

Calcium Dependence of the Activation and Inactivation Kinetics of the Light-activated Phosphodiesterase of Retinal Rods

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ABSTRACT The Ca^{2+} dependence of the kinetics and light sensitivity of light-activated phosphodiesterase was studied with a pH assay in toad and bovine rod disk membranes (RDM), and in a reconstituted system containing GTP-binding protein, phosphodiesterase and rhodopsin kinase. Three statistics, peak hydrolytic velocity, turnoff time, and time to peak velocity, were measured. ATP decreased phosphodiesterase light sensitivity nearly 10-fold and accelerated the dim-flash kinetics of cGMP hydrolysis when compared to those with GTP alone. Ca^{2+} reversed all of the effects of ATP, Ca^{2+} increased peak velocity, turnoff time, and time to peak velocity, to the values obtained with GTP alone. The Ca^{2+} dependence of peak velocity and turnoff time can be characterized as hyperbolic saturation functions with a $K_{0.5}$ for Ca^{2+} of 1.0–1.5 mM in toad RDM. In bovine RDM the Ca^{2+} dependence of peak velocity and turnoff time has a $K_{0.5}$ of 0.1 mM Ca^{2+} . The Ca^{2+} dependence in the reconstituted system is similar to that in bovine RDM for peak velocity ($K_{0.5} = 0.1$ mM Ca^{2+}) but differs for turnoff time ($K_{0.5} = 2.5$ mM Ca^{2+}). We tested the hypothesis that a soluble modulator, normally required to confer submicromolar Ca^{2+} sensitivity, was too dilute in our assay by comparing data obtained at one RDM concentration with those obtained at 10-fold higher RDM, and therefore a constituent protein, concentration. We observe no difference and present a formal analysis of these data that excludes the hypothesis that the soluble modulator binds its target protein with $K_d < 5 \mu\text{M}$. The lack of submicromolar Ca^{2+} dependence of any of the steps in the cGMP cascade that underlie cGMP phosphodiesterase activation and inactivation *in vitro* argues against Ca^{2+} regulation of these steps having a significant role in the light adaptation of the intact rod.

INTRODUCTION

Despite signal advances in the understanding of the biochemical steps leading to the closure of the rod light-sensitive conductance, changes in the magnitude and light

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sensitivity of the rod photocurrent produced by altered intracellular calcium (Cobbs and Pugh, 1985; Matthews et al., 1985; Lamb et al., 1986; Torre et al., 1986) remain unexplained in the current framework of the cGMP hypothesis of phototransduction. Nevertheless, recent experiments suggest that Ca^{2+} plays an important role as a diffusible messenger in a feedback loop that regulates rod light sensitivity (Yau and Nakatani, 1985; Torre et al., 1986).

In the fully dark-adapted rod, steady-state Ca^{2+} activity is 0.5–1.0 μM (Lamb et al., 1986; McNaughton et al., 1986) and is maintained primarily by a balance between Ca^{2+} influx through the light-sensitive conductance (Yau and Nakatani, 1984*a, b*) and Ca^{2+} efflux via Na/Ca exchange (Yau and Nakatani, 1985). Upon suppression of the dark current by light, $[\text{Ca}^{2+}]_i$ decreases (Yau and Nakatani, 1985; McNaughton et al., 1986) as Na/Ca exchange continues to extrude cytoplasmic Ca^{2+} . After an intense flash produces a rapid and prolonged dark current suppression, $[\text{Ca}^{2+}]_i$ decreases to a very low steady state activity with a time constant of ~ 0.5 s (Yau and Nakatani, 1985; McNaughton et al., 1986). The similar time scales of the calcium decrease and the linear, dim-flash photocurrent (Baylor et al., 1979; Lamb et al., 1981) indicate that these light-induced Ca^{2+} changes are rapid enough to affect the kinetics and/or light sensitivity of the normal rod photocurrent. If such a relationship exists, manipulations that alter intracellular $[\text{Ca}^{2+}]$ are expected to produce corresponding changes in the rod light response.

Rods infused with the calcium buffer BAPTA (1,2-bis (*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid) have increased light sensitivity and greatly slowed photocurrents with respect to normal rods (Matthews et al., 1985; Lamb et al., 1986; Torre et al., 1986). To account for these observations Torre et al. (1986) presented a theory in which the normal dynamic changes in free Ca^{2+} determine the state of light adaptation and the kinetics of the physiological light response by controlling one or more recovery steps. According to this theory, the retardation of the light-induced calcium decrease by BAPTA-buffered calcium increased the light sensitivity and prolonged the photocurrents of the BAPTA-infused rods. In principle, the BAPTA-induced hypersensitivity could be mediated via cGMP synthesis by guanylate cyclase and/or cGMP hydrolysis by light-activated phosphodiesterase (PDE); either activation of guanylate cyclase and/or inactivation of PDE might be delayed.

In addition to having submicromolar calcium dependence, an adaptation mechanism must satisfy formidable requirements: in the presence of background lights adaptation mechanisms decrease rod light sensitivity and extend the rod's response range nearly three orders of magnitude (Kleinschmidt and Dowling, 1975). If the fraction of the light-sensitive conductance open is determined by a binding relation between free cGMP and the closed conductance, adaptation mechanisms must, therefore, be capable of regulating light-activated cGMP metabolism over a large range. This requirement, in conjunction with the calcium hypothesis of light adaptation outlined above, requires that Ca^{2+} , as the adaptational messenger, be able to shift the light sensitivity of the cGMP cascade equivalently.

In systems characterized by considerable amplification, an efficient locus for gain control is any stage before the amplification, a strategy not unfamiliar in biology (Gavin et al., 1974; Stock and Koshland, 1981; Bertics and Gill, 1985). It is thus reasonable to hypothesize that the ATP-dependent quench of light-activated, rod

PDE (Liebman and Pugh, 1979, 1980; Kawamura and Bownds, 1981), thought to be mediated by phosphorylation of photoisomerized rhodopsin by rhodopsin (Rh) kinase (Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983; Sitaramayya, 1986) and/or binding of 48-kD protein (Zuckerman et al., 1985; Wilden et al., 1986), may be a locus of variable gain control.

In light of the physiological consequences of BAPTA infusions upon the rod photocurrent kinetics and light sensitivity, and the proposed role for calcium ions as determining the rod light sensitivity (Torre et al., 1986), the present experiments investigate the calcium dependence of the ATP-dependent quench of light-activated PDE in native toad and bovine rod disk membranes (RDM). These experiments generate predictions for the Ca²⁺ dependence of the quench effected by purified proteins of the cGMP cascade. We test these predictions for Rh kinase in a reconstituted system containing rhodopsin, G protein (GTP-binding protein), PDE, and Rh kinase.

METHODS

Preparation of Toad RDMs

The methods for RDM preparation are essentially those of Barkdoll et al. (1988) and will only be briefly summarized here. Under infrared illumination the retinas dissected from four to six dark-adapted toad eyes (*Bufo marinus*) were placed receptor side up in a plastic Petrie dish containing 1.5–2.0 ml of MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer (100 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol [DTT], 100 μM EDTA, and 10 or 20 mM MOPS, pH 8.0). The receptor surface of each piece was gently brushed with a fine artist's brush to remove the rod outer segments (ROS). Of the ROS isolated by this procedure, fluorescence microscopy of the ROS in a 100 μM DDC (*N,N'*-didansyl-L-cysteine) solution (Yoshikami et al., 1974) indicated that ~50% had intact plasma membranes.

