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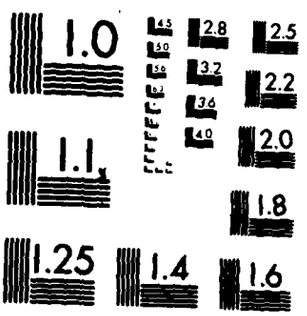

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PRIMARY EVENTS IN VISION — INVESTIGATION OF BASIC EYE RESPONSES

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The focus of this investigation is on the inter-relationship between two chemical mediators in the amplification of light in vision - calcium and cyclic GMP. Two questions were studied - whether calcium affects both the activation and deactivation of the light dependent hydrolysis of cyclic GMP by phosphodiesterase (PDE) in visual cells and the possible role of calmodulin in this activation and deactivation of PDE.

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ANNUAL REPORT

INTRODUCTION

Over the past year we have focused on the inter-relationship between two important chemical mediators in the amplification of light in vision. The mediators we have considered are calcium and cyclic GMP. Light absorption by rhodopsin is known to cause calcium translocation (1, 2) and a decrease in cyclic GMP (3) in vertebrate photoreceptors. In many systems the function of both of these chemical mediators of transduction are inter-related by the calcium binding protein calmodulin (4). Our detection of this protein in bovine photoreceptors by using rhodamine labelled calmodulin antibodies (5) led us to investigate two questions: first, whether calcium affects both the activation and deactivation of the light dependent hydrolysis of cyclic GMP by phosphodiesterase (PDE) in visual cells; second, the possible role of calmodulin in this activation and deactivation of PDE.

METHODS

Bovine brain calmodulin was obtained from Calbiochem-Behring(La Jolla, CA) and LKB Instruments(Baltimore, MD), trifluoperazine was a gift from Smith, Kline, and French(Philadelphia, PA), and all other reagents were obtained from Sigma(St. Louis, MO).

Bovine retinas were obtained from American Stores(Lincoln, NE) and kept in liquid nitrogen until use. Retinal rod outer segments were isolated by conventional sucrose flotation techniques, with brief centrifugation times to avoid increasing the dark PDE activity(9). Isolation procedures were carried out at 4°C or with reagents on ice using infrared illumination ($\lambda > 750$ nm) and an image convertor (NI-Tec, Inc., Niles, IL). Typically, 100 bovine retinas were removed from liquid nitrogen and thawed at room temperature for 30 minutes, followed by immersion of the vials in cool water for further thawing as necessary. Retinas were placed in 45% sucrose (w/v) in a buffer (60 mM KC1, 30 mM NaC1, 2 mM $MgC1_2$, 1 mM dithiothreitol, and 10 mM HEPES at pH 7.8) and manually homogenized by four passages through a teflon-glass test tube homogenizer (Wheaton), followed by 45-60 seconds of agitation on a tabletop vortexer operated at full speed. Thirty-two cc of this material was placed in each of 3 cellulose nitrate tubes, overlayed with 1-2 cc of the above buffer, and centrifuged at 22K for 20 minutes in an SW 25.1 swinging bucket rotor (Beckman, Palo Alto, CA). Crude ROS harvested from the sucrose-buffer interface were diluted 4:1 with buffer and centrifuged at 5K for 20 minutes in a fixed-angle SW 30 rotor (Beckman). The resulting pellet was resuspended in 96 cc of 38% sucrose (w/v) in the above buffer, divided into 3 cellulose nitrate tubes each overlayed with 1-2 cc of buffer, and centrifuged at 22K for 20 minutes as above. Purified ROS harvested from the sucrose-buffer interface were diluted 4:1 with buffer and pelleted at 5K for 20 minutes. The pellet was resuspended in several cc of buffer, divided into 200 μ1 aliquots, and stored in liquid nitrogen until further use. The rhodopsin concentration was determined by placing a 10 μ1 aliquot in 1 cc of 1.5% Ammonyx Lo (Onyx Chemicals, Jersey City, NJ) in 10 mM HEPES, 50 mM hydroxylamine at pH 7.8, using E=42,700 $M^{-1}cm^{-1}$ at 500 nm(22). A_{280}/A_{500} was typically 1.9-2.3.

