Effects of magnesium on cyclic GMP hydrolysis by the bovine retinal rod cyclic GMP phosphodiesterase

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Knowledge of the kinetics of the rod cyclic GMP phosphodiesterase is essential for understanding the kinetics and gain of the light response. Therefore, the interactions between Mg^{2+} , cyclic GMP, and purified, trypsin-activated bovine rod cyclic GMP phosphodiesterase (EC 3.1.4.17) were examined. The effects of Mg^{2+} and of cyclic GMP on the rod phosphodiesterase activity were mutually concentration-dependent. Formation of a free Mg -cyclic GMP complex is unlikely due to its high dissociation constant ($K_d = 19$ mM). Plots of $1/\text{velocity}$ versus $1/[\text{cyclic GMP}]$ as a function of $[Mg^{2+}]$ and $1/\text{velocity}$ versus $1/[Mg^{2+}]$ as a function of $[\text{cyclic GMP}]$ intersected to the left of

the $1/\text{velocity}$ axis. This is consistent with the formation of a ternary complex between the phosphodiesterase, Mg^{2+} , and cyclic GMP. A competitive inhibitor of the phosphodiesterase relative to cyclic GMP, 3-isobutyl-1-methylxanthine, non-competitively inhibited the enzyme relative to Mg^{2+} . Pb^{2+} , a competitive inhibitor of the phosphodiesterase relative to Mg^{2+} [D. Srivastava, R. L. Hurwitz and D. A. Fox (1995) *Toxicol. Appl. Pharmacol.*, in the press] non-competitively inhibited the enzyme relative to cyclic GMP. Collectively these results are suggestive of a rapid equilibrium random binding order of Mg^{2+} and cyclic GMP to the rod phosphodiesterase.

INTRODUCTION

In vertebrate rod photoreceptor outer segments, light triggers a G-protein-coupled enzymic cascade that results in the closure of cyclic GMP-gated cation channels and in the subsequent hyperpolarization of the photoreceptor cell [1–4]. The target enzyme of the activated G-protein in this visual signal transduction cascade is a cyclic GMP-specific phosphodiesterase (PDE). The hydrolysis of cyclic GMP by activated cyclic GMP PDE decreases the local cytoplasmic concentration of the cyclic nucleotide. Cyclic GMP dissociates from the channels and the channels close.

Knowledge of the kinetics of the rod cyclic GMP PDE is essential for understanding the kinetics and gain of the light response [5]. To address this issue, we (i) examined the dependence of initial rates of cyclic GMP hydrolysis on the concentrations of Mg^{2+} and cyclic GMP, and (ii) identified the mechanism by which this reaction occurs. If there are any Mg^{2+} - or cyclic GMP-dependent changes in cyclic GMP hydrolysis by the rod PDE, then the intracellular concentrations of both Mg^{2+} and cyclic GMP must be included in any analysis of the light response. The total and free concentrations of cyclic GMP in dark-adapted amphibian rod outer segments are $60 \mu\text{M}$ and $3.5 \mu\text{M}$ cyclic GMP, respectively [6]. These cyclic GMP levels change in a light-sensitive manner [5]. The total concentration of Mg^{2+} also changes in a light-sensitive manner [7], but the effect of light on the free concentration of Mg^{2+} is unknown.

Magnesium is required as a co-factor for maximum PDE activity in purified rod outer segments isolated from bovine retinas [8]. In bovine retinal outer segment preparations, cyclic GMP PDE activity, measured using $5 \mu\text{M}$ cyclic GMP as the substrate, was abolished with EDTA. The inhibition of activity by EDTA was reversed in a concentration-dependent manner with the addition of Mg^{2+} [9]. In another study, cyclic GMP PDE

was hypotonically extracted from light-exposed bovine rod outer segments and assayed with 1 mM cyclic GMP. Magnesium stimulated the hydrolysis of cyclic GMP by the isolated enzyme with maximal activity at a Mg^{2+} concentration of 0.5 mM [10].