Further purification of the ROS was performed with a modification of Nagao et al.'s method (1987). The ROS obtained by the brushing procedure outlined above were layered on top of a discontinuous gradient of 65, 50, 45, and 30% Percoll (wt/wt) in MOPS buffer and centrifuged in a refrigerated (0°C) centrifuge (Beckman Instruments, Inc., Palo Alto, CA) for 25 min at 3,000 rpm (SW 27.1 rotor). ROS formed two bands; one (band I) between 50 and 65% Percoll and the other (band II) between 45 and 50%. Occasionally a minor band was also detected at the 30%/45% interface, however, the amount of material in this third band was insignificant. More than 99% of the ROS sampled from band I had intact plasma membranes as judged by their inability to incorporate DDC. Band I ROS were resuspended in MOPS buffer and centrifuged for 30 min at 24,000 rpm. The ROS formed a very loose layer on top of a denser pellet of the residual Percoll and were resuspended in 1.5–2.0 ml of MOPS buffer. All pipetting and transfer of the ROS during the centrifugation procedures were performed under infrared illumination.

ROS obtained by either brushing alone or brushing and centrifugation were permeabilized in total darkness by syringing the buffer containing the ROS 10 times through 18, 20, and 22 gauge needles, in that order. For the remainder of the experiment the resulting RDM were kept on ice in a light-tight container that could be opened briefly in the dark to obtain aliquots.

Preparation of Bovine RDM and Reconstituted Membranes

Dark bovine RDM, RDM stripped of peripheral proteins, and Rh kinase were prepared according to Sitaramayya (1986). PDE and G-protein were prepared according to Baehr et al.

(1979, 1982) and were reconstituted with dark, stripped RDM in their native proportions: rhodopsin:G protein:PDE in the reconstituted membranes was 100:7:1.5 (Sitaramayya et al., 1986). Rh kinase was added to the reconstituted membranes in amounts that produced kinase activity equivalent to that of normal RDM (Sitaramayya, 1986).

PDE Assay

The catalyzed hydrolysis of GMP by light-activated PDE was assayed with a pH assay (Barkdoll et al., 1988) based on the methods of Liebman and Evanczuk (1982), which measures the acidification of the reaction medium as protons are stoichiometrically released upon cGMP hydrolysis at pH 8.0. In brief, RDM were added under infrared illumination to a thermostatted (24°C) reaction cuvette containing MOPS buffer (pH 8.0), 10 mM cGMP and 1 mM GTP bringing the final volume including reagents and buffer to 100 μ l. Unless otherwise stated the final rhodopsin concentration in the cuvette was 4 μ M. Calibrations of the recording device were performed during each experiment by injecting known aliquots of strong acid into the cuvette and measuring the pH excursion. Changes in pH produced by cGMP hydrolysis were measured with a Lazar pH electrode (PHM-146) referenced to an Ag/AgCl junction via an agar/KCl bridge. The time constant of the electrode was <0.3 s. Experiments were controlled by a LSI-11/23 computer, which triggered the flash (Vivitar 3700) that initiated the reactions, and displayed the data in real time and stored the traces after low-pass filtering at 100 Hz.

Bleach Calibration

The fraction of the rhodopsin bleached per flash was determined from a series of pre- and postflash bleach measurements under conditions identical to those used during experiments. 100 μ l of 4 μ M rhodopsin in MOPS buffer with 1 mM GTP and 20 mM NH_2OH at pH 8.0 were exposed in the reaction cuvette to a series of flashes attenuated by neutral density filters.

We define those flashes that produce the same hydrolytic velocity when scaled by the fraction rhodopsin isomerized as linear or "dim" flashes. Accordingly, the responses produced by these flashes are referred to as linear- or dim-flash responses.

Analysis of cGMP Hydrolysis Curves

With 10 mM cGMP, a concentration much greater than the K_m of toad (0.6 mM cGMP; Barkdoll et al., 1988) bovine (1.0 mM cGMP; Sitaramayya et al., 1986) rod light-activated PDE, present during all experiments, PDE remains saturated and the change in hydrolytic velocity after dim-flash activation is produced by a decrease in the total number of active PDE molecules. That is, assuming Michaelis-Menten kinetics are obeyed:

$$V(t) = [\text{PDE}^*](t)k_{\text{cat}}[\text{cGMP}]/([\text{cGMP}] + K_m), \quad (1)$$

and that for $[\text{cGMP}] \gg K_m$, $V(t) = [\text{PDE}^*](t)k_{\text{cat}}$, where $[\text{PDE}^*](t)$ is the time-dependent total molar amount of active PDE and k_{cat} is the PDE turnover number with the units s^{-1} . For the small acidifications (i.e., <0.1 pH unit) produced by dim-flash PDE activation the reaction progress curve is the integral of the product, i.e., proton, formation and can be represented by:

$$k_{\text{cat}} \int_0^t [\text{PDE}^*](\tau) d\tau \quad (2)$$

for $[\text{cGMP}] \gg K_m$, which upon differentiation yields the enzyme velocity curve, $k_{\text{cat}} [\text{PDE}^*](t)$. Thus, differentiation of the reaction progress curve yields a function proportional to the

time-dependent active enzyme concentration. The proportionality constant is the PDE turnover number, $k_{cat} = 500\text{--}2,000\text{ s}^{-1}$ (Miki et al., 1975; Baehr et al., 1979).

Dim-flash-activated PDE exhibits distinctive kinetics, shown in the inset to Fig. 1, with a measurable delay to maximum activity followed by a relatively slow inactivation. Three statistics of the hydrolysis curves measured in this study are t_{peak} , the time to reach maximum hydrolytic velocity, $V_p = V(t_{peak})$, the maximum velocity in molar s^{-1} and τ_{off} , the turnoff time in seconds. The turnoff or integration time is related to the duration of the reaction and is defined to be $\tau_{off} = \Delta[cGMP]_{t \rightarrow \infty} / V_0$, where $\Delta[cGMP]_{t \rightarrow \infty}$ is the amount of cGMP hydrolyzed at the end of the reaction. For a purely exponential decay process the turnoff time is equal to the decay time constant.

The time-dependent amplification, $g(t)$, of PDEs activated per R^* (the catalytic, photo-isomerized form of rhodopsin) can be estimated from (a) $[Rh](M)$, the total rhodopsin concentration in the experiment, (b) $V(t)$ (M cGMP s^{-1} ; i.e., $k_{cat}[PDE^*](t)$), the hydrolytic velocity of a dim-flash response, (c) F_1 , the fraction of rhodopsin isomerized by the dim flash, and (d) k_{cat} (s^{-1}), the PDE* turnover number. Thus, $g(t) = V(t)/k_{cat}(F_1[Rh])$ and has the units (mol PDE*)/(mol R^*), attaining its maximum value at $t = t_{peak}$. Throughout the paper the value of $g(t_{peak})$ determined from the linear dim-flash response will be referred to by the term absolute sensitivity. Note that a particular $g(t)$, e.g., $g(t_{peak})$, provides an instantaneous estimate of PDE*/ R^* and does not by itself indicate the rate with which the PDE*s were activated.

