CALCIUM-DEPENDENT ACTIVATION AND DEACTIVATION OF ROD OUTER
SEGMENT PHOSPHODIESTERASE IS CALMODULIN-INDEPENDENT

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SUMMARY: ATP-dependent activation and deactivation of retinal rod outer
segment phosphodiesterase is affected by calcium [Kawamura, S. and Bownds,
M. D., J. Gen. Physiol. 77:571-591(1981)]. Our data demonstrate that
although calmodulin has been found in rod outer segments [Liu, Y. P. and
Schwartz, H., Biochim. Biophys. Acta 526:186-193(1978); Kohnken, R. E. et
al, J. Biol. Chem. 256:12517-12522(1981)], this protein is not involved in
calcium-dependent phosphodiesterase activation at light levels at which
calcium clearly affects this enzyme's activity. Furthermore, calmodulin
does not mediate the calcium-dependent deactivation of phosphodiesterase.

Light absorption by rhodopsin is known to cause calcium translocation

(1,2) and a decrease in cellular cyclic GMP (3-10) in vertebrate retinal rod

outer segments. It is also known that calcium affects both the activation

and deactivation of the light-dependent hydrolysis of cyclic GMP by

phosphodiesterase (EC 3.1.4.17) in visual cells (4). In many systems these

calcium-dependent cyclic nucleotide alterations are mediated by the

calcium binding protein calmodulin(11). The detection of this protein in

bovine ROS (12,13), albeit at low concentrations, has led us to investigate

the role of CaM in the calcium-dependent activation and deactivation of PDE.

Of these two calcium-dependent processes,the low ratio of CaM to PDE

(≈1:10) (12) makes it more probable that CaM could be affecting the

deactivation rather than activation of this enzyme. The deactivation

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Abbreviations: AMPPNP = adenosine-5'-(β,γ-imino)triphosphate; ATP =
adenosine-5'-triphosphate; CaM = calmodulin; EGTA = Ethyleneglycol-
bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; cyclic GMP = cyclic
guanosine-3',5'-monophosphate; GMPPNP = guanosine-5'-
(β,γ-imino)triphosphate; GDP = guanosine-5'-diphosphate; GTP = guanosine-
5'-triphosphate; HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic
acid; ROS = rod outer segment(s); PDE = phosphodiesterase.

process requires ATP with a transferable γ-phosphate (10). Since rhodopsin

is phosphorylated in response to light (14-18), it was suggested that

rhodopsin kinase may play a role in PDE deactivation(10). This hypothesis

was further strengthened by the observation that ATP-dependent PDE

activation and deactivation appear to be regulated by phosphorylation of

rhodopsin on either side of the C-terminus thermolysin cleavage site (19).

Thus, the low concentration of CaM in ROS which may be insufficient to

regulate PDE activation may be sufficient to regulate this enzyme's

deactivation. In this paper we address the question of calmodulin involve-

ment in the calcium-dependent deactivation and activation of PDE. Although

previous experiments on the role of CaM in this system have used fully

bleached samples(12,20,21), we have been careful to perform our experiments

at light levels at which calcium clearly influences PDE activity.

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 ob-
tained 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
(λ>750 nm) and an image convertor (NI-Tec, Inc., Niles, IL). Typically, 100
bovine retinas were removed from liquid nitrogen and thawed at room tempera-
ture 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 KCl, 30 mM NaCl, 2 mM MgCl$_2$, 1 mM dithiothreitol, and 10 mM
HEPES at pH 7.8) and manually homogenized by four passages through a tef-
lon-glass test tube homogenizer (Wheaton), followed by 45-60 seconds of agi-
tation on a tabletop vortexer operated at full speed. 32 cc of this mater-
ial 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 μl aliquots, and stored in liquid nitrogen until further use. The
rhodopsin concentration was determined by placing a 10 μl aliquot in 1 cc of
1.5% Ammonyx Lo (Onyx Chemicals, Jersey City, NJ) in 10 mM HEPES, 50 mM hy-
droxylamine 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 µl of ROS to a total volume of 250 µl 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 µl of 100 mM $CaCl_2$ 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. 5 µl 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 µl 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 AND DISCUSSION

It is well established that a single proton is released for each molecule of cyclic GMP hydrolyzed at pH 7.8 (3,4,9,10). 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 ROS samples prepared on different days, the initial % PDE activity elicited by a dim flash was reproducible from preparation to preparation to within $\pm 5\%$ as previous workers have noted (4). 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%
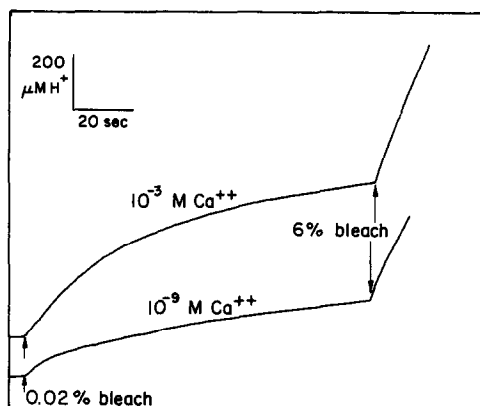
FIGURE 1: pH Assay of Phosphodiesterase (PDE) Activity: In 10^{-3}M Ca^{++} (upper trace), bleaching 0.02% of the rhodopsin present leads to ≈40% activation of the enzyme (compare the initial slopes at 0.02% and 6% bleach). In 10^{-9}M Ca^{++} (lower trace), the same flash elicits ≈20% of the maximum 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.