To characterize the effects of Mg^{2+} on cyclic GMP hydrolysis, purified, trypsin-activated bovine cyclic GMP PDE was used to examine the concentration-dependent interaction between Mg^{2+} and cyclic GMP and its effect on the initial rates of cyclic GMP hydrolysis. The binding of Mg^{2+} to cyclic GMP was measured using the fluorescent indicator Magnesium Green. Competitive inhibitors of the enzyme relative to cyclic GMP and Mg^{2+} were used in PDE assays to define the binding order of cyclic GMP and Mg^{2+} to the enzyme. Finally, a kinetic model was used to examine the experimental data.

EXPERIMENTAL

Materials

BSA (fraction V), sucrose, soybean trypsin inhibitor, trypsin, cyclic AMP, cyclic GMP, PMSF, *Crotalus atrox* venom, glycerol, 3-isobutyl-1-methylxanthine (IBMX), and EDTA were purchased from Sigma. $[8\text{-}^3\text{H}]$ Cyclic GMP ($5\text{--}15$ Ci/mmol) and $[2,8\text{-}^3\text{H}]$ cyclic AMP ($30\text{--}50$ Ci/mmol) were purchased from ICN Pharmaceuticals. Ultrapure Tris and dithiothreitol (DTT) were purchased from Boehringer Mannheim. Ultrapure NaCl, KCl and $MgCl_2 \cdot 6H_2O$ were obtained from Aldrich. Additional sources of materials were: Bio-Rad protein assay dye reagent, Bio-Rad; $Pb(C_2H_3O_2)_2 \cdot 3H_2O$, Fisher Scientific; Sephadex A-25, Pharmacia; Ultima Gold XR scintillation cocktail, Packard; Magnesium Green fluorescent indicator, Molecular Probes. All solutions were prepared using distilled deionized water further purified through a series of Milli-Q (Millipore) cartridges to produce water with a resistance of 18.3 M Ω . The concentration

of cationic stock solutions was verified using a Wescor 5100C Vapor Pressure Osmometer (Logan, UT, U.S.A.) and the concentration of stock cyclic nucleotide solution was determined spectrophotometrically using an LKB Ultrospec II Model 4050.

Isolation of rod outer segments

All procedures were carried out at 4 °C and under dim red light unless otherwise indicated. Fifty frozen, dark-adapted bovine retinas (Hormel, MN, U.S.A.) were thawed and homogenized in an isotonic buffer (10 mM Tris, 60 mM KCl, 30 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 200 µM PMSF) at pH 7.5, using an SDT-182E probe on an Ultra-Turrax Model SDT-1810 homogenizer (Tekmar) at 35% power for 30 s. The homogenate was centrifuged in a 40.5% sucrose/isotonic buffer solution (w/w) for 2 h at 4000 g in a Sorvall Superspeed RC-2 centrifuge. The supernatant containing crude rod outer segments was resuspended in isotonic buffer and centrifuged for 1 h at 16000 g.

Purification of rod photoreceptor cyclic GMP PDE

The rod outer segments were washed twice in isotonic buffer. The washed pellet was resuspended in hypotonic buffer (10 mM Tris, 1 mM EDTA, 1 mM DTT, 200 µM PMSF) at pH 7.5 under normal room illumination and centrifuged in a Beckman L8-M ultracentrifuge for 20 min at 100000 g. The hypotonic extraction was repeated and the supernatants were pooled. The pooled supernatants were injected on to a Mono-Q (Pharmacia) anion-exchange HPLC column (5 mm × 50 mm bed size) equilibrated with column Buffer (10 mM Tris, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl) at pH 7.5. The column was controlled by an LKB 2152 HPLC controller and 2150 HPLC pump system. The pump was run at a flow rate of 0.5 ml/min and the PDE was eluted using a linear NaCl gradient (50–500 mM) in Column Buffer. Fractions were collected at 0.5 min intervals at 4 °C using an LKB 2211 SuperRac fractionator. Isolation of the rod photoreceptor enzyme was confirmed by the retention time of the enzymic activity [11], by an anti-PDE antibody (ROS-1) that recognizes the rod photoreceptor enzyme [12], and by the presence of 88 and 84 kDa subunits [13] using silver-stained SDS/PAGE. No other polypeptides were visualized on SDS/PAGE. Those fractions containing rod PDE activity were pooled, diluted 1:1 (v/v) with glycerol, and stored at –20 °C.

