandwidth DC-100 Hz). The currents have been normalized with respect to their initial values and synchronized at the time of switching the bath solution to 0 cGMP. The slope (dashed line) of the linear part of each trace gives the rate of the current decay, and is 8.55 s^{-1} at $1 \mu\text{M} \text{Ca}^{2+}$ —first exposure (trace 1), 4.11 s^{-1} at $300 \text{ nM} \text{Ca}^{2+}$ (trace 2), 2.49 s^{-1} at $100 \text{ nM} \text{Ca}^{2+}$ (trace 3), 6.80 s^{-1} at $1 \mu\text{M} \text{Ca}^{2+}$ —second exposure (trace 4), and 0.77 s^{-1} at $10 \text{ nM} \text{Ca}^{2+}$ (trace 5). (B) Measurement of the rate of cGMP loss through diffusion. Same outer segment as in A. This is essentially the same kind of experiment as in Fig. 1 and Fig. 2 A, but it was carried out in darkness and in the presence of 0.5 mM IBMX, which inhibited the phosphodiesterase activity. The current decline rate (nr) is 0.39 s^{-1} . (C) Calculated phosphodiesterase activity plotted as a function of free Ca^{2+} concentration. The value at $1 \mu\text{M} \text{Ca}^{2+}$ is the one obtained with the first exposure.

present, giving $nr = 0.39 \text{ s}^{-1}$ for the contribution from diffusion (Fig. 2 B). The phosphodiesterase activity calculated from these measurements is plotted as a function of Ca^{2+} concentration in Fig. 2 C. This activity includes the basal (i.e., light-independent) rate. Altogether, four experiments of this kind were carried out.

The dependence of the phosphodiesterase activity on Ca^{2+} was slowly lost in these experiments, especially after exposure to low Ca^{2+} concentrations. This phenomenon suggests the involvement of one or more soluble factors that gradually

became washed out, as also observed by Kawamura and Murakami (1991). In the experiment shown in Fig. 1 A, 80% of the original activity at 1 μM Ca^{2+} remained after exposure to 300 and 100 nM Ca^{2+} for ~ 2 min (from comparing the rates for the first and second exposure to 1 μM Ca^{2+}); after subsequent exposure to 10 nM Ca^{2+} for another minute, only 45% of the original activity at 1 μM Ca^{2+} remained. In general, exposures to Ca^{2+} concentrations < 300 nM during the course of an experiment (typically ~ 8 min) resulted in 20–70% loss of the initial hydrolytic activity at 1 μM Ca^{2+} . As a rule, we truncated an outer segment in a solution containing 1 μM Ca^{2+} and measured the hydrolytic activity in 1 or 10 μM Ca^{2+} within 1 min after truncation in order to obtain a relatively unperturbed baseline; exposure to these Ca^{2+} concentrations for 1 min resulted in $< 10\%$ activity loss. Since the major loss of mediating factors occurred during the 10 nM Ca^{2+} exposures, this Ca^{2+} concentration was usually applied last. The washout may have led to an underestimation of the enzyme activity at 10 nM Ca^{2+} by a factor of 2, but this does not significantly affect the subsequent calculations, because 10 nM approaches the lower limit of the physiological Ca^{2+} concentration in the rod outer segment, reached only at high light intensities (see below). Finally, rhodopsin kinase and arrestin, both soluble proteins, did not appear to wash out over the course of these experiments; otherwise, the light-stimulated phosphodiesterase activity would increase with time, which we have not observed.

In two other experiments on different cells, we measured the phosphodiesterase activity at a lower light intensity of $11.9 \text{ h}\nu \mu\text{m}^{-2} \text{ s}^{-1}$ and found that, at a given Ca^{2+} concentration, this activity was approximately half that elicited at $27.8 \text{ h}\nu \mu\text{m}^{-2} \text{ s}^{-1}$. Thus, the activity scaled approximately linearly with light intensity, at least over the intensity range we used. This linearity is expected from the results of Barkdoll, Pugh, and Sitaramayya (1989).

To extract the light-stimulated enzyme activity, the basal activity was measured in separate experiments ($\sim 0.3 \text{ s}^{-1}$, see below) and subtracted from the measurements in the light. Collected results on the light-stimulated phosphodiesterase activity and its Ca^{2+} dependence from all six experiments are shown in Fig. 3 (*filled triangles*). The points represent averages of individual measurements that have been normalized by the light intensity; the error bars indicate standard errors. The enzyme activity increases by ~ 30 -fold when the Ca^{2+} concentration increases from 10 nM to 10 μM . The solid curve is drawn according to Eq. 2 with $\beta_{\text{max}}^* = 0.16 \text{ s}^{-1}/(\text{h}\nu \mu\text{m}^{-2} \text{ s}^{-1})$, $K_p = 400 \text{ nM}$ Ca^{2+} , and $w = 1.0$. This is strictly an empirical fit to the data to be used for subsequent calculations and may not apply to Ca^{2+} concentrations $> 10 \mu\text{M}$, for which we have no measurements. Indeed, based on the trend of the data points in Fig. 3, the enzyme activity at such Ca^{2+} concentrations may well rise above the β_{max}^* value of $0.16 \text{ s}^{-1}/(\text{h}\nu \mu\text{m}^{-2} \text{ s}^{-1})$ we have adopted for the empirical curve. For the same reason, the Hill coefficient of unity in the curve fit is also empirical, and does not imply necessarily a single site of interaction with Ca^{2+} . However, these concerns do not affect our calculations because the free Ca^{2+} concentration in the rod outer segment never reaches such high values (see below). Estimates for the free Ca^{2+} concentration in the outer segment in darkness, C_0 , vary from 220 to 550 nM (Ratto, Payne, Owen, and Tsien, 1988; Korenbrot and Miller, 1989; Lagnado et al., 1992; Gray-Keller and Detwiler, 1994; McCarthy, Younger, and Owen, 1994).