A second measure of sensitivity is F_{e-1} , the fraction rhodopsin isomerized that produces $0.63 V_{max}$, is referred to as the relative sensitivity. Together, F_{e-1} and $g(t_{peak})$ determine the position of the function relating light-activated PDE velocity and the fraction of rhodopsin isomerized.

Rhodopsin Measurements: Enzyme Activity Units

Unbleached RDM were diluted in a 1–2% Ammonyx Lo (vol/vol) solution. The absorbance of this suspension at 500 nm was measured before and after bleaching, and the rhodopsin concentration was determined from the difference between these two values using the extinction coefficient $40,000\text{ cm}^2\text{ mmol}^{-1}$. We report PDE activity in units of (M substrate hydrolyzed)/(M rhodopsin)/s. These units may be converted to units of (M substrate hydrolyzed)/(M enzyme)/s by multiplying by the proportionality constant 50–100, since there is one PDE for every 50–100 rhodopsins (Baehr et al., 1979).

Free Ca^{2+} Determination

During Ca^{2+} experiments free Ca^{2+} was buffered to the desired concentrations by 5 mM BAPTA. Because of significant Ca^{2+} and Mg^{2+} buffering by the millimolar concentrations of nucleotide triphosphates, determination of free Ca^{2+} required the simultaneous solution of six binding equations for BAPTA, ATP, GTP, and the two metal ions, Mg^{2+} and Ca^{2+} . These equations were solved by an iterative algorithm using the following logarithmic stability constants at pH 8.0: Ca-BAPTA = 6.96 and Mg-BAPTA = 1.77 (Tsien, 1980), and Ca-ATP = 3.77, Ca-GTP = 3.58, Mg-ATP = 4.04, and Mg-GTP = 4.02 (Wallas, 1958; cited in Bartfai, 1979). Below $\sim 10\text{ }\mu\text{M}$ free Ca^{2+} , BAPTA was the principle determinant of $[Ca^{2+}]$, (the presence of ATP and GTP had minimal effects) as expected on the basis of the much lower Ca^{2+} -nucleotide stability constants.

The solutions used in these experiments have a finite calcium contamination which in the absence of BAPTA, nucleotides, or added $CaCl_2$ was below the limit reliably detected by a standard Ca^{2+} electrode (Ionetics STAT calcium electrode) i.e., $\sim 10\text{ }\mu\text{M}$. Assuming that Ca^{2+} contamination is $10\text{ }\mu\text{M}$, addition of 5 mM BAPTA will reduce free Ca^{2+} to below 1.0 nM. The 0 Ca^{2+} condition in this paper is defined to have 5 mM BAPTA and no added $CaCl_2$ and is therefore expected to have $< 1.0\text{ nM}$ free Ca^{2+} .

Materials

Cyclic GMP, ATP, GTP, and BAPTA were obtained from Sigma Chemical Co., St. Louis, MO). Toads (*Bufo marinus*) were obtained from West Jersey Biological, Wenonah, NJ.

RESULTS

Characteristics of the ATP-dependent Quench of PDE

The dependence of PDE V_p upon the fraction rhodopsin isomerized is shown in Fig. 1 for the two conditions: 1 mM GTP alone (filled circles) and 1 mM GTP with 2 mM ATP (open circles). The most notable difference between the two sets of data is the

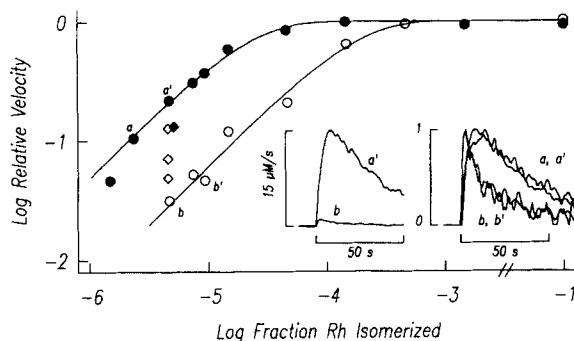


FIGURE 1. Log relative peak velocity vs. log fraction rhodopsin isomerized with 1 mM GTP alone (solid circles) or 1 mM GTP and 2 mM ATP (open circles). Velocities have been normalized to the saturated velocity ($F_1 = 1.0$) with GTP alone. Each point is the average of data from two or more experiments, excepting the leftmost solid circle (one

experiment). The velocities represented by the diamonds at $\log(F_1) = -5.3$ were obtained in the presence of 2 mM ATP, 1 mM GTP, and increasing $[Ca^{2+}]$. From the lowest diamond to the uppermost free $[Ca^{2+}]$ was 0.08 mM, 0.4 mM, 3.13 mM, and 4.44 mM Ca^{2+} and 9.5 mM free $MgCl_2$. The solid diamond has been offset for clarity. The left-hand inset shows dim-flash activated velocity traces obtained by numerical differentiation of cGMP hydrolysis records in the presence of 1 mM GTP (a'), and 1 mM GTP and 2 mM ATP (b). The flash intensity is the same for the two responses. The right-hand inset shows normalized PDE velocity traces obtained in the presence of 1 mM GTP (a, a'), and 1 mM GTP and 2 mM ATP (b, b'). The corresponding flashes differed twofold in intensity for each condition, and are indicated by the positions of the letters adjacent to the curves. The response to the dimmer flash of each pair has been scaled up twofold corresponding to the difference in flash intensity. The a and b traces have been normalized requiring a factor of 13 to scale the height of the GTP/ATP response to the GTP alone response at the same flash intensity (see left-hand inset).

1.0 log unit shift of the curve induced by ATP. The smooth curves drawn through the data are Poisson saturation functions that have been translated laterally to fit the two sets of data and are defined by the equation $\log(V_p/V_{max}) = \log[1 - \exp(-F_1/F_{e-1})]$. The saturated hydrolytic velocity produced by very bright flashes ($F_1 > 10^{-4}$) with 1 mM GTP, exceeds 10 M cGMP (M rhodopsin s) $^{-1}$ (12.4 ± 4.6 M [M s] $^{-1}$, SD, $n = 9$ experiments). In the absence of ATP, F_{e-1} , the fraction of rhodopsin isomerized that produces $0.63 V_{max}$, is $10^{-4.7}$. As the fraction isomerized decreases below F_{e-1} , peak velocity becomes proportional to the number of isomerized rhodopsins, seen in Fig. 1 as the portion of the left-most curve with unity slope.