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 0.02% 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, confirming earlier results(4).

Although three previous attempts(12,20,21) have failed to detect any effect of exogenous calmodulin on ROS PDE activity, we have reinvestigated this question in light of the data presented in Figure 2. All previous experiments aimed at detecting calmodulin-dependent activation have used tot-
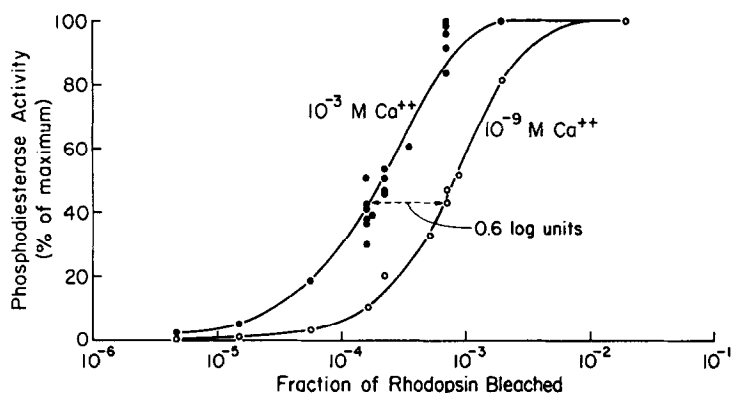
FIGURE 2: Effect of Calcium on % Phosphodiesterase Activity [(rate$_{dim}$-rate$_{dark}$)/rate$_{6\% bleach}$]: Data for this figure is obtained from many tracings similar to Figure 1 with the bleaching flash attenuated by calibrated neutral density filters as necessary. When the free Ca^{++} concentration is decreased from 10^{-3} M($\bullet$) to 10^{-9} M(o), the intensity-response curve for enzyme activation is shifted to higher light levels by $\approx$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.

ally bleached samples. However, the major effect of calcium is at low light

levels where 0.02% of the rhodopsin present is bleached. Using a ratio of

CaM:rhodopsin of 1:1000 (12) and a concentration of 3 mM rhodopsin in ROS,

we calculate an approximate calmodulin concentration of 3 μM in ROS. 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 96 μM on % PDE activation (Table 1). Similarly, 100 μM trifluopera-

zine, a highly specific calmodulin inhibitor (23), has no effect on % PDE

activation (Table 1). We conclude that ROS PDE activation is calcium-depen-

dent but calmodulin-independent. This is consistent with the finding that

although CaM is present in ROS, the $\approx$1:10 ratio of CaM to PDE is lower than

in other cells where CaM regulates the activation of this enzyme (11,12).

We have also investigated the effect of adding calmodulin or trifluo-

perazine on the deactivation of PDE in the presence of ATP and GTP. Al-

though exact values varied from preparation to preparation, we have found

that deactivation of PDE in 0.5 mM ATP and 0.5 mM GTP was consistently more

rapid in 10^{-9} M Ca^{++} than 10^{-3} M Ca^{++} by a factor of >2, in agreement with

other workers (4). As seen in Table 1, neither 100 μM trifluoperazine nor

TABLE 1: 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

At light levels bleaching 0.02% 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.

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 con-

centrations 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 ROS enzymes by excess exo-

genous protein. This hypothesis is further supported by a decrease in the

absolute PDE activity under these conditions (data not shown).

The phosphorylation of rhodopsin in response to light may be at least

partially responsible for PDE deactivation (10,19). The non-hydrolyzable

analogs AMPPNP and GMPPNP cannot substitute for the ATP and GTP cofactors respectively required for the quenching of flash-activated PDE(10). Bleached rhodopsin catalyzes the exchange of GDP bound to the G-protein of ROS for GTP, and the slow hydrolysis of GTP($\approx$ minutes) required for PDE deactivation appears to be too slow for a role in visual adaptation(9). 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(24), Liebman and Pugh(10) have suggested that phosphorylation of rhodopsin may play an important role in deactivating PDE. This original 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(19). 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 V_0 and τ_0 can be decreased by phosphorylation of rhodopsin on opposite sides of the thermolysin cleavage site (19). However, Hermolin and Bownds have evidence suggesting rhodopsin phosphorylation may occur too slowly and only at high light levels to account for PDE deactivation(16).

Even if rhodopsin kinase does not play a role in deactivating PDE, the requirement for ATP with a hydrolyzable 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 conditions. It remains to be determined if calmodulin may affect PDE at higher rhodopsin concentrations, and if.there is any physiological role for the presence of this calcium-binding protein in mature ROS. Chader et al (21) have observed that the activity of PDE in ROS of 9 day old Irish setters is affected by calmodulin, but this enzyme changes to a calmodulin-independent

form in 31 day old control animals. The activity of PDE in ROS from Irish

setters affected by an inherited retinal degeneration remains calmodulin-

dependent from 9 days of age until at least 48 days of age (21). Therefore,

the presence of CaM in mature bovine ROS (12,13) may be a vestige of the

regulation of PDE by calmodulin in immature ROS.

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