Applying $C_D = 200$ or 500 nM Ca^{2+} to Eq. 2, we obtain $\beta^*(C_D) = 0.05$ or $0.09 \text{ s}^{-1} / (\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1})$, respectively. In Fig. 3 we have also included averaged results (*open circles*) from seven rods dialyzed with a ChCl intracellular solution (solution pair A in Methods). Five of these experiments were carried out at a light step intensity of $105.7 \text{ h}\nu \mu\text{m}^{-2} \text{s}^{-1}$ and two at $56.8 \text{ h}\nu \mu\text{m}^{-2} \text{s}^{-1}$. These experiments indicated hardly any effect of Ca^{2+} on the light-stimulated phosphodiesterase activity, which remained low throughout. Thus, the Ca^{2+} effect appears to be sensitive to ionic conditions, but we have not pursued this point further.

Fig. 4 A shows an experiment to measure the basal phosphodiesterase activity in darkness. In this experiment, the dialyzing K^+ -gluconate solution did not contain ATP and GTP. The current decays upon removing bath cGMP are shown expanded and synchronized in Fig. 4 B. The decay rate was 1.13 s^{-1} in $1 \mu\text{M}$ (first exposure, trace 1), 0.73 s^{-1} in 300 nM (trace 2), 0.77 s^{-1} in 100 nM (trace 3), and 1.07 s^{-1} in

FIGURE 3. Collected results on the Ca^{2+} dependence of the light-stimulated phosphodiesterase activity in salamander rods. Basal activity in darkness has been subtracted. The enzyme activity has been normalized by the light intensity. Experiments were carried out in a K^+ -gluconate (*filled triangles*; six cells) or ChCl (*open circles*; seven cells) intracellular solution. The solid curve has been drawn according to Eq. 2 with $\beta^*_{\text{max}} = 0.16 \text{ s}^{-1} / (\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1})$, $K_p = 400 \text{ nM } \text{Ca}^{2+}$, and $w = 1.0$.

$10 \text{ nM } \text{Ca}^{2+}$ (trace 4), respectively. In the same experiment, the decay rate in 0.5 mM IBMX (i.e., the nr value) was 0.47 s^{-1} (trace 5). Again, as in the experiment shown in Figs. 1 and 2, the latency for a given current decline was not always correlated with the decline rate, because the cGMP concentration in the outer segment had not necessarily reached steady state before the bath cGMP was switched off. The calculated basal phosphodiesterase activity is plotted against free Ca^{2+} concentration in Fig. 4 C, with the point at $1 \mu\text{M } \text{Ca}^{2+}$ being calculated from the average of three measured current decay rates (1.13 s^{-1} for the first exposure, 0.54 s^{-1} for the second, and 0.87 s^{-1} for the third). The plot suggests a slight dependence of the hydrolytic rate on the Ca^{2+} concentration, but this is not borne out by the averaged data from five rods, shown in Fig. 5 A (*open circles*). There was also no systematic time-dependent change in the basal enzyme activity observed in these experiments, suggesting that it was not affected by the washout of any soluble factor, at least over several minutes. To assess any possible contribution from spontaneous activations of rhodopsin and transducin to the basal activity in darkness, similar experiments

A

Ca²⁺ (nM) 1000 10 1000 300 100 1000

1 mM cGMP

Current (pA)

Time (sec)

0.5 mM IBMX

B

Current (pA)

Time (sec)

1 4 2 3 5

C

Basal PDE Activity (sec⁻¹)

[Ca²⁺] (nM)

FIGURE 4. Measurement of basal phosphodiesterase activity in darkness as a function of Ca^{2+} concentration. (A) Membrane current recording from a truncated salamander rod outer segment. The pipette contained a ChCl solution, and a K^{+} -gluconate solution was used for intracellular dialysis, without added nucleotide triphosphates. The arrow indicates time of truncation. Junction currents have not been subtracted. The break in the recording is to remove an artifact caused by valve switching. Bandwidth DC-20 Hz. (B) Expanded records of the decays of the cGMP-elicited currents from A. The traces have been synchronized at the time of switching the bath cGMP to 0 (time 0). Ca^{2+} concentrations were as follows: 1 μM (trace 1), 300 nM (trace 2), 100 nM (trace 3), and 10 nM (trace 4). Trace 5 was obtained in the presence of 0.5 mM IBMX to measure the rate of cGMP loss through diffusion. In each trace, the latency before the current begins to decline is not necessarily correlated with the decline rate, because each trace may not have started with the same initial cGMP concentration in the outer segment (see text). The cause of the drop in current in trace 1 immediately after switching the cGMP concentration in the bath to zero is unknown; nevertheless, this drop does not affect the measurement of phosphodiesterase activity. (C) Basal phosphodiesterase activity, calculated from the current decays shown in B, plotted as a function of Ca^{2+} concentration. The value at 1 μM Ca^{2+} was derived from the average of three measurements, all shown in A.

200–500 nM, would be $\sim 0.3 \text{ s}^{-1}$. At higher light intensities, with a Ca^{2+} decrease in the intact rod outer segment, the basal rate might decrease slightly according to Fig. 5 A, but it would in any case be negligible compared with the light-stimulated activity. Thus, for simplicity, we can take the basal phosphodiesterase activity, β_D , to be 0.3 s^{-1} and independent of Ca^{2+} . The guanylate cyclase activity in darkness, $\alpha(C_D)$, was calculated from Eq. 1 to be 1.92 and $0.32 \mu\text{M cGMP s}^{-1}$ at $C_D = 200$ and 500 nM Ca^{2+} , respectively. Thus, from Eq. 13, the resting cGMP concentration in darkness should be $G_D = \alpha(C_D)/\beta_D = 6.4$ or $1.1 \mu\text{M}$. These values are in broad agreement with the cGMP concentration estimated from the fraction of open