PDE activated by these dim flashes manifests distinctive activation and inactivation characteristics: in contrast to the bright-flash responses these dim-flash

responses inactivate within ~ 1 min. This inactivation reflects exhaustion of neither GTP nor cGMP, but rather the slow decay of R^* , as reactivation is observed upon subsequent flash stimulation (Liebman and Pugh, 1980). The time course of PDE activation and inactivation is shown in the left-hand inset to Fig. 1. The traces were obtained by numerically differentiating cGMP hydrolysis curves. The velocity curve marked "a" was obtained with 1 mM GTP alone and returns nearly to zero within 100 s. In the linear portion of the velocity saturation function, the complete time course of PDE activation and inactivation is the same, that is velocities $V(t)$, and therefore amplification $g(t)$, are invariant in this region when normalized by the fraction rhodopsin isomerized. The right-hand inset to Fig. 1 shows this property of linearity, the upper velocity traces marked a and a' are responses to flashes that differ by a factor of two in intensity; the response to the dimmer flash has been scaled by this difference and superimposes on the brighter flash response. The flash intensities at which these responses were obtained are indicated by the corresponding letters on the left-hand curve of Fig. 1. The dim-flash PDE velocity with 1 mM GTP typically accelerates slowly, peaking in ~ 10 – 15 s (13.0 ± 3.9 s, \pm SD, $n = 6$ responses) and decays with a turnoff time of 40 – 60 s (49.2 ± 7.5 s, \pm SD, $n = 5$). The maximum velocity per R^* is $5 \pm 30 \times 10^5$ (\pm SD) cGMP $s^{-1} R^{*-1}$ for five velocity vs. bleach experiments. Thus, if the PDE turnover number is 500 – $2,000 s^{-1}$ (Miki et al., 1975; Baehr et al., 1979), the absolute sensitivity of PDE activation in the presence of GTP alone is ~ 250 – $1,000$ PDE*/ R^* by $t_{\text{peak}} = 13$ s.

PDE light sensitivity with 2 mM ATP and 1 mM GTP, shown by the open circles in Fig. 1, decreases nearly 10-fold relative to that with 1 mM GTP alone, though the maximum light-activated velocity (14.8 ± 3.1 M [M s] $^{-1}$, SD, $n = 3$ experiments) is indistinguishable from that with GTP alone (15.9 ± 3.6 M [M s] $^{-1}$, SD, same experiments). In the presence of 2 mM ATP, F_{e-1} is $10^{-3.8}$. Underlying the shift in relative light sensitivity in the presence of ATP are markedly accelerated dim-flash PDE kinetics shown in the inset of Fig. 1. In the left-hand inset, the presence of ATP (b) decreases V_p more than 10-fold compared with GTP alone (a'). The right-hand inset shows normalized ATP/GTP and GTP alone responses. The velocity traces marked b and b' were obtained with 1 mM ATP and should be compared with the GTP alone traces a and a' . The velocity traces with ATP were produced by flashes differing by twofold in intensity (indicated by the corresponding letters on the right-hand curve of Fig. 1), and the dimmer flash response has been scaled by this factor. In addition, the ATP/GTP responses have been scaled to the height of the GTP alone responses, which facilitates comparison of the kinetics under the different conditions. A factor of 13 was required to match the peak velocity produced by the same bleach, $F = 10^{-5.3}$. The time scale of the response is considerably shortened by ATP such that $t_{\text{peak}} = 2$ – 6 s (4.5 ± 1.3 s, SD, $n = 26$ responses) and the velocity decays with $\tau_{\text{off}} = 12$ – 20 s (15.7 ± 4.5 s, SD, $n = 26$ responses). In the presence of ATP the hydrolytic velocity per R^* is $6.5 \pm 1.5 \times 10^4$ (SD, $n = 5$ experiments) cGMP $s^{-1} R^{*-1}$, which corresponds to an absolute sensitivity of 30 – 130 PDE*/ R^* at the peak velocity, depending upon the value of PDE turnover number assumed.

In summary, ATP decreases the velocity, and therefore the gain PDE* per R^* decreases the total [cGMP] hydrolyzed and accelerates the kinetics of dim-flash PDE inactivation, when compared with the kinetics with GTP alone.

The following experiments examine the effects of Ca^{2+} on the dim flash, linear PDE kinetics.

Ca^{2+} Inhibits the ATP-dependent Quench in Toad RDM

Each of the effects of ATP upon light-activated PDE hydrolysis are reversed by Ca^{2+} . The decrease in light sensitivity produced by ATP is reversed by Ca^{2+} in a concentration-dependent manner as indicated by the diamonds in Fig. 1. Varying free Ca^{2+} between 80 μM and 4.4 mM at a constant fraction of rhodopsin isomerized, $10^{-5.3}$, increases the PDE velocity to that observed in the absence of ATP. The filled diamond indicates a measurement with 4.4 mM Ca^{2+} and 10 mM additional MgCl_2 and will be further discussed below. Upon increasing the flash intensity, the GTP alone and ATP/GTP velocities approach one another and the effects of Ca^{2+} diminish until at velocity saturation no dependence upon Ca^{2+} is seen. That is, V_{max} is the same for the three conditions GTP alone, GTP/ATP, and GTP/ATP with 5 mM Ca^{2+} . Without ATP the peak velocity of the dim-flash response is unaffected by Ca^{2+} (data not shown).

Complete cGMP hydrolysis curves obtained at different free Ca^{2+} concentrations are shown in Fig. 2 A. The kinetic consequences of increasing Ca^{2+} upon cGMP hydrolysis in the presence of ATP are threefold: (a) an increase in the total amount of cGMP hydrolyzed, (b) an increase in initial velocity, and (c) a general slowing of the inactivation of light-activated hydrolysis. The expanded traces of the inset reveal that Ca^{2+} increases the hydrolytic velocity as early as the responses are measurable. These early kinetic differences are maintained at 10-fold higher membrane concentrations: hydrolysis in the presence of 1 mM GTP/2 mM ATP/4 mM Ca^{2+} or 1 mM GTP alone is distinct from that with 1 mM GTP/2 mM ATP/0 Ca^{2+} throughout the dim-flash response (data not shown).

The calcium-dependent increase in initial velocity is more readily apparent from the PDE velocity curves in Fig. 2 B, obtained by numerical differentiation of the hydrolysis curves in Fig. 2 A. The time to peak velocity of the lowest trace with 1 mM GTP/0.5 mM ATP/0 Ca^{2+} is 3 s and increases to 11–13 s with 1 mM GTP alone or 1 mM GTP/0.5 mM ATP/4.7 mM Ca^{2+} (uppermost two traces).

Fig. 3 quantifies the effects of Ca^{2+} and PDE dim-flash activation and inactivation kinetics. To describe the ability of Ca^{2+} to increase peak velocity V_p from the fully quenched velocity V_Q observed with ATP, GTP, and 0 Ca^{2+} , to the velocity with GTP alone, V_G , we define a normalized velocity $(V_p - V_Q)/(V_G - V_Q)$. The average GTP alone and fully quenched velocities are therefore forced to be equal to 1.0 and 0.0, respectively, and are indicated by the open circles above *GTP Alone* and < -9 . The most notable feature of the graph is the absence of any significant effect of calcium on the peak velocity below ~ 50 – $100 \mu\text{M}$ free Ca^{2+} . Varying free calcium between 10^{-8} and 10^{-5} M is without effect and the PDE velocities in this range are virtually identical to the velocity in 0 Ca^{2+} . Above 10 μM , free Ca^{2+} velocity increases and approaches the GTP alone velocity near 5 mM free Ca^{2+} . The smooth curve drawn through the data is a hyperbolic saturation function having a $K_{0.5}$ for Ca^{2+} of 1.0 mM.