PDE assay

A modification of the original assay method of Butcher and Sutherland was utilized [14]. Aliquots of purified rod photoreceptor cyclic GMP PDE (9–69 fmol) were treated with trypsin (0.1 mg/ml) for 5 min on ice to remove the inhibitory subunits of the PDE [15]. The trypsin activation procedure was optimized for our assays and was independent of the enzyme concentrations used in our assays (data not shown). The reaction was terminated with soybean trypsin inhibitor (0.5 mg/ml). With the exception of greater enzyme activity observed with trypsin treatment, the initial velocity patterns reported here were similar to those obtained using histone activation of the enzyme (data not shown). The activated PDE was assayed at 30 °C in the presence of the indicated concentrations of Mg²⁺, Pb²⁺, IBMX and cyclic GMP or cyclic AMP and ³H-labelled cyclic nucleotide in an isotonic buffer (30 mM Tris, 130 mM KCl, 15 mM NaCl, 0.25 mg/ml BSA, 0.1 mM DTT) at pH 7.5. All assays were conducted in triplicate. All rates of hydrolysis were constant with respect to time (range: 3–40 min). The enzyme was inactivated by boiling for 1 min. The reaction mixtures were treated with *Crotalus atrox* venom (0.25 mg/ml) for 15 min at 30 °C to convert all the 5'-

nucleotide formed by the PDE into the nucleoside. Complete conversion was confirmed with control experiments. The samples were chromatographed using Sephadex A-25, the anion-exchange column which binds the charged, unreacted nucleotide monophosphate and allows the free passage of the less charged nucleoside through the column. More than 95% of the non-adsorbed nucleoside was recovered, scintillation cocktail was added, and the samples were analysed using a Packard 1900 TR liquid scintillation counter.

Protein determination

Protein concentration was determined spectrophotometrically (LKB Ultrospec II Model 4050) by the method of Bradford [16] using BSA as a standard. This assay may overestimate the protein concentration, when compared with the amino acid composition, by a factor of 1.2 [17]. A molecular mass of 194 kDa was assumed for the PDE [5].

Mg²⁺ binding studies

All measurements were made with an SLM-Aminco SPF-4800 spectrofluorimetric system (Urbana, IL, U.S.A.) using the fluorescent magnesium indicator, Magnesium Green. The cuvette was maintained at 20 °C in a thermostatic cuvette holder. Magnesium Green was excited at 475 nm and the emission intensity spectrum was monitored between 500 and 650 nm. Emission intensity measurements were recorded at the observed maximum of 530 nm. To generate a standard curve, aliquots of a magnesium solution (10 mM Tris, 37.5 mM MgCl₂, 10 µM Magnesium Green, pH 7.5) were titrated into a fluorescent dye solution (10 mM Tris, 10 µM Magnesium Green, pH 7.5). To measure binding of Mg²⁺ to cyclic GMP, aliquots of a magnesium solution (10 mM Tris, 50 mM MgCl₂, 30 mM cyclic GMP, 10 µM Magnesium Green, pH 7.5) were titrated into a solution of fluorescent dye and cyclic GMP (10 mM Tris, 30 mM cyclic GMP, 10 µM Magnesium Green, pH 7.5).