FIGURE 5. Collected results on Ca^{2+} dependence of basal phosphodiesterase activity from truncated salamander rod outer segments. Experimental conditions were as follows: (A) intracellular dialysis with K^+ -gluconate solution in the presence (*filled triangles*; four cells) and absence (*open circles*; five cells) of nucleotide triphosphates. (B) Intracellular dialysis with ChCl in the presence (*filled triangles*; five cells) and absence (*open circles*; three cells) of nucleotide triphosphates. Error bars indicate standard errors.

cGMP-gated channels in intact rods in darkness and the apparent affinity of the channels for cGMP (Nakatani and Yau, 1988*b*; see also Rispoli, Sather, and Detwiler, 1993). Finally, Fig. 5 B shows averaged results for the basal phosphodiesterase activity obtained with an intracellular ChCl solution, in either the absence (*open circles*; three rods) or the presence (*filled triangles*; five rods) of ATP and GTP. The values are not significantly different from those obtained with the K^+ -gluconate solution. The apparently higher activity in the absence of GTP and ATP may also reflect measurement uncertainty.

Ca²⁺ Influx as a Function of the cGMP-gated Current

To calculate the effect of the Ca²⁺ feedback on rod response and sensitivity, it is necessary to know whether the fraction of inward current carried by Ca²⁺ through the cGMP-gated channels changes in the light. If the ion selectivity of the channel does not change with illumination, then the Ca²⁺ influx should be proportional to the total current. To address this question, we used the Na⁺/Ca²⁺, K⁺ exchange current visible at the initial plateau of a cell's response to saturating light as a measure

FIGURE 6. Experiment to test any dependence of Ca²⁺ permeability of the cGMP-gated channels on background light. (A) Response to an intense test light, recorded from an intact toad rod, under two conditions: (a) with no background (trace 1), and (b) in the presence of a nonsaturating background (trace 2). The outer segment of the cell was drawn into the suction pipette for recording membrane current. Both pipette and bath solutions contained normal Ringer's solution. Both test and background lights were at 500 nm. The saturating test light, lasting 0.5 s, delivered 1,003 $\text{h}\nu \mu\text{m}^{-2}$. The background light had an intensity of 4.1 $\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$. Each trace is the average of 10 trials. Bandwidth DC-20 Hz. (B) Expanded records of the initial plateaus of the two test responses in A to show the Na⁺/Ca²⁺, K⁺ exchange current transients. Dashed curves are single-exponential fits, both with a time constant of 0.5 s. Extrapolations of the exponentials to the time at half-height of the responses (time 0) give an initial exchange current of 1.7 pA in trace 1 and 0.7 pA in trace 2. (C) Initial exchange current measured from test response plotted against steady state cGMP-gated current at background light intensities of 0 (dark), 0.5, 1.1, 1.9, 4.1, 12.8, and 27.4 $\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$. Collected results from eight toad rods, each indicated by a different symbol. For each cell, the exchange current and the steady state cGMP-gated current have both been normalized with respect to the cGMP-gated current in darkness. The slope of the linear regression fit is 0.086.

of the Ca^{2+} influx (Yau and Nakatani, 1984, 1985*a*; Nakatani and Yau, 1988*a*) and examined how this exchange current varied with light intensity. Fig. 6 shows such an experiment. The outer segment of an intact toad rod was drawn into the suction pipette for recording of membrane current, and a brief, saturating test light was delivered in either darkness (trace 1) or the presence of a nonsaturating steady light (trace 2). The time courses of the exchange current at the initial plateau of the response to the saturating test light under the two conditions are shown expanded in Fig. 6 *B*. Both of them can be broadly fitted with a single-exponential decline of 0.5 s time constant (Nakatani and Yau 1988*a*); the initial current dip in the traces is due to a transient capacitive current (Nakatani and Yau, 1988*a*). Because of the small magnitude of the exchange current, the fit with the single exponential is only approximate. Indeed, recent measurements by Gray-Keller and Detwiler (1994) have suggested a sum of two exponentials for the decay of the exchange current. Nevertheless, this point does not affect the final conclusion of this experiment (see below). Extrapolations of the exponentials to the time at half-height of the light responses gave initial exchange current amplitudes of 1.7 pA in the dark and 0.7 pA in the nonsaturating light. From Fig. 6 *A*, the total light-suppressible current was 19.9 pA in darkness and 8.6 pA in steady light. These currents represent the sum of the cGMP-gated and exchange currents. Thus, the cGMP-gated currents were 18.2 and 7.9 pA in the two cases, which are roughly proportional to the exchange currents. Collected results from eight experiments with multiple light intensities (see Fig. 6 legend) are shown in Fig. 6 *C*, in which the initial exchange current amplitude is plotted against the cGMP-gated current, both having been normalized against the cGMP-gated current in darkness. The graph shows that the exchange current is proportional to the cGMP-gated current regardless of light intensity, with a proportionality constant of 0.086. The initial amplitude of the exchange current represents the steady state exchange current just before the delivery of the saturating test light; from the exchange stoichiometry, the steady state Ca^{2+} influx is equal to twice this exchange current (Yau and Nakatani, 1984, 1985*a*). Thus, the Ca^{2+} influx comprised about $0.086 \times 2 = 0.17$ of the cGMP-gated current, which is in reasonable agreement with previous measurements (Yau and Nakatani, 1985*a*; Nakatani and Yau, 1988*a*). It should be pointed out that the general conclusion of the above experiment, namely, a proportionality between Ca^{2+} influx and the dark current, does not really depend on the single-exponential fits we have used. The only requisite is that the overall profile of the exchange current scales with the dark current, which from Fig. 6 *B* is approximately the case. Direct measurements of the Ca^{2+} concentration in the intact rod outer segment (Younger, McCarthy, and Owen, 1992; Gray-Keller and Detwiler, 1994) have also indicated an approximate proportionality between steady state Ca^{2+} concentration and cGMP-gated current during a light step, suggesting that the ion selectivity of the cGMP-gated channel does not change with illumination.