Experiments were performed at room temperature by diluting 20-40 μ1 of ROS to a total volume of 250 μ1 in low or high calcium buffer, and adding calmodulin or trifluoperazine as indicated (final rhodopsin concentration 10-15 μM). For 10^{-3} M Ca^{++}, 2.5 μ1 of 100 mM $CaC1_2$ was added to the ROS solution; for 10^{-9} M free Ca^{++}, the ROS solution was made 0.1 mM in calcium and 2.78 mM in EGTA(5). The ROS solution was placed in a small test tube and rapidly stirred with a magnetic spin bar. Five μ1 of a stock solution 25 mM in ATP and GTP in buffer (prepared daily to avoid decomposition) was added to the test tube 3-5 minutes prior to the onset of flash illumination, and the ROS solution was made 2 mM in cyclic GMP (5 μ1 of a 100 mM stock solution)

immediately before the flash. Initial pH was adjusted when necessary by titrating with 0.1N NaOH. A 2 msec flash was delivered through a 520 nm transmission filter by a Vivitar photoflash unit placed 20 cm from the test tube and attenuated by calibrated neutral density filters as necessary. Flash-to-flash intensity variation was less than 3% as measured by electronically integrating the output of a power meter (Coherent Radiation, Palo Alto, CA). Flash bleaching was determined by absorption difference spectroscopy before and after illumination.

The protons released by complete hydrolysis of 2 mM cyclic GMP led to a pH change of 0.3-0.4 units. This change in pH was recorded by displaying the output of a portable pH meter equipped with a microelectrode (Markson Scientific, Phoenix, AZ) on a strip chart recorder. Hydrolytic velocities were determined before the pH had changed by 0.15 units. Buffering capacities of the individual media were determined by back titration with 0.1N NaOH.

RESULTS

It is well established that a single proton is released for each molecule of cyclic GMP hydrolyzed at pH 7.8 (3). We use this pH change to kinetically monitor the light-induced hydrolysis of cyclic GMP by PDE. In Figure 1 the moles of H^+ released are recorded versus time. The PDE activity (moles cyclic GMP hydrolyzed/mole rhodopsin-sec) is determined by dividing the slope of this tracing by the rhodopsin concentration of the sample (15 μM). In our experiments, an unattenuated photoflash bleaching 6% of the rhodopsin present maximally activates PDE in both low and high calcium. The % PDE activity elicited by a flash attenuated with neutral density filters is defined as $(rate_{dim} - rate_{dark})/rate_{6\%\ bleach}$. Although the absolute PDE activity varied considerably among samples prepared on different days, the initial % PDE activity elicited by a dim flash was reproducible from preparation to preparation to within ±5% as noted (3), and we have thus focused on this parameter in our measurements.

In the presence of 10^{-3} M Ca^{++} a green flash bleaching 0.02% of the rhodopsin present stimulates ≈40% of the maximum PDE activity elicited by a 6% saturating bleach (Figure 1). In 10^{-9} M Ca^{++} the same flash elicits ≈20% of the maximum PDE activity. The deactivation of PDE in the presence of 0.5 mM ATP and 0.5 mM GTP is demonstrated in Figure 1 by the decreasing slope of each tracing following a 1.9×10^{-4} bleach. Assuming first order deactivation kinetics the time constant for deactivation was typically 28-42 secs for 10^{-3} M Ca^{++} and 11-18 secs for 10^{-9} M Ca^{++}. Therefore, in the presence of ATP and GTP, calcium affects the activity of ROS PDE in two ways, by controlling both PDE activation and ATP-dependent deactivation.

Figure 2 shows the effect of calcium on % PDE activation in further detail. A change in calcium concentration from 10^{-3} M to 10^{-9} M results in a shift of the intensity-response curve to higher light levels by 0.6 log units. This extremely reproducible shift appears to be a principal mechanism by which calcium affects PDE activation.

In light of our detection of calmodulin in vertebrate visual cells (5) and the data presented in Figure 2 we have investigated the role of calmodulin in the inter-relationship of these two chemical mediators of transduction. All previous experiments aimed at detecting calmodulin-dependent activation have used totally bleached samples (6, 7). However, the major effect of calcium is at low light levels where 0.02% of the rhodopsin present is bleached (see Figure 2). Using a ratio of CaM: rhodopsin of 1:1000 based on our fluorescence antibody experiments and a concentration of 3 mM rhodopsin in bovine photoreceptors we calculate an approximate calmodulin concentration of 3 μM in these photoreceptors. Using flashes bleaching 0.02% of the rhodopsin present, we have detected no effect of bovine brain calmodulin at concentrations of 0.48 μM, 4.8 μM, 12 μM, 48 μM, or