RESULTS

Cyclic GMP-dependence of the EC₅₀ of Mg²⁺

The Mg²⁺-dependent hydrolysis of cyclic GMP was quantitatively examined by determining the dependence of initial velocities on the concentration of Mg²⁺ in the presence of different concentrations of cyclic GMP. The concentration of Mg²⁺ that resulted in half-maximal stimulation of activity (EC₅₀) of the rod photoreceptor cyclic GMP PDE increased as the concentration of cyclic GMP increased (Figure 1). When the rod photoreceptor cyclic GMP PDE was assayed using 1 mM cyclic GMP, an apparent EC₅₀ (±S.D.) of 112.7 ± 12.3 µM Mg²⁺ was derived using non-linear least-squares analysis [18]. When the rod photoreceptor cyclic GMP PDE was assayed using 100 nM cyclic GMP, an apparent EC₅₀ (±S.D.) of 12.0 ± 1.4 µM Mg²⁺ was found. A similar experiment using 690 pM rod photoreceptor cyclic GMP PDE and 100 nM cyclic AMP also revealed a EC₅₀ of ~10 µM Mg²⁺ (data not shown). These results indicated that at low substrate concentration the EC₅₀ was independent of the cyclic nucleotide used.

Cyclic GMP increased the velocity at saturating concentrations of Mg²⁺. As the concentration of cyclic GMP increased from 100 nM to 1 mM, there was a 2.9 log unit increase in the maximal activity of the PDE from 280 nmol to 221 µmol of cyclic GMP hydrolysed/min per mg of protein. Thus, cyclic GMP increased both the apparent EC₅₀ of Mg²⁺ and the velocity at saturating concentrations of Mg²⁺.

Figure 1 Cyclic GMP-dependence of the EC_{50} of Mg^{2+}

The rod photoreceptor cyclic GMP PDE was assayed using procedures described in the Experimental section with the indicated concentrations of Mg^{2+} and cyclic GMP. All data have been normalized to 100% (maximal activity = 280 nmol of cyclic GMP hydrolysed/min per mg of protein, 100 nM cyclic GMP; 221 μ mol of cyclic GMP hydrolysed/min per mg of protein, 1 mM cyclic GMP). 90 and 340 pM rod photoreceptor cyclic GMP-PDE was assayed using 100 nM cyclic GMP and 1 mM cyclic GMP respectively. Each data point represents the mean $\pm$ S.E.M. for 4–6 separate experiments. Curves were fitted using non-linear least-squares hyperbolas.

Mg^{2+} increases the apparent K_m and the apparent V_{max} of the cyclic GMP PDE

Cyclic GMP PDE (190 pM) was assayed using 1–1000 μ M cyclic GMP in the presence of 50 μ M–10 mM Mg^{2+} . To avoid loss of enzyme activity observed during long incubation times at low concentrations of Mg^{2+} (10 μ M) and high concentrations of cyclic GMP (≥ 500 μ M), 690 pM PDE was used for these assays instead of 190 pM. As the concentration of Mg^{2+} increased from 10 μ M to 10 mM, there was an 8-fold increase in the maximal activity of the PDE from 26 to 222 μ mol of cyclic GMP hydrolysed/min per mg of protein (Figure 2).

The K_m values were determined from the data in Figure 2 using

Figure 2 Magnesium increases the apparent V_{max} of the cyclic GMP-PDE

Rod photoreceptor cyclic GMP-PDE (190 pM) was assayed using procedures described in the Experimental section with the indicated concentrations of cyclic GMP and 50 μ M–10 mM Mg^{2+} . PDE (690 pM) was used for assays at 10 μ M Mg^{2+} . The graphic symbols and their respective Mg^{2+} concentrations are: ●, 10 μ M; ○, 50 μ M; ▲, 100 μ M; □, 500 μ M; △, 10 mM. Each data point represents the mean $\pm$ S.E.M. for 3–6 separate experiments. Curves were fitted using non-linear least-squares hyperbolas.

Table 1 Mg^{2+} effects on the K_m of the cyclic GMP phosphodiesterase

The derived apparent K_m values $\pm$ S.D. for each concentration of magnesium were determined from the data in Figure 2 using non-linear least-squares analysis.