Step Response and Sensitivity of Rods

In this section, we compare the step response and sensitivity predicted from the Ca^{2+} feedback to direct measurements from intact rods. We first describe the ex-

perimental response–intensity relation and the associated step sensitivity as a function of background light. Similar experiments have been performed previously (Baylor, Matthews, and Yau, 1980; Matthews, Murphy, Fain, and Lamb, 1988; Nakatani and Yau, 1988c; Fain, Lamb, Matthews, and Murphy, 1989). Fig. 7 A shows the responses of an intact salamander rod to steps of different light intensities. In this experiment, the outer segment of the cell was in the pipette, and both the pipette and the bath contained Ringer's solution. The steady state response amplitudes, normalized with respect to the saturated value, are plotted in Fig. 7 B against light

FIGURE 7. Step response–intensity relation and dependence of sensitivity on background measured from two intact salamander rods in normal Ringer's solution. (A) Responses of a rod to steps of light at different intensities. Photocurrents have been normalized with respect to the 34-pA amplitude of the dark current. Outer segment was in the pipette. Both the pipette and the bath contained Ringer's solution. (B) Step response–intensity relation in steady state obtained from A. The amplitude of each response was measured just before the light step was turned off. (C) Responses elicited from a different rod with dim flashes superimposed on different background lights. The response amplitudes have been normalized with respect to the dark current (32 pA), and the DC level of each flash response represents the amplitude of the steady state response to the corresponding background light. The flash intensities were 1.3, 2.7, 5.4, 20, 79, and 1,173 $\text{h}\nu \mu\text{m}^{-2}$, and the corresponding background intensities were 0 (dark), 1.9, 7, 48, and 173 $\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$, respectively. (D) Step sensitivity, in units of $(\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1})^{-1}$, plotted as a function of background intensity, obtained from the records in C. The step sensitivity is calculated as the product of flash sensitivity and the integration time of the corresponding response. The smooth curve is a least-squares fit according to the Weber–Fechner law with $I_0 = 2.7 \text{ h}\nu \mu\text{m}^{-2} \text{s}^{-1}$.

intensities. Fig. 7 *C* shows an experiment from another salamander rod, in which incremental responses were elicited with dim flashes superimposed on different background lights. These flash responses have again been normalized with respect to the saturated light response, with the DC level of each trace representing the amplitude of the steady state response to the corresponding background light. In the present work, we focus only on step sensitivities, obtained by multiplying the flash sensitivity (peak amplitude of each flash response divided by the flash intensity) by the response integration time (Baylor and Hodgkin, 1973). The calculated step sensitivity is plotted as a function of background light intensity in Fig. 7 *D*. For this cell, the step sensitivity in darkness, S_s^D , was $0.078 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$, similar to what was measured previously (Nakatani and Yau, 1988c). The solid curve is a least-squares fit with the Weber–Fechner relation (Baylor et al., 1980):

$$S_s = S_s^D \cdot \frac{I_0}{I_s + I_0} \quad (34)$$

with $I_0 = 2.7 \text{ h}\nu \mu\text{m}^{-2} \text{ s}^{-1}$, in broad agreement with the value obtained by Matthews et al. (1988).

Similar experiments were carried out in the absence of Ca^{2+} feedback, obtained by preventing the Ca^{2+} concentration from falling during illumination. In these experiments, the inner segment of a rod was drawn into the suction pipette containing Ringer's solution, while the bath was transiently switched from Ringer's to a 0 Na^+ solution containing nominal 0 or $5 \mu\text{M}$ Ca^{2+} in order to temporarily clamp the intracellular Ca^{2+} concentration during illumination (Nakatani and Yau, 1988c). The results from representative experiments are shown in Fig. 8 (*A* and *B* from one rod and *C* and *D* from another); again, calculated incremental step sensitivities are plotted in Fig. 8 *D*. The curve fitted to the steady state response–intensity relation in Fig. 8 *B* is drawn according to Eq. 18, with $\beta^*(C_D)/\beta_D = 0.13 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$, which is close to the range of 0.17 – $0.3 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$ predicted from the measured $\beta^*(C_D)$ of 0.05 – $0.09 \text{ s}^{-1}/\text{h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$ for $C_D = 200$ – 500 nM Ca^{2+} and the measured β_D of 0.3 s^{-1} . The curve in Fig. 8 *D* is drawn according to Eq. 19 with $\beta^*(C_D)/\beta_D = 0.21 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$, again within the predicted range. The measured step sensitivity in darkness, $0.38 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$, was also close to $S_s^D = n \cdot \beta^*(C_D)\beta_D = 0.34$ – $0.6 \text{ (h}\nu \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$ predicted from the phosphodiesterase measurements.

Fig. 9 shows collected results from the above experiments and the comparison with predictions. Fig. 9 *A* shows the averaged steady state response–intensity relation in the presence (*filled triangles*; six rods) and absence (*open circles*; six rods) of the Ca^{2+} feedback. The error bars represent standard errors. Fig. 9 *B* shows the dependence of step sensitivity on background light intensity, with the same symbols designating the two situations. The smooth curves are drawn according to Eqs. 14, 15, and 18, in *A* and Eqs. 14–16 and 19 in *B*. The parameters used for generating the curves in the presence of Ca^{2+} feedback are given in the Theory subsection, quantifying the Ca^{2+} modulations of the guanylate cyclase, the cGMP–phosphodiesterase and the cGMP-gated channel, all having been measured experimentally. All of the solid curves in Fig. 9 were calculated with $C_D = 200 \text{ nM}$ Ca^{2+} . In the presence of Ca^{2+} feedback, the predicted half-saturation intensity is about twice the ex-