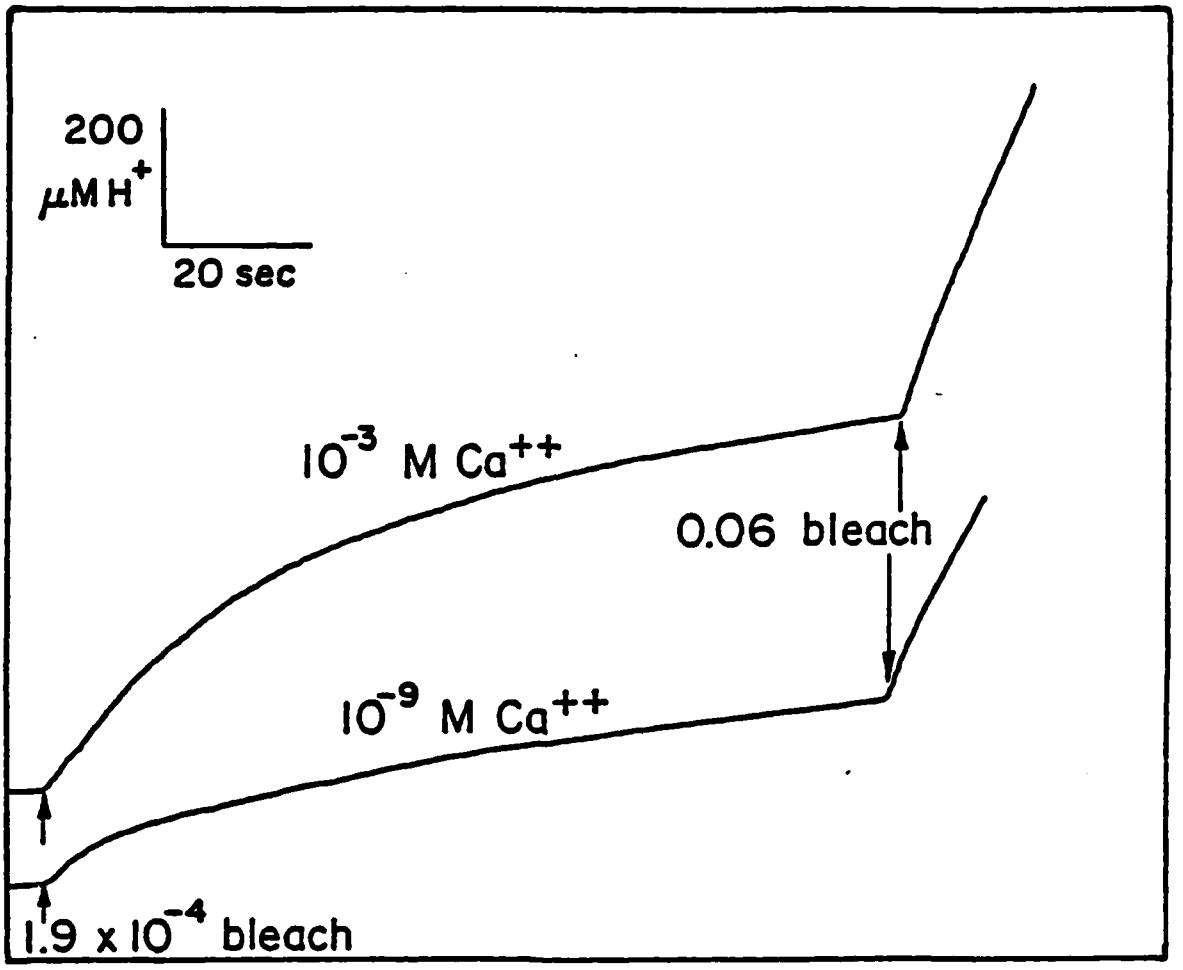

FIGURE 1: pH Assay of Phosphodiesterase (PDE) Activity: In 10^{-3}M Ca^{++} (upper trace), bleaching 0.02% of the rhodopsin present leads to ≈40% activation of the enzyme (compare the initial slopes at 0.02% and 6% bleach). In 10^{-9}M Ca^{++} (lower trace), the same flash elicits ≈20% of the maximum enzyme activity. The absolute slopes in the two tracings should not be compared directly, as the absolute phosphodiesterase activity (moles cyclic GMP hydrolyzed/mole rhodopsin-sec) varied from preparation to preparation. Since the % phosphodiesterase activity elicited by a dim flash [($rate_{dim}$-$rate_{dark}$)/$rate_{6\%\ bleach}$] was reproducible to within +5% from sample to sample, this parameter was monitored in these investigations. All experiments were performed at room temperature with 0.5 mM ATP, 0.5 mM GTP and 15 μM rhodopsin with 2 mM cyclic GMP as substrate. Initial pH = 7.8., final pH 7.5.