$[Mg^{2+}]$	K_m (μ M cyclic GMP)
10 μ M	10.4 $\pm$ 2.9
50 μ M	13.5 $\pm$ 1.9
100 μ M	29.3 $\pm$ 2.9
500 μ M	40.3 $\pm$ 1.9
10 mM	53.2 $\pm$ 4.5

Figure 3 Binding of Mg^{2+} to cyclic GMP

Binding studies using the fluorescent magnesium indicator, Magnesium Green, were conducted using procedures described in the Experimental section. Each data point represents the mean of 30 readings from a representative experiment. The conditions are: ●, 10 μ M dye and ○, 10 μ M dye + 30 mM cyclic GMP. Curves were fitted using non-linear least squares hyperbolas.

non-linear least-squares analysis (Table 1). The apparent K_m increased from 10 to 53 μ M cyclic GMP when the concentration of Mg^{2+} increased from 10 μ M to 10 mM. Thus, Mg^{2+} increased both the apparent K_m of cyclic GMP and the apparent V_{max} .

Mg^{2+} binding to cyclic GMP

The mutual concentration dependence of Mg^{2+} and cyclic GMP and the resultant effects on the hydrolysis of cyclic GMP raised the possibility that Mg^{2+} and cyclic GMP formed an intermediate Mg–cyclic GMP complex. To test this hypothesis, the binding of Mg^{2+} to cyclic GMP was examined using Magnesium Green, a fluorescent magnesium indicator. Magnesium Green, excited at 475 nm, has an emission peak at 530 nm upon Mg^{2+} binding. The observed dissociation constant (K_d) of the indicator (~ 200 μ M Mg^{2+}) was different than that reported by Molecular Probes (~ 900 μ M Mg^{2+}). This discrepancy may be due to differences in the buffer composition since the K_d reported by Molecular Probes was determined at pH 7.2 in the presence of 150 mM KCl and 20 mM NaCl, whereas our measurements were performed at pH 7.5 in the absence of additional ions.

The emission intensity measurements from a representative experiment (Figure 3) were corrected for quench by cyclic GMP using the Stern–Volmer equation:

$$K_Q[\text{cyclic GMP}] = (f_0/f) - 1 \quad (1)$$

Figure 4 The hydrolysis of cyclic GMP is a bireactant (ordered or random) sequential reaction

Rod photoreceptor cyclic GMP-PDE (100 pM) was assayed using procedures described in the Experimental section with the indicated concentrations of cyclic GMP and Mg^{2+} . The data were re-plotted as (a) $1/\text{velocity}$ versus $1/[\text{cyclic GMP}]$ as a function of the indicated concentrations of Mg^{2+} and (b) $1/\text{velocity}$ versus $1/[\text{Mg}^{2+}]$ as a function of the indicated concentrations of cyclic GMP.

In this equation, K_q is the bimolecular quench constant, and f_0 and f are the fluorescence intensities in the absence and presence of cyclic GMP respectively [19]. At the plateau of the titration curve ($\text{Mg}^{2+} \geq 4 \text{ mM}$), K_q was computed to be 0.06 mM^{-1} . Each data point of the titration in the presence of cyclic GMP was corrected for this factor by using eqn. (2) where F is the observed fluorescence intensity in the presence of cyclic GMP and F_0 is the fluorescence intensity after correction for quench by cyclic GMP:

$$F_0 = F(K_q[\text{cyclic GMP}] + 1) \quad (2)$$

The dissociation constant (K_d) for Mg^{2+} and cyclic GMP can be computed from Figure 3 using the following equilibrium equation:

$$K_d = [\text{Mg}^{2+}]_{\text{free}}[\text{cyclic GMP}]_{\text{free}}/[\text{Mg-cyclic GMP}] \quad (3)$$

The ratio of the K_d values (derived from Figure 3) for the indicator in the absence of cyclic GMP (0.204 mM Mg^{2+}) to that in the presence of cyclic GMP (0.525 mM Mg^{2+}) represents the fraction of free Mg^{2+} in the presence of cyclic GMP at any point on the curve (eqn. 4).

$$[\text{Mg}^{2+}]_{\text{free}} = 0.204/0.525 = 0.389 \quad (4)$$

Therefore, the fraction of Mg^{2+} that is bound to cyclic GMP at any point on the curve can be computed using eqn. (5).

$$[\text{Mg-cyclic GMP}] = 1 - [\text{Mg}^{2+}]_{\text{free}} = 1 - 0.389 = 0.611 \quad (5)$$

Figure 5 Mg^{2+} and cyclic GMP bind in a random order

100 pM rod PDE was assayed using procedures described in the Experimental section with the indicated concentrations of (a) cyclic GMP, Mg^{2+} and IBMX; and (b) cyclic GMP, Mg^{2+} and Pb^{2+} .