FIGURE 8. Step response-intensity relation and dependence of sensitivity on background measured from two intact salamander rods in the absence of Ca^{2+} feedback. (A) Responses of a rod to light steps of different intensities. Photocurrents have been normalized with respect to the instantaneous amplitude of the dark current, which varied between 16 and 26 pA during the course of the experiment. Inner segment was in pipette. The pipette contained Ringer's solution and the bath contained a 0 Na^+ /5 μM Ca^{2+} guanidinium solution. (B) Step response-intensity relation in steady state obtained from A. The amplitude of each response was measured as the average of the plateau region. The smooth curve is a least-squares fit according to Eq. 18, giving $\beta^*(C_D)/\beta_D = 0.13$ ($\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$)⁻¹. (C) Responses elicited from a different rod with dim flashes superimposed on different background lights. The response amplitudes have been normalized with respect to the instantaneous dark current (8–17 pA), and the DC level of each flash response represents the amplitude of the steady response to the corresponding background light. The flash intensities were 0.7, 1.3, 5.4, 5.4, and 11 $\text{h}\nu \mu\text{m}^{-2}$, and the corresponding background intensities were 0 (dark), 0.9, 1.9, 3.4, and 7 $\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$, respectively. (D) Step sensitivity, in units of ($\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$)⁻¹, plotted as a function of background intensity, obtained from the records in C. The step sensitivity is calculated as the product of flash sensitivity and the integration time of the corresponding response. The smooth curve is a least-squares fit according to Eq. 19, giving $\beta^*(C_D)/\beta_D = 0.21$ ($\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1}$)⁻¹.

perimental value (Fig. 9 A), a reasonable agreement considering the complexity of the control of phototransduction. Likewise, there is good agreement between prediction and measurement for the dependence of step sensitivity on background light intensity (Fig. 9 B). There is also good agreement between the predicted curves and the experimental points in the absence of the Ca^{2+} feedback for $C_D = 200$ nM Ca^{2+} . The dashed curves in Fig. 9 were calculated with $C_D = 500$ nM Ca^{2+} ,

and are not greatly different from the solid curves. In Fig. 9 A, in the absence of feedback, the slight leftward shift of the predicted response–intensity relation for $C_D = 500$ nM Ca^{2+} relative to that for $C_D = 200$ nM Ca^{2+} is due to the higher light-stimulated phosphodiesterase activity at 500 nM Ca^{2+} ; still, it is within a factor of two from the experimental data points. Thus, the Ca^{2+} feedback pathways, as measured, can account for the response behavior of the intact rod quite well.

The contribution of each Ca^{2+} feedback pathway can now be individually as-

FIGURE 9. Comparison between predicted and measured step responses and sensitivities for salamander rods in the presence and absence of Ca^{2+} feedback. (A) Averaged step response–intensity relation in steady state measured from intact rods in the absence (*open circles*; six rods) and presence (*filled triangles*; six rods) of Ca^{2+} feedback. Error bars indicate standard errors. The solid curves are calculated according to Eq. 18 (no feedback) and 14 and 15 (with feedback), respectively, with $C_D = 200$ nM Ca^{2+} . The dashed curves are corresponding calculations with $C_D = 500$ nM Ca^{2+} . (B) Averaged step sensitivity, in units of $(\text{hv } \mu\text{m}^{-2} \text{ s}^{-1})^{-1}$, as a function of background light intensity from intact rods in the absence (*open circles*; six rods) and presence (*filled triangles*; five rods) of Ca^{2+} feedback. Error bars indicate standard errors. The solid curves are calculated according to Eq. 19 (no feedback) and 14–16 (with

feedback), respectively, with $C_D = 200$ nM Ca^{2+} . The dashed curves are corresponding calculations with $C_D = 500$ nM Ca^{2+} .

essed by omitting the others in the calculations (see Theory subsection). Fig. 10 shows the contribution of the Ca^{2+} modulation of the cGMP-gated channel. Fig. 10, A and B, show calculations with a C_D value of 200 nM Ca^{2+} . In Fig. 10 A, the step response–intensity relation in steady state was calculated under several conditions: (a) without any Ca^{2+} modulation (trace 1, which is identical to the left solid curve in Fig. 9 A); (b) with Ca^{2+} modulation of the channel only (trace 2, calculated from Eq. 22); (c) with Ca^{2+} modulations of the guanylate cyclase and the phosphodi-

esterase (trace 3, calculated from Eq. 23); and (d) with all three Ca^{2+} modulations active (trace 4, which is identical to the right solid curve in Fig. 9 A). Corresponding plots for the dependence of incremental step sensitivity on background light intensity are shown in Fig. 10 B. From comparing traces 1 and 2, as well as traces 3 and 4, it is clear that the channel modulation has little effect on the light response and sensitivity in both the absence and presence of the modulations of the cyclase and the phosphodiesterase. On the other hand, the latter two modulations are pre-

FIGURE 10. Assessment of the contribution of the Ca^{2+} modulation of the cGMP-gated channel to light adaptation. (A) Predicted step response–intensity relations in steady state. $C_D = 200 \text{ nM Ca}^{2+}$ adopted. Curve 1, corresponding to no feedback at all, is identical to the left solid curve in Fig. 9. Curve 4, corresponding to the situation with all three feedback pathways active, is identical to the right solid curve in Fig. 9. Curve 2 shows the effect of the channel modulation alone. Curve 3 shows the combined effect of the cyclase and phosphodiesterase modulations, but no channel modulation. See Theory subsection for details. (B) Corresponding plots of the relation between step sensitivity, in units of $(\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1})^{-1}$, and background light intensity. (C and D) Same calculations as in A and B, but with $C_D = 500 \text{ nM Ca}^{2+}$.

dominantly responsible for light adaptation. Calculations with $C_D = 500 \text{ nM Ca}^{2+}$ (Fig. 10, C and D) led to the same conclusions.