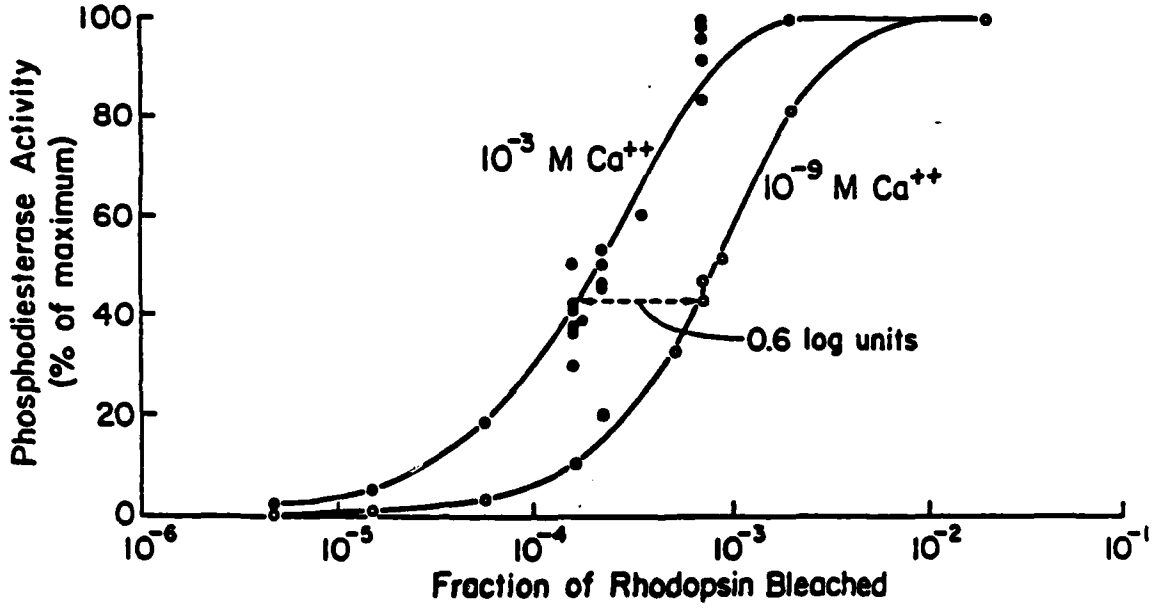

FIGURE 2: Effect of Calcium on % Phosphodiesterase Activity [$(rate_{dim}-rate_{dark})/rate_{6\%\ bleach}$]: Data for this figure is obtained from many tracings similar to Figure 1 with the bleaching flash attenuated by calibrated neutral density filters as necessary. When the free Ca^{++} concentration is decreased from 10^{-3} M(o) to 10^{-9} M(o), the intensity-response curve for enzyme activation is shifted to higher light levels by ≈0.6 log units. Experiments were performed at room temperature with 0.5 mM ATP, 0.5 mM GTP and 10-15 μM rhodopsin with 2 mM cyclic GMP as substrate.

96 μM on % PDE activation (Table I). Similarly, 100 μM trifluoperazine, a highly specific calmodulin inhibitor (23), has no effect on % PDE activation (Table 1). We conclude that ROS PDE activation is calcium-dependent but calmodulin-independent.

TABLE I: EFFECT OF CALMODULIN AND TRIFLUOPERAZINE ON PHOSPHODIESTERASE ACTIVATION AND DEACTIVATION

	Phosphodiesterase Activity (0.02% Bleach)	Time Constant for Deactivation
10^{-9} M Ca^{++}	20%	12 secs.
10^{-3} M Ca^{++}	40%	29
10^{-3} M Ca^{++} with:		
100 μM Trifluoperazine	42%	35
0.48 μM Calmodulin	38%	33
4.8 μM Calmodulin	35%	32
12 μM Calmodulin	34%	41
48 μM Calmodulin	33%	39
96 μM Calmodulin	34%	61

TABLE I: Calcium-Dependent PDE Activation and Deactivation are Calmodulin-Independent: At light levels bleaching 1.9×10^{-4} of the rhodopsin present, increasing the free calcium concentration from 10^{-9} M to 10^{-3} M results in an increase in the % PDE activation and the time constant for PDE deactivation. Neither parameter is affected by adding 100 μM trifluoperazine, a potent calmodulin inhibitor, or up to 48 μM calmodulin. At calmodulin concentrations > 48 μM the PDE deactivation time was increased, but this non-specific excess protein effect could not be reversed by trifluoperazine and could be observed by adding 23 μM bovine serum albumin. Initial pH was chosen at 7.5 because trifluoperazine binding to calmodulin decreases rapidly above pH 7.5 (23). The measurements were performed at room temperature in 0.5 mM ATP, 0.5 mM GTP and 13 μM rhodopsin with 2 mM cyclic GMP as substrate. The data shown in this table were all obtained in one day using the same preparation of rod outer segments to minimize sample-to-sample variation. However, multiple experiments (> 4) have been performed with identical results for % PDE activation. There was variation in the absolute value of the deactivation time constant from day to day, but in every trial this time constant was consistently longer in 10^{-3} M Ca^{++} (typically 28-42 secs.) than in 10^{-9}M CA^{++} (11-18 secs.) and was unaffected by trifluoperazine or < 48 μM CaM.