The free concentration of cyclic GMP can be computed using eqn. (6); however, since the concentration of cyclic GMP was much greater than the concentration of a Mg-cyclic GMP complex, we assumed that $[\text{cyclic GMP}]_{\text{free}} = [\text{cyclic GMP}]_{\text{total}}$.

$$[\text{Cyclic GMP}]_{\text{free}} = [\text{cyclic GMP}]_{\text{total}} - [\text{Mg-cyclic GMP}] \quad (6)$$

The K_d for Mg^{2+} and cyclic GMP can now be computed using eqn. (3) where $\text{Mg}^{2+}_{\text{free}} = 0.389$, cyclic GMP = 30 mM , and Mg-cyclic GMP = 0.611 .

$$K_d = (0.389 \times 30)/0.611 = 19 \text{ mM}$$

A K_d of 19 mM represents a weak association between Mg^{2+} and cyclic GMP. Therefore, the formation of an intermediate Mg-cyclic GMP complex prior to binding to the catalytic site of the enzyme appears unlikely.

Mg^{2+} and cyclic GMP function as co-reactants

Further experiments were performed resulting in plots of $1/\text{velocity}$ versus $1/[\text{cyclic GMP}]$ as a function of Mg^{2+} concentration (Figure 4a) and $1/\text{velocity}$ versus $1/[\text{Mg}^{2+}]$ as a function of cyclic GMP concentration (Figure 4b). Both plots exhibited points of intersection in the third quadrant. The point of convergence is a diagnostic criterion of a bireactant (ordered or random) sequential reaction mechanism [20]. Furthermore, the quadrant of convergence suggests that the K_m of cyclic GMP is greater than the K_d of cyclic GMP for the PDE (Figure 4a) and that the EC_{50} of Mg^{2+} is greater than the K_d of Mg^{2+} for the PDE (Figure 4b). Thus, when modelling the reaction, both Mg^{2+} and cyclic GMP can be treated as co-reactants.

Cyclic GMP and Mg²⁺ bind in a random order

Competitive inhibitors of substrates that alone do not promote the formation of product can be used to differentiate between random and ordered reaction mechanisms [21]. IBMX, a competitive inhibitor of the PDE relative to cyclic GMP in our own experiments (data not shown) and in those of others [22,23], was a non-competitive inhibitor of the enzyme relative to Mg²⁺ (Figure 5a). Pb²⁺, a competitive inhibitor relative to Mg²⁺ [23a], non-competitively inhibited the PDE relative to cyclic GMP (Figure 5b). These results are consistent with a random binding order of Mg²⁺ and cyclic GMP to the enzyme [21].

The points of convergence of the double-reciprocal plots occurred in the third quadrant (Figures 5a and 5b). This result implies that the affinity of the inhibitor (IBMX or Pb²⁺) for the free enzyme was less than the affinity of IBMX or Pb²⁺ for the free enzyme–Mg²⁺ or free enzyme–cyclic GMP complexes, respectively [21].

DISCUSSION

Cyclic nucleotide PDEs belong to a family of enzymes that require Mg²⁺ for activity. Magnesium maximally stimulated cyclic nucleotide hydrolysis when measuring PDE activity from rabbit brain [24], dog heart [25], rat brain [26], and bovine retina [27] whereas Mn²⁺ and Co²⁺ only partially enhanced hydrolysis. Additionally, Ca²⁺ and Zn²⁺ had no significant effect on any of these isoenzymes.