The relative contributions of the Ca^{2+} modulations of the guanylate cyclase and the phosphodiesterase to rod response and sensitivity can be further dissected. Fig. 11 shows similar plots as in Fig. 10, calculated with $C_D = 200 \text{ nM Ca}^{2+}$ (A and B) and 500 nM Ca^{2+} (C and D). The conditions were (a) in the absence of any Ca^{2+} modulation (trace 1); (b) with Ca^{2+} modulation of the phosphodiesterase only

(trace 2, calculated from Eq. 21); (c) with Ca^{2+} modulation of the guanylate cyclase only (trace 3, calculated from Eq. 20); and (d) with both modulations active (trace 4, same as trace 3 in Fig. 10). A comparison between traces 1, 3, and 4 in Fig. 11, A or C, shows that the Ca^{2+} modulation of the cyclase is important in setting the absolute sensitivity of the rod at low light intensities, but contributes progressively less at higher light intensities. On the other hand, the Ca^{2+} modulation of the phosphodiesterase provides an increasingly significant contribution at higher intensities

FIGURE 11. Assessment of the relative contributions of Ca^{2+} modulations of guanylate cyclase and phosphodiesterase to light adaptation. (A) Predicted step intensity–response relations in steady state. $C_D = 200 \text{ nM } \text{Ca}^{2+}$ adopted. Curve 1, corresponding to no feedback, is identical to curve 1 in Fig. 10. Curve 2 shows the effect of phosphodiesterase modulation alone. Curve 3 shows the effect of cyclase modulation alone. Curve 4 shows the combined effect of the two modulations, and is identical to curve 3 in Fig. 10. (B) Corresponding plots of the relation between step sensitivity, in units of $(\text{h}\nu \mu\text{m}^{-2} \text{s}^{-1})^{-1}$, and background light intensity. (C and D) Same calculations as in A and B, but with $C_D = 500 \text{ nM } \text{Ca}^{2+}$.

and extends the operating range of the rod. Similar information is provided in Fig. 11, B and D, showing that the phosphodiesterase modulation brings the dependence of step sensitivity on background light intensity closer to the Weber–Fechner relation (Eq. 34), as well as extends this behavior to higher intensities before response saturation is reached. The contribution of the phosphodiesterase modulation is more important for $C_D = 200 \text{ nM}$ than 500 nM .

Another way of evaluating the different Ca^{2+} modulations is to calculate their

fractional contributions to threshold, defined as the reciprocal of step sensitivity. The threshold is the light intensity required to produce a criterion response. To produce the criterion response, the incremental light has to reduce the concentration of cGMP and to overcome the effect of the three Ca^{2+} feedback pathways. Eq. 32 gives the threshold expressed as a linear combination of four terms that represent these different constituents. In Fig. 12, the fractional contribution of each term to the reciprocal sensitivity calculated from Eqs. 28–31 is shown as a function of the background light intensity. Again, both $C_D = 200 \text{ nM Ca}^{2+}$ (A) and $C_D = 500$

FIGURE 12. Plot of reciprocal sensitivity to show the relative contributions by the three feedback pathways to increment threshold. See Theory subsection for details. $C_D = 200 \text{ nM Ca}^{2+}$ in A and 500 nM in B.

nM Ca^{2+} (B) were used for calculations, but there is no qualitative difference in results between the two situations. The cGMP term contributes a fairly constant amount, 20–30%, over the entire intensity range. The rest comes from the Ca^{2+} feedback. The modulation of the channel contributes <10% of the reciprocal sensitivity value throughout, in accord with the result shown in Fig. 10. The Ca^{2+} feedback via the guanylate cyclase constitutes most of the threshold at low-intensity backgrounds, whereas feedback via the phosphodiesterase becomes more important, and eventually takes over, at higher light intensities, again consistent with the results shown in Fig. 11.

DISCUSSION

Using the truncated rod outer segment, we have measured the cGMP-phosphodiesterase activity and its Ca^{2+} dependence under conditions that are near physiological. The basal enzyme activity does not show any physiologically significant Ca^{2+} dependence. Its value of 0.3 s^{-1} we found is in approximate agreement with the $0.5\text{--}1.0 \text{ s}^{-1}$ measured in the intact salamander rod (Hodgkin and Nunn, 1988; Cornwall and Fain, 1994), and below the upper limit of $1\text{--}2 \text{ s}^{-1}$ measured biochemically (Arshavsky, Dumke, and Bownds, 1992; Dumke et al., 1994). The light-stimulated cGMP-phosphodiesterase activity, on the other hand, varies by ~ 30 -fold when Ca^{2+} changes between 10 nM and $10 \text{ }\mu\text{M}$. The Ca^{2+} concentration in the outer segment in darkness is $220\text{--}550 \text{ nM}$ (Ratto et al., 1988; Korenbrot and Miller, 1989; Lagnado et al., 1992; Gray-Keller and Detwiler, 1994; McCarthy et al., 1994), and in bright light decreases to 50 nM (Gray-Keller and Detwiler, 1994) or much lower (Cervetto, Lagnado, Perry, Robinson, and McNaughton, 1989; McCarthy et al., 1994). Over this Ca^{2+} range, the enzyme activity varies by ≥ 10 -fold, a modulation large enough for a significant feedback control on phototransduction. At $10 \text{ }\mu\text{M}$ Ca^{2+} , the absolute enzymatic activity per unit light intensity is $0.16 \text{ s}^{-1}/(\text{h}\nu \text{ }\mu\text{m}^{-2} \text{ s}^{-1})$. This rate is four times lower than the value of $\sim 0.6 \text{ s}^{-1}/(\text{h}\nu \text{ }\mu\text{m}^{-2} \text{ s}^{-1})$ inferred from measurements on intact salamander rods using weak background lights (Hodgkin and Nunn, 1988; see also Cornwall and Fain, 1994). Since the free Ca^{2+} concentrations associated with the latter measurements are unknown, however, a strict comparison with ours cannot be made. Nonetheless, the light-stimulated phosphodiesterase activity we obtained here with the truncated rod outer segment preparation can predict quite well the observed behavior of intact rods in the simple situation of no feedback (see Fig. 9, *A* and *B*, *open circles*, and Eqs. 18 and 19). The total phosphodiesterase activity in the outer segment that can be stimulated by light is $\sim 1,000 \text{ s}^{-1}$ in bullfrog (Dumke et al., 1994). At the same time, the light-stimulated phosphodiesterase activity in bright light according to our data should decrease to $\sim 0.01 \text{ s}^{-1}/(\text{h}\nu \text{ }\mu\text{m}^{-2} \text{ s}^{-1})$ or lower because of the Ca^{2+} decline (see Fig. 3). From these numbers, it follows that the light-stimulated phosphodiesterase activity should remain far below saturation for light intensities at least up to $10^4 \text{ h}\nu \text{ }\mu\text{m}^{-2} \text{ s}^{-1}$, which is the highest intensity used in most of our calculations. Thus, our assumption of a linear increase of enzyme activity with light intensity seems justified. This is also in agreement with the results of Barkdoll et al. (1989).