The effect of Ca^{2+} on the turnoff times of dim-flash-activated PDE, shown in Fig. 3 B, is similar to this effect upon the peak velocity. Between 10^{-8} and 10^{-5} free Ca^{2+}

the turnoff times are indistinguishable from the zero Ca^{2+} response. At very high Ca^{2+} (i.e., free $Ca^{2+} > 4$ mM) the turnoff time actually exceeds the observed with 1 mM GTP alone indicated by the o above *GTP Alone*. Insight into this phenomenon can be gained by considering the τ_{off} of light-activated PDE with 0.1 mM GTP indicated by the x above *GTP Alone*. Liebman and Pugh (1980) reported that GTP could partially substitute for ATP in quenching light-activated PDE, although the $K_{0.5}$ for the GTP effect was 1.4 mM, which is nearly 1,000-fold higher than that for ATP. Therefore, 1 mM GTP, the concentration used in this study, accelerates the turnoff

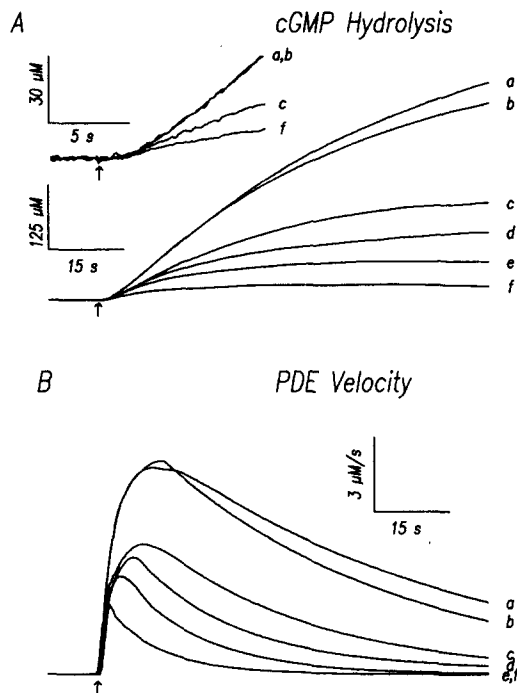


FIGURE 2. Ca^{2+} inhibits the ATP-dependent inactivation of PDE. (A) cGMP hydrolysis by dim-flash-activated PDE is increased by Ca^{2+} in the presence of 0.5 mM ATP and 1 mM GTP. The reaction was initiated impulsively at the arrow by a flash that bleached 5.0×10^{-6} fraction of the rhodopsin. Traces a (1 mM GTP alone) and f (1 mM GTP, 0.5 mM ATP and 0 Ca^{2+}) are the limits between which Ca^{2+} exerts its effect. The Ca^{2+} concentrations corresponding to the remaining traces are: a, 4.7 mM; c, 0.79 mM; d, 0.15 mM; e, 0.02 mM. This notation is the same for the inset and B. (Inset) cGMP hydrolysis is shown in the upper set of traces on an expanded time base. The rate of cGMP hydrolysis with 1 mM GTP alone or 1 mM GTP, 0.5 mM ATP, and 4.7 or 0.79 mM Ca^{2+} is greater than with 1 mM GTP, 0.5 mM ATP, and 0 Ca^{2+} within 2.0 s

after the flash. (B) Increasing Ca^{2+} increases t_{peak} , V_p , and τ_{off} . PDE velocity curves were obtained by numerical differentiation of the curves in A. The decay phase of each response is the exponential best fitting the raw derivative traces starting from a point on each curve after V_p from where the velocity decays with apparent first-order kinetics. The letters to the right of the traces are as in A.

of light-activated PDE, whereas the turnoff induced by 0.1 mM GTP is insignificant. In light of this observation, the Michaelis-Menten saturation curve drawn through the data is constrained to pass through the 0.1 mM GTP point. The curve has a $K_{0.5}$ for Ca^{2+} of 1.0 mM.

Another measure of the speed of the dim-flash activation/inactivation sequence is t_{peak} , the time required to achieve V_p . As can be seen qualitatively in Fig. 2 B, Ca^{2+} increases t_{peak} from the ATP/GTP/0 Ca^{2+} response to that of the GTP alone response. Although the Ca^{2+} dependence of t_{peak} appears similar to that of V_p and

τ_{off} , the variability of the t_{peak} data preclude estimation of the $K_{0.5}$ for Ca^{2+} . T_{peak} with 4.5 mM free Ca^{2+} is 10.1 ± 2.4 s (SD, $n = 2$).

Ca^{2+} Inhibits the ATP-dependent Quench with High Membrane Concentration

To address the possibility that a soluble modulator that may be too dilute in our reaction cuvette is required for submicromolar regulation of the ATP-dependent quench, we increased the membrane, and concomitantly any soluble protein, con-

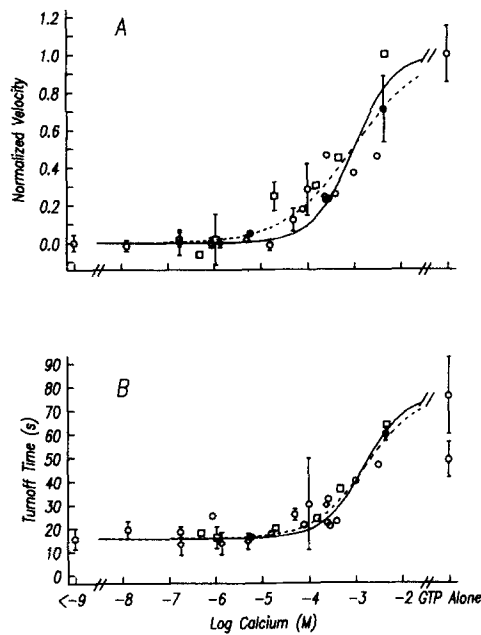


FIGURE 3. (A) Ca^{2+} increases the dim-flash-activated peak velocity in the presence of ATP in toad RDM. Normalized velocity measured with 2.0 mM ATP (\circ , \circ) or 0.5 mM ATP (\square) with 0 Ca^{2+} is increased by Ca^{2+} to the GTP alone velocity. The curves have the form: $V = [\text{Ca}^{2+}]^N / ([\text{Ca}^{2+}]^N + K_{0.5}^N)$, where V is the normalized velocity, N is the Hill coefficient, and $K_{0.5}$ is the $[\text{Ca}^{2+}]$ that produces 50% inhibition. The dashed curve has been fitted by the method of least squares with $K_{0.5} = 1.0$ mM Ca^{2+} and $N = 0.6$. The continuous curve has $K_{0.5} = 1.0$ mM Ca^{2+} and $N = 1.0$. [Rhodopsin] is 3–4 μM for all symbols except the diamonds, for which [rhodopsin] = 40 μM . The filled circles at $\log[\text{Ca}^{2+}] = -2.35$ in each panel are measurements in the presence of 12 mM MgCl_2 . No dif-

ferences were observed in the Ca^{2+} dependence of “brushed” and “brushed/centrifuged” RDM. Therefore, the data from these preparations have been combined in A and B. (B) Ca^{2+} increases the τ_{off} of light-activated PDE in the presence of ATP. τ_{off} with ATP and 0 Ca^{2+} is 15.7 ± 3.6 s (± 1 SD, $n = 25$ responses) and increases to 49.2 ± 7.5 s (± 1 SD, $n = 5$) with 1 mM GTP alone. The x indicates the turnoff time with 0.1 mM GTP alone and is 76.3 ± 16.2 s (\pm SD, $n = 3$). The dashed curve has $K_{0.5} = 1.6$ mM Ca^{2+} and $N = 0.8$. The continuous curve has a $K_{0.5} = 1.0$ mM Ca^{2+} and $N = 1.0$. The curves are constrained to pass through the ATP/GTP/0 Ca^{2+} point and the 0.1 mM GTP alone value.

centration 10-fold ([rhodopsin] = 40 μM). Data from these experiments are represented by the diamonds in Fig. 3. We find the control kinetics with ATP and 0 Ca^{2+} to be no different than those with 3–4 μM : $t_{\text{peak}} = 3.6 \pm 0.8$ s (SD, $n = 3$ responses, $P > 0.1$) and $\tau_{\text{off}} = 15.6 \pm 3.0$ s (SD $n = 4$ responses, $P > 0.1$). The Ca^{2+} dependence of these measures coincides with that of the lower membranes concentration: normalized velocity, τ_{off} , and t_{peak} determined at 40 μM rhodopsin are within the experimental error of measurements obtained at 3–4 μM rhodopsin. The signifi-

cance of these data can be best appreciated in the context of a theory of the affect of increasing the hypothetical modulator concentration.