In this paper, we report that the effects of Mg²⁺ and of cyclic GMP on the rod PDE activity are mutually concentration-dependent. Increasing concentrations of cyclic GMP increased the apparent EC₅₀ for Mg²⁺ as well as the velocity observed at saturating concentrations of Mg²⁺. Likewise, increasing concentrations of Mg²⁺ increased both the apparent K_m of cyclic GMP and the apparent V_{max}. These alterations occurred without an intermediate Mg–cyclic GMP complex. Double-reciprocal plots indicated that the hydrolysis of cyclic GMP was a bireactant reaction that proceeded with Mg²⁺ and cyclic GMP binding in a random order to the free enzyme.

The EC₅₀ for Mg²⁺ ranged from ~10 μM to ~100 μM Mg²⁺ depending on the concentration of cyclic GMP (Figure 1). In comparison, half-maximal PDE activity from rat brain was found at 13 μM Mg²⁺ when using 2 mM cyclic AMP as the substrate [26]. Because this value was not corrected for contaminating Mg²⁺, which accounted for 30% of their maximal activity in the absence of added Mg²⁺, their half-maximal activity was probably at a much greater concentration of Mg²⁺. For four soluble PDE isoenzymes from rat hepatocytes, the concentration of Mg²⁺ that resulted in half-maximal activation ranged from 20 to 200 μM Mg²⁺ when using 1 μM cyclic AMP or cyclic GMP as the substrate [28]. In addition, the existence of a soluble 33 kDa, Mg²⁺-insensitive PDE isolated from rat hepatocytes, termed PDE-MQ-I, was reported.

An 8-fold increase in the apparent V_{max} and a 5-fold increase in the apparent K_m for cyclic GMP was observed when the Mg²⁺ concentration was increased from 10 μM to 10 mM (Figure 2 and Table 1). These results are similar to the brain PDE where Mn²⁺ increased both the apparent V_{max} and the apparent K_m [29]. The K_m for the purified, trypsin-activated bovine rod cyclic GMP-PDE has ranged from 17 μM [30] to 150 μM cyclic GMP [13]. Our data suggest that the differences in K_m values for cyclic GMP may result in part from the different levels of Mg²⁺ present in the assays. The V_{max} for the purified, trypsin-activated bovine rod cyclic GMP-PDE ranges from a low of 168 [31] to a high of

2200 μmol of cyclic GMP hydrolysed/min per mg of protein [30] with values in between [13,15]. In some of our enzyme preparations, the V_{max} value was ~500 μmol of cyclic GMP hydrolysed/min per mg of protein. The V_{max} reported here is less and may represent degraded enzyme within our assays. The degraded enzyme may lead to an overestimation of the amount of protein and consequently an underestimation of the V_{max}. However, it would not affect the proposed reaction mechanism.

The mutual dependence of Mg²⁺ and cyclic GMP was probably not due to an intermediate Mg–cyclic GMP complex since Mg²⁺ and cyclic GMP bound with a K_d of 19 mM at a pH of 7.5 (Figure 3). This result is consistent with the reported affinities of other nucleotide monophosphates for Mg²⁺. A K_d of 24 mM was determined for a Mg–cyclic AMP complex based on thermodynamic formation constants measured at a pH of 9.1 with an ion-selective membrane electrode [32]. Additionally, a K_d of 17 mM was calculated for a Mg–GMP complex based on thermodynamic constants that were determined at a pH of 8.5 using a microcalorimeter [33]. Because of the low pK_a of the only dissociable proton of the phosphate moiety [34] of cyclic GMP, its K_d for Mg²⁺ is not expected to be strongly dependent on the pH in the range 7.5–9.1.

The EC₅₀ and K_m were 2–3 orders of magnitude less than the K_d for formation of a Mg–cyclic GMP complex. This suggests that the PDE may have increased any interactions between Mg²⁺ and cyclic GMP. This may have occurred within the constraints of a catalytic pocket where, as observed with other enzymes [35,36], metal ion(s) render the reactant susceptible to nucleophilic attack. This hypothesis may be confirmed when more is known about the structure of the catalytic pocket and site(s) of metal binding for the rod PDE.