The possibility of a Ca^{2+} modulation of the phosphodiesterase was suggested many years ago by Kawamura and Bownds (1981) and later by Torre, Matthews, and Lamb (1986). More recently, this modulation was demonstrated directly in truncated bullfrog rod outer segments by Kawamura and Murakami (1991), though no quantitative measurements were made in their work. Kawamura and Murakami (1991) also found that this modulation requires a soluble factor, which they called S-modulin. This protein was subsequently found to be a homologue of recoverin, a Ca^{2+} -binding protein purified and cloned from bovine retina (Dizhoor, Ray, Kumar, Niemi, Spencer, Brolley, Walsh, Philipov, Hurley, and Stryer, 1991; Kawamura, Takamatsu, and Kitamura, 1992; Kawamura, Hisatomi, Kayada, Tokunaga, and Kuo, 1993). S-modulin/recoverin acts by inhibiting the phosphory-

lation of photoisomerized rhodopsin in a Ca^{2+} -dependent manner (Kawamura, 1993; Kawamura et al., 1993; Chen and Hurley, 1994; see also Gray-Keller, Polans, Palczewski, and Detwiler 1993). Phosphorylation of the active pigment leads to its deactivation and shut-off of phototransduction (Liebman and Pugh, 1980; Wilden, Hall, and Kühn, 1986; Palczewski, Rispoli, and Detwiler, 1992; Chen, Makino, Peachey, Baylor, and Simon, 1995). Thus, high Ca^{2+} enhances light-stimulated phosphodiesterase activity by permitting a longer lifetime for activated rhodopsin, whereas low Ca^{2+} , reached during illumination, decreases phosphodiesterase activity by shortening the pigment's active lifetime. From biochemical experiments by Kawamura (1993), we infer the S-modulin/recoverin-dependent phosphodiesterase activity to increase by only twofold when raising Ca^{2+} from 10 nM to 10 μM , compared with the 30-fold increase found by us over the same Ca^{2+} range. One explanation for this discrepancy is that protein concentrations are diluted in the biochemical preparation, a condition that might result in the diminution of low-affinity interactions. Another possibility is that there are several Ca^{2+} targets in the phosphodiesterase activation pathway, only one of which involves S-modulin/recoverin. For example, Lagnado and Baylor (1994) have recently shown a recoverin-independent effect of Ca^{2+} on the rising phase of the flash response in truncated salamander rod outer segments. On the other hand, since basal phosphodiesterase activity is independent of Ca^{2+} concentration, the enzyme itself is unlikely to be another Ca^{2+} target.

Our calculations suggest that the combined Ca^{2+} modulations of the guanylate cyclase, the phosphodiesterase, and the cGMP-gated channel that we have measured (see also Koutalos et al., 1995a; Nakatani et al., 1995) can explain the step response and sensitivity behavior of the intact rod quite well. The contribution from the channel modulation is small throughout the intensity range, a finding expected considering that the maximal change in the channel affinity for cGMP due to Ca^{2+} is less than a factor of two. In contrast, the feedback pathways via the guanylate cyclase and the cGMP phosphodiesterase are both important for sensitivity regulation. Broadly speaking, these two feedbacks operate at different light ranges. At low light intensities, the cyclase modulation by Ca^{2+} is predominant in setting the sensitivity of the cell, reducing its step sensitivity in darkness by four- to fivefold compared with the situation of no feedback. On the scale of threshold, this feedback contributes >70% of the total. At higher light intensities, especially above the half-saturation point, the phosphodiesterase modulation by Ca^{2+} becomes progressively important, eventually contributing >60% of the threshold. This growing influence of phosphodiesterase modulation at brighter light levels is due to the fact that total Ca^{2+} -sensitive phosphodiesterase activity increases with light intensity, whereas the total cyclase activity regulated by Ca^{2+} remains unchanged. The plots in Fig. 11 and Fig. 12 show that the relative contributions of these two feedback pathways depend to some degree on the free Ca^{2+} concentration in darkness. The measurements of this parameter have so far given values of 220–550 nM. Although this range is fairly narrow, it nonetheless affects the predictions because the IC_{50} for the inhibition of the cyclase is ~ 90 nM Ca^{2+} (Koutalos et al., 1995a). When Ca^{2+} drops below the IC_{50} , the effect of the relief of the cyclase from inhibition rapidly decreases (see also Tamura et al., 1991). If the free Ca^{2+} concentration in the rod