The calcium dependence of V_p at 40 μ M rhodopsin is replotted in Fig. 4 with predictions of the model outlined in Fig. 4. Briefly, a target protein, T, which quenches light-activated PDE is inhibited directly by calcium, or via a soluble calcium-binding protein, M_s ($K_{0.5} = 0.5 \mu$ M Ca^{2+}), that confers submicromolar Ca^{2+} regulation. The direct Ca^{2+} inhibition of the target protein in the absence of M_s is constrained by the low [rhodopsin] data of Fig. 3 A to have $K_{0.5} = 1.0$ mM Ca^{2+} . The target protein is assumed to have a concentration equal to that of Rh kinase (i.e., 1 per 150 [Okada and Ikai, 1988] to 300 rhodopsins [Sitaramayya, 1986]), which has the lowest molar concentration of any protein known to participate in the turnoff of

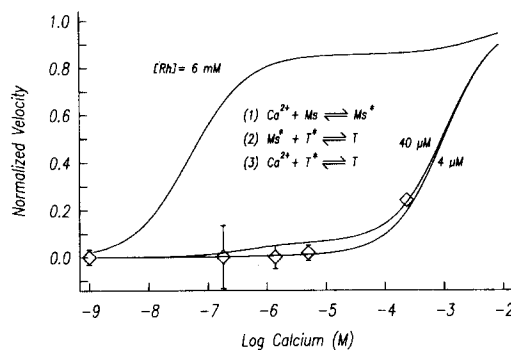


FIGURE 4. Observed Ca^{2+} dependence of normalized PDE velocity, and predictions based on a Ca^{2+} -dependent soluble modulator hypothesis. The open symbols are the normalized velocity data with 40 μ M rhodopsin replotted from Fig. 3 A with 95% confidence intervals indicated by the error bars. The curves show the Ca^{2+} dependence predicted for membrane concentrations corresponding to 4 μ M, 40 μ M, and 6 mM rhodopsin, the latter being the physiological value. The 4- μ M rhodopsin

curve is constrained by the 4- μ M rhodopsin data of Fig. 3 A to have $K_{0.5} = 1.0$ mM Ca^{2+} and no Ca^{2+} dependence below 1 μ M Ca^{2+} . To generate the regulation below 1 μ M Ca^{2+} expected in vivo (6 mM curve) and to be compatible with the 40- μ M rhodopsin data, such a theory requires that the K_d between the modulator and its target protein be greater than 5 μ M. The specific reaction scheme is defined by reactions 1-3 of the inset. For simplicity we assume noncooperative binding between Ca^{2+} and the modulator. The curves have been generated with reaction 2, $K_d = 5 \mu$ M. M_s and T are the modulator and target proteins, respectively, the "*" indicates the active forms of each. The ratio of total M_s and T with respect to rhodopsin are 1:75 and 1:150. The $K_{0.5}$'s for reactions 1 and 3 are 0.5 μ M Ca^{2+} and 1.0 mM Ca^{2+} (see text).

light-activated PDE. This target protein concentration generates a lower bound for the K_d between the modulator and target proteins. The theory requires that the K_d be greater than 5 μ M, a lower value exceeds the 95% confidence interval for the 40 μ M rhodopsin data.

Ca^{2+} Inhibits the ATP-dependent Quench in Bovine RDM and in a Reconstituted System

Partially purified Rh kinase quenches light-activated PDE in a reconstituted system containing ATP, G-protein, PDE, and rhodopsin in a manner very similar to the ATP dependent quench of RDM (Sitaramayya, 1986). Nevertheless, the quench

effected by Rh kinase in a reconstituted system need not have a Ca^{2+} dependence similar to the ATP-dependent quench in RDM: the RDM may contain a component lacking in the reconstituted membrane that confers the observed Ca^{2+} dependence.

Kinase and ATP decrease the relative light sensitivity of the reconstituted system V_p , as shown in Fig. 5 by the difference in the lateral position of the GTP alone (●) and the ATP/GTP/0 Ca^{2+} data (○). Addition of 3.1 mM free calcium (◇) to the ATP/GTP condition increases the relative light sensitivity to near that of the GTP alone data. Saturation curves are fit to the ATP/GTP/0 Ca^{2+} , GTP alone and ATP/GTP/3.1 mM Ca^{2+} data with $F_e^{-1} = 10^{-2.8}$, $10^{-3.7}$, and $10^{-3.5}$, respectively.

Quantification of the effects of calcium on the dim-flash PDE activation and inactivation kinetics in bovine RDM and the reconstituted system is shown in Fig. 6. As with toad RDM, Ca^{2+} has virtually no effect upon the normalized velocity (Fig. 6 A) in bovine RDM (○) or in the reconstituted system (x) in the range of calcium concentrations most likely to be obtained in the rod, that is, below $\sim 10 \mu\text{M}$ free Ca^{2+} . Above $10 \mu\text{M}$ free Ca^{2+} the normalized velocity of the dim-flash response increases and saturates hyperbolically having a $K_{0.5}$ for Ca^{2+} of $\sim 0.1 \text{ mM}$ for both RDM and

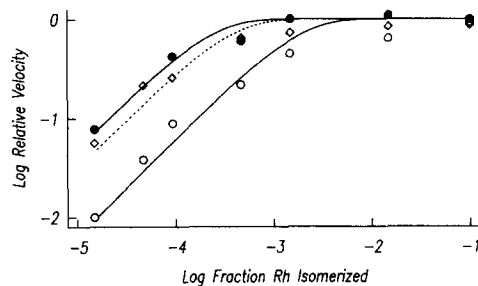


FIGURE 5. Log peak velocity of a reconstituted system vs. log fraction rhodopsin isomerized with 1 mM GTP alone (solid circles), 1 mM GTP and 2 mM ATP (open circles), or 1 mM GTP, 2 mM ATP, and 3.1 mM Ca^{2+} (diamonds). The continuous curves are Poisson saturation functions with $F_e = 10^{-2.8}$ and $10^{-3.7}$ for ATP/GTP and GTP alone, respectively; the dashed curve fitted to the ATP/GTP/5 mM Ca^{2+} data has $F_e = 10^{-3.5}$.

the reconstituted system. Unlike toad RDM, however, the normalized velocity at saturating calcium concentrations is only $\sim 70\text{--}80\%$ of the GTP alone response.