We have also investigated the effect of adding calmodulin or trifluoperazine on the deactivation of PDE in the presence of ATP and GTP. As seen in Table I, neither 100 μM trifluoperazine nor up to 48 μM CaM have any effect on the deactivation of ROS PDE. The kinetic deactivation of this enzyme is therefore calcium-dependent and calmodulin-independent. For the sake of completeness, we note that extremely high concentrations of CaM 96 μM can prolong the PDE deactivation time. However, this effect could not be reversed by adding excess trifluoperazine, and could be duplicated

by adding 23 μM bovine serum albumin. It is possible that this effect may be due to a perturbation of photoreceptor enzymes by excess exogenous protein. This hypothesis is further supported by a decrease in the absolute PDE activity under these conditions (data not shown).

DISCUSSION

In view of the recent demonstration of calmodulin in bovine retinal we have investigated the role of this calcium-binding protein in the calcium-dependent activation and deactivation of PDE. Previous attempts at detecting the effect of calmodulin on cGMP hydrolysis have been hampered by using fully bleached samples (6.7). We felt it was important to reinvestigate this question using light levels at which we have shown that calcium has a maximum influence on PDE activation and employing techniques that allow the detection of the effect of calmodulin on the deactivation of this enzyme. At bleaches of 1.9×10^{-4}, changing the calcium concentration from 10^{-9} M to 10^{-3} M results in a two fold increase in light-activated PDE activity (Figure 2), but neither exogenous calmodulin nor trifluoperazine affects this activity. Thus, the effects of calcium on this enzyme must be mediated by calmodulin-independent mechanisms.

Deactivation of PDE after flash illumination is clearly calcium and ATP-dependent. Although exact values varied from preparation to preparation, we have found that deactivation of PDE in .5 mM ATP and .5 mM GTP was consistently more rapid in 10^{-9} M Ca^{++} than 10^{-3} M Ca^{++} by a factor of ≈2. The nonhydrolizable analogs AMPPNP and GMPPNP cannot substitute for the ATP and GTP cofactors respectively required for the quenching of flash-activated PDE. Bleached rhodopsin catalizes the exchange of GDP bound to the G-protein of ROS for GTP, and the slow hydrolysis of GTP (≈ minutes) required for PDE deactivation appears to be too slow for a role in visual adaptation. In view of the requirement for ATP with a hydrolyzable γ-phosphate (10) and the observation that rhodopsin phosphorylated in response to light cannot stimulate PDE, it can be suggested that phosphorylation of rhodopsin may play an important role in deactivating PDE. This hypothesis was recently strengthened by the observation that ATP does not affect either the initial hydrolytic velocity (Vo) or the deactivation time constant (τ_0) for PDE in rod disc membranes devoid of rhodopsin kinase activity (5). Adding rhodopsin kinase in the presence of ATP decreases both Vo and τ_0. By using thermolysin digestion to remove a 12 amino acid segment from the C-terminus of rhodopsin, Sitaramayya and Liebman were able to demonstrate that phosphorylation of rhodopsin proximal and distal to the cleavage site decreases Vo and τ_0, respectively (9). However, Hermolin and Bounds have evidence suggesting rhodopsin phosphorylation occurs too slowly and only at high light levels to account for PDE activation (10).

Although the exact role of rhodopsin phosphorylation in PDE activation is still open to some question, we have investigated the role of calmodulin in this calcium-dependent process for several reasons. Even if rhodopsin kinase does not play a role in deactivating PDE, the requirement for ATP with a hydrolizable γ-phosphate suggests that some protein kinase may be involved in this regulation. Since calmodulin regulates myosin light chain kinase, phosphorylase kinase, glycogen synthase kinase, and NAD kinase(23), we investigated the role of this protein in calcium-dependent PDE deactivation. Significantly, we have demonstrated that PDE deactivation is also calmodulin-independent under our experimental condition. Thus, the physiological role for the presence of CaM in ROS remains to be established.

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