outer segment in darkness is 200 nM, then it will already decrease below the IC_{50} of the cyclase at light intensities slightly higher than half saturating. In contrast, if free Ca^{2+} in darkness is at 500 nM, then the step response will have to reach 80% saturation before the IC_{50} is reached. In general, the higher the C_D/IC_{50} ratio, the more effective is the Ca^{2+} feedback via the cyclase. The plots in Fig. 11 and Fig. 12 essentially reflect this point. The Ca^{2+} modulation of the phosphodiesterase can be viewed as a way to ensure Weber–Fechner behavior of the rod over a broad intensity range, regardless of the value of the C_D/IC_{50} ratio. Previously, Tamura et al. (1991) have shown that, in primate rods, the Ca^{2+} feedback on the guanylate cyclase alone can predict physiological light adaptation quite well. These earlier calculations, however, adopted a steep dependence of the enzyme on Ca^{2+} concentration (with a Hill coefficient of 4; see Koch and Stryer, 1988) and a much larger dynamic range for the enzyme modulation (~ 80 -fold change in enzyme activity between Ca^{2+} concentrations of 300 nM and very low values), both of which are now deemed inappropriate in light of the new measurements (Koutalos et al., 1995a; Gorczyca, Gray-Keller, Detwiler, and Palczewski, 1994). At the same time, Tamura et al. (1991) assumed that the free Ca^{2+} concentration in the rod outer segment did not decrease below 100 nM even in very bright light (Ratto et al., 1988), which is also unlikely on the basis of the more recent Ca^{2+} measurements (see above).

All of the calculations described here are for diffuse, step illumination in the steady state, so that Ca^{2+} buffering in the outer segment need not be considered. The implicit assumption is that the kinetics of Ca^{2+} buffering are sufficiently fast that at the time instants when the response amplitudes were measured (typically 20 s after light onset), the Ca^{2+} concentration had already reached steady state. Since the step responses except for those at near-saturating intensities have leveled off by this time (see Fig. 7 A), this is a reasonable assumption, supported also by the recent work of Gray-Keller and Detwiler (1994) on gecko rods. Another advantage of considering a diffuse, continuous light step is that photon averaging over space and time allows the outer segment to be approximated as a spatially homogeneous compartment over a wide range of light intensities. The integration time of the single-photon response in salamander rods is ~ 1 s, and the effective collecting area of the outer segment for unpolarized light is $\sim 15 \mu m^2$ (Nakatani and Yau, 1988c). Thus, at intensities as low as $2 h\nu \mu m^{-2} s^{-1}$, there is the equivalent of ~ 30 photoisomerizations distributed over the rod outer segment at any time instant during the light step. This average level of excitation covers essentially the entire outer segment, because a single photoisomerization spreads over a minimum of 3% of the length of the outer segment (Lamb et al., 1981). At still lower light intensities, such as down to 1 or 2 photoisomerizations occurring over the entire outer segment at a given time instant, the assumption of spatial homogeneity can be an issue. Nonetheless, our predictions may still provide reasonable approximations in this limiting case, because calculations by Lamb and Pugh (1992) have indicated that the single-photon response may not be as spatially restricted as previously thought (Lamb et al., 1981).

The good agreement between the predictions and the experimental measurements indicates that the Ca^{2+} feedback pathways considered here are probably the

predominant mechanisms for sensitivity regulation in rods, at least over the range of light intensities we have examined. Recent experiments by Matthews (1995) on intact rods have suggested that the Ca^{2+} feedback underlies the changes in the kinetics of the light response during light adaptation as well. Several other processes have been proposed to affect phototransduction. These processes include a phospholipase C- IP_3 pathway (Ghalayini and Anderson, 1984), the phosphorylation of photoactivated and nonphotoactivated rhodopsin by protein kinase C (Newton and Williams, 1991, 1993), a cGMP modulation of the phosphodiesterase through binding to noncatalytic sites on the enzyme (Arshavsky and Bownds, 1992; Cote, Bownds, and Arshavsky, 1994), and the phosphorylation/dephosphorylation of the cGMP-gated channel (Gordon, Brautigan, and Zimmerman, 1992). At this point, the extent of the contribution of each of these processes to light adaptation is unclear. One possibility is that one or more of these mechanisms are involved in rod adaptation at much higher, such as bleaching, light levels.

APPENDIX

We provide here a more rigorous derivation of Eq. 33 from diffusion theory. Upon switching the bath cGMP concentration to 0, the concentration of cGMP inside the truncated rod outer segment, $G(x, t)$, at distance x and time t , is given by (Koutalos, Nakatani, and Yau, 1995b):

$$G(x, t) = e^{-\beta t} \sum_{i=0}^{\infty} \left\{ e^{-(2i+1)^2 r t} \cos \frac{(2i+1)\pi x}{2L} \left[\int_0^L f(z) \cos \frac{(2i+1)\pi z}{2L} dz \right] \right\}, \quad (\text{A1})$$

where the closed end of the outer segment is at $x = 0$ and the open end at $x = L$, D is the cGMP diffusion coefficient in the rod outer segment, $f(x) = G(x, 0)$ is the initial cGMP distribution, and

$$r = \frac{\pi^2 D}{4 L^2}. \quad (\text{A2})$$

The infinite sum in Eq. A1 converges rapidly, with the first term providing a good approximation for t longer than 1–2 s, which holds for the measurements. Thus,

$$G(x, t) \approx e^{-(\beta + r)t} \left\{ \cos \frac{\pi x}{2L} \left[\int_0^L f(z) \cos \frac{\pi z}{2L} dz \right] \right\}. \quad (\text{A3})$$

For low cGMP concentrations, that is, $G(x, t) \ll K_{1/2}$,

$$J(t) \propto \int_0^{L-s} [G(x, t)]^n dx, \quad (\text{A4})$$

where s is the effective seal position between the outer segment and the pipette, and $L - s$ is the length of the outer segment contributing current. Eq. 33 follows after substituting Eq. A3 into Eq. A4.

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