Qualitatively similar effects of Ca^{2+} upon τ_{off} are observed in the reconstituted system (Fig. 6 B) and in bovine RDM (Fig. 6 C). Below $100 \mu\text{M}$ in the reconstituted system and $10 \mu\text{M}$ free Ca^{2+} in bovine RDM τ_{off} is independent of calcium. Increasing Ca^{2+} above these concentrations causes τ_{off} to increase and saturate hyperbolically with $K_{0.5} = 0.1 \text{ mM}$ and 2.5 mM calcium for bovine RDM and the reconstituted system. As with toad RDM, the τ_{off} in very high free Ca^{2+} exceeds that with 1 mM GTP alone and approaches the 0.1 mM GTP alone responses in bovine RDM or the τ_{off} with no added kinase in the reconstituted system.

As in toad RDM, the calcium-induced increase in T_{peak} is variable in bovine RDM and the reconstituted system, and is not readily described by the hyperbolic saturation functions (data not shown).

Quench Inhibition Is Not Due to Altered [Metal-Nucleotide]

In these experiments involving two metal ions, two nucleotides and the resulting metal-nucleotide complexes, altering one of these components will result in an

adjustment of the others' concentrations. For example increasing total Ca^{2+} concentration may increase free Ca^{2+} , Mg^{2+} , $CaATP$, and $CaGTP$ while decreasing $MgATP$ and $MgGTP$. Therefore, what appears to be a Ca^{2+} -dependent phenomenon may be so only indirectly through an effect upon some other chemical species.

The nearly universal substrate in phosphorylation reactions, such as that of rhodopsin by Rh kinase, is $MgATP$. A consequence of increasing Ca^{2+} is competitive with Mg^{2+} for binding to ATP, thereby decreasing $[MgATP]$. The kinetics of PDE activation and inactivation could approach the GTP alone condition simply because

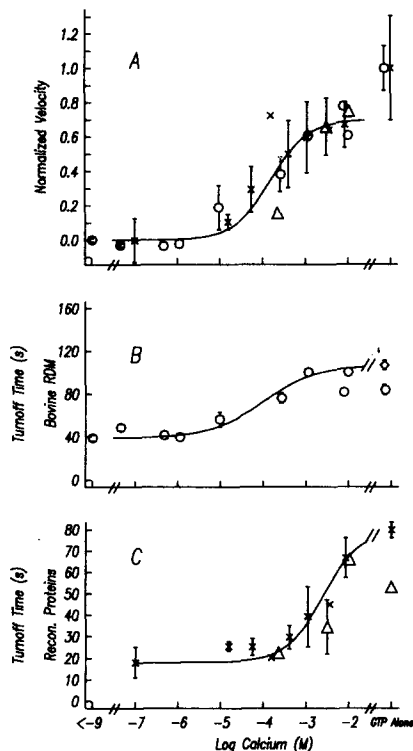


FIGURE 6. (A) Ca^{2+} increases the dim-flash-activated peak velocity with 2.0 mM ATP and GTP in bovine RDM (x) and the reconstituted system (O, Δ). $F_1 = -3.8$. Normalized velocity measured with 1.0 mM GTP (x, O) or 0.1 mM GTP (Δ) with 0 Ca^{2+} is increased by Ca^{2+} . The smooth curve is a hyperbolic saturation function with a $K_{0.5}$ of 0.1 mM and a maximum value of 0.7. (B) Ca^{2+} increases the τ_{off} of light-activated PDE of bovine RDM in the presence of ATP. τ_{off} with ATP and 0 Ca^{2+} is 39.0 ± 2.8 s (± 1 SD, $n = 2$ responses) and increases to 83.8 ± 5.0 s (± 1 SD, $n = 2$) with 1 mM GTP alone. The diamond above GTP alone was obtained with 0.1 mM GTP. The smooth curve has a $K_{0.5} = 0.1$ mM free Ca^{2+} , and is constrained to pass through the ATP/GTP/0 Ca^{2+} point and the 0.1 mM GTP alone value. (C) Ca^{2+} increases the τ_{off} of light-activated PDE in the reconstituted system in the presence

of 2.0 mM ATP. The x's indicate measurements with 0.1 mM GTP and the Δ 's were obtained with 1.0 mM GTP. τ_{off} with ATP and 0 Ca^{2+} is 18.1 ± 7.1 s (± 1 SD, $n = 7$ responses) and increases to 79.7 ± 3.8 s (± 1 SD, $n = 2$) with 0.1 mM GTP alone and no Rh kinase, the latter being indicated by the x above GTP alone. The smooth curve has a $K_{0.5} = 2.5$ mM free Ca^{2+} .

$[MgATP]$ decreases significantly. The $K_{0.5}$'s of the ATP-dependent quench for added ATP are 1–4 μM for the peak velocity (Liebman and Pugh, 1980; Kawamura and Bownds, 1981; Kawamura, 1983) and 50 μM for the turnoff time (Kawamura, 1983). Of the data reported in this paper none were obtained with free $MgATP < 0.2$ mM. Yet, the peak velocity increases up to 10-fold over a range of $MgATP$ concentrations the lowest of which is 100-fold greater than the $K_{0.5}$. Finally, a three-fold increase in peak velocity was observed over a range of Ca^{2+} , which produced virtually no change in calculated free $MgATP$. Therefore, the calcium-dependent

inhibition of the ATP-dependent quench is not due simply to a decrease in Rh kinase substrate, and is even observed if [MgATP] remains constant.

Another possibility is that Ca^{2+} may complex with ATP to form poor substrates that compete with MgATP, as has been observed in the Ca-ATPase of sarcoplasmic reticulum (Shigekawa et al., 1983). According to a purely competitive model of quench inhibition, decreasing the inhibitor CaATP concentration of increasing the substrate MgATP concentration cannot increase the degree of inhibition, that is, increase the hydrolytic velocity. If Michaelis-Menten kinetics are obeyed, these statements can be verified by differentiating the Michaelis-Menten equation for competitive inhibition: $V = S/[S + K_m(1 + I/K_i)]$; $\delta V/\delta I < 0$ and $\delta V/\delta S > 0$ for all I, S , where S and I are the substrate (MgATP) and inhibitor (CaATP) concentrations. Empirically, however, increased inhibition can be observed when [CaATP] is lowered and [MgATP] are increased. Consider $\log[\text{Ca}^{2+}] = -3.6$ and -2.35 in Fig. 3. At $\log[\text{Ca}^{2+}] = -3.6$, $[\text{CaATP}] = 0.6 \pm 0.14 \text{ mM}^1$ (SD, $n = 4$), $[\text{MgATP}] = 1.0 \pm 0.02 \text{ mM}$ (SD, $n = 4$) and the normalized velocity = 0.22 ± 0.1 (SEM, $n = 4$). The data indicated by the filled circles at $\log[\text{Ca}^{2+}] = -2.35$ were obtained by increasing added CaCl_2 to 5 mM and MgCl_2 to 12 mM. The corresponding [CaATP] decreased to $0.44 \pm 0.05 \text{ mM}$ (SD, $n = 5$) and [MgATP] increased to $1.52 \pm 0.01 \text{ mM}$ (SD, $n = 4$), yet the normalized velocity increased to 0.7 ± 0.2 (SEM, $n = 4$), that is, inhibition of the quench increased ($P < 0.01$). Thus, under these conditions the effects of Ca^{2+} on the peak velocity and τ_{off} are not produced by simple competition between CaATP and MgATP. Similar arguments can be made against competition by MgGTP or CaGTP.

DISCUSSION

Qualitatively, the results of the present study agree with prior studies (Kawamura and Bownds, 1981; Del Priore and Lewis, 1983) showing that increasing Ca^{2+} and 10^{-9} – 10^{-3} M increases light sensitivity and slows ATP-dependent inactivation of light-activated rod PDE (Liebman and Pugh, 1980). Quantitatively, our results characterize Ca^{2+} inhibition of the ATP-dependent inactivation mechanism as having a $K_{0.5}$ for Ca^{2+} of 0.1–2.5 mM, with virtually no Ca^{2+} dependence of PDE below 10 μM Ca^{2+} in native toad or bovine disk membranes, or in bovine disk membranes reconstituted with peripheral proteins. There are two points upon which our results and previous reports do not agree. First, we found that the calcium dependences of the measures τ_{off} and $V_p = V(t_{\text{peak}})$ are described by a saturation function with a single $K_{0.5}$ rather than a more complicated function with two $K_{0.5}$'s, one less than one greater than 10^{-6} M Ca^{2+} (Kawamura and Bownds, 1981). Second, we find V_{max} of flash-activated PDE ($F_1 > 10^{-3}$) to be independent of $[\text{Ca}^{2+}]$ from 10^{-9} – 10^{-2} M, in contrast to the prior report that V_{max} is greater in 1 mM Ca^{2+} than in 10^{-9} M Ca^{2+} (Kawamura and Bownds, 1981). We have no explanation for these differences.

The experimental technique used in the present experiments measures the composite kinetics of activation and inactivation of the first three steps of the light-activated cGMP cascade, viz., of rhodopsin, G-protein, and PDE. If the only mecha-

¹ The variability reported for the calculated metal-nucleotide concentrations reflect results from different experimental conditions (e.g., 2.0 vs. 0.5 mM total ATP).

nism by which Ca^{2+} can affect the kinetics of the first three steps of the cascade is that characterized here, our results categorically reject the hypothesis that under the physiological conditions that prevail in the rod, changes in $[Ca^{2+}]_i$ modulate the down-regulation of any of these three steps in the cascade. This conclusion follows because normal resting $[Ca^{2+}]_i$ in the rod is $<1 \mu M$ (Lamb et al., 1986; McNaughton et al., 1986), and diminishes during the light response (Yau and Nakatani, 1985), which means that $[Ca^{2+}]_i$ remains 2–3 orders of magnitude below the level required for producing any significant effect on PDE kinetics in the reaction cuvette.

The hypothesis that changes in $[Ca^{2+}]_i$ modulate the kinetics of one or more of the first three steps of the cGMP cascade *in situ* could be consistent with, although unsupported by, our data, if there exists a soluble modulator that confers submicromolar Ca^{2+} sensitivity to the enzyme(s) effecting the ATP-dependent quench but which is too dilute in our membrane suspensions. A general prediction of this “soluble modulator” hypothesis is that increasing the membrane concentration in the reaction cuvette will increase the fraction of target enzyme with high Ca^{2+} sensitivity. For the data obtained with 10-fold greater membrane concentration to be consistent with the model outlined in Fig. 4, the K_d for the binding of the modulator to the target protein would have to be greater than $\sim 5 \mu M$. Such a binding interaction between the hypothetical modulator and its target protein is atypically weak. The K_d 's between calcium binding proteins and their target proteins typically range from 10^{-9} – 10^{-8} M (Picton et al., 1980; Adelstein and Klee, 1981; Kohnken et al., 1981; Vallet et al., 1981), that is, 2–3 orders of magnitude lower than that which would be consistent with these data. Although the model is cast in terms of a target protein with a concentration equal to that of Rh kinase, we point out that the conclusion applies equally well to all the cGMP cascade components that compositely determine the dim-flash or linear PDE kinetics of Fig. 2. We find no Ca^{2+} regulation below $10 \mu M$ Ca^{2+} of any components—rhodopsin, GTP-binding protein/GTPase, PDE, Rh kinase; or 48K protein—in the experiments with high membrane concentration.

The hypothesis that the ATP-dependent inactivation or “quench” of PDE (Liebman and Pugh, 1980; Kawamura and Bownds, 1981) is effected primarily by Rh kinase (Liebman and Pugh, 1981; Sitaramayya and Liebman, 1983; Wilden et al., 1986) predicts that a membrane system reconstituted with Rh kinase could be inhibited by Ca^{2+} in a manner similar to that observed for native membranes. This prediction was borne out (Fig. 6) for peak velocity, $V_p:K_{0.5} \sim 0.1$ mM Ca^{2+} in both bovine RDM and the reconstituted system, but failed for $\tau_{off}:K_{0.5} \sim 0.1$ mM in bovine RDM and 2.5 mM Ca^{2+} in the reconstituted system. Nonetheless, the similarity between the $K_{0.5}$ that we observe for peak velocity in bovine RDM and the reconstituted system provides additional evidence that Rh kinase is a necessary part of the ATP-dependent quench in RDM. Previous work has shown that the Ca^{2+} -dependent inhibition of phosphorylation by partially purified Rh kinase (Palczewski et al., 1988) has a $K_{0.5}$ (0.1–0.5 mM Ca^{2+}) very similar to those reported here.

Although our results militate against any role for Ca^{2+} in regulating the first three steps of the cGMP cascade under normal physiological conditions, we believe that they do have a physiological counterpart. It can be inferred that under conditions in which the cGMP-activated conductance of the outer segment is greatly increased by

the infusion of cGMP, $[Ca^{2+}]_i$ rises to 100 μM or higher, based upon the following premises: (a) infusion of cGMP can increase the light-sensitive current to 500 pA or higher for at least 10 s (Cobbs and Pugh, 1985; Matthews et al., 1985; Hestrin and Korenbrot, 1987), (b) ~15% of the light-sensitive current is carried by Ca^{2+} (Yau and Nakatani, 1985), and (c) 90–95% of Ca^{2+} loadings are buffered within the rod cytoplasm (McNaughton et al., 1986). Accordingly, in an outer segment with cytoplasmic volume of ~1 pl, a cGMP-induced current of 500 pA would correspond to an increase in free $[Ca^{2+}]_i$ of 20–40 $\mu\text{M/s}$, with free Ca^{2+} rising up to a level of several hundred micrometers over 10 s. This inferred rise in internal Ca^{2+} has been confirmed by measurement of the luminescence of aequorin in rods whose cGMP is elevated by the PDE inhibitor IBMX (3-isobutyl-1-methylxanthine) (McNaughton et al., 1986). Photoresponses subsequent to cGMP elevation manifest markedly slowed inactivation kinetics and increased light sensitivity with respect to responses prior to cGMP infusion (Miller, 1982; Cobbs and Pugh, 1985; Cobbs et al., 1985; Matthews et al., 1985), effects that cannot be due to guanylate cyclase, because cGMP is supplied exogenously. We believe that the correct biochemical interpretation of the slowed inactivation of the photoresponses under such conditions is that the ATP-dependent inactivation of rhodopsin has been inhibited by the rise in $[Ca^{2+}]_i$.

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