The points of convergence of the double-reciprocal plots (Figures 4a and 4b) are consistent with a bireactant (ordered or random) sequential reaction mechanism [20]. Therefore, when modelling the reaction, Mg²⁺ and cyclic GMP function as co-reactants for the rod PDE. Competitive inhibitors relative to either substrate (IBMX for cyclic GMP and Pb²⁺ for Mg²⁺) non-competitively inhibited the enzyme relative to the other substrate (Figures 5a and 5b). This result is consistent with cyclic GMP and Mg²⁺ binding to the PDE in a random order and ultimately forming a ternary complex with the PDE. This reaction mechanism may also apply to PDEs in general. The results do not allow us to speculate on the order of release of the product, 5'-GMP, and Mg²⁺ from the enzyme, although this information may help define *in vivo* regulation [37].

If the binding of cyclic GMP and Mg²⁺ to the rod cyclic GMP-PDE obeys a rapid equilibrium random mechanism, as the data suggest, then eqn. (7) can be used to model the data [38].

$$\frac{1}{v} = \frac{\alpha K_{Mg}}{V_{max}} \times \left(\frac{1 + K_{cyclic\ GMP}}{[cyclic\ GMP]} \right) \times \frac{1}{[Mg^{2+}]} + \frac{1}{V_{max}} \left(\frac{1 + \alpha K_{cyclic\ GMP}}{[cyclic\ GMP]} \right) \quad (7)$$

where $\alpha = [PDE][Mg^{2+}][cyclic\ GMP]/(K_{Mg})(K_{cyclic\ GMP})[PDE Mg-cyclic\ GMP]$, $K_{Mg} = [PDE][Mg^{2+}]/[PDE Mg]$ and $K_{cyclic\ GMP} = [PDE][cyclic\ GMP]/[PDE\ cyclic\ GMP]$ (see Figure 6). When all of the data from Figure 2 are re-plotted as 1/velocity versus 1/[cyclic GMP] as a function of Mg²⁺ concentration and 1/velocity versus 1/[Mg²⁺] as a function of cyclic GMP concentration, the constants can be evaluated using secondary plots [38]. Computations from the former plot yielded V_{max} = 296 μmol of cyclic GMP hydrolysed/min per mg of protein, K_{Mg} = 9.3 μM, and $\alpha = 8.7$. Computations from the latter plot revealed V_{max} = 298 μmol of cyclic GMP hydrolysed/min per mg of protein, K_{cyclic GMP} = 8.5 μM and $\alpha = 9.8$. The calculated V_{max} of 296–298 μmol of cyclic GMP hydrolysed/min per mg of protein

Figure 6 Proposed reaction mechanism

The results of this investigation are supportive of a rapid equilibrium random mechanism for the bovine rod cyclic GMP PDE. This mechanism, with its derived binding constants, is summarized here. The description of the binding constants appears in the Discussion. Abbreviation: cGMP, cyclic GMP.

suggests that the experimentally determined $V_{\max}$ of 221 μmol of cyclic GMP hydrolysed/min per mg of protein (Figure 2) was not under completely saturating conditions. The derived K_{Mg} of 9.3 μM and $K_{\text{cyclic GMP}}$ of 8.5 μM are consistent with the lower limit of the apparent affinities, EC_{50} and K_m , of 12 μM Mg^{2+} (Figure 1) and of 10 μM cyclic GMP (Table 1) respectively. The quadrant of the points of convergence in Figures 4(a) and 4(b) suggests that the binding constants of cyclic GMP for the PDE and Mg^{2+} for the PDE are less than or equal to the K_m of cyclic GMP and EC_{50} of Mg^{2+} respectively [20]. This is confirmed by the calculated K_{Mg} and $K_{\text{cyclic GMP}}$ values. The two derived values for α (8.7 and 9.8) are in relatively good agreement with each other (13% difference). Therefore, the mathematical analysis of all the data, using eqn. (7), supports the conclusion of a rapid equilibrium random mechanism for the binding of cyclic GMP and Mg^{2+} to the rod cyclic GMP-PDE.

An $\alpha > 1$ suggests that the affinity of the second co-reactant for the enzyme is decreased by the binding of the first co-reactant [38]. This is in contrast to the results presented in Figures 5(a) and 5(b) where, based on the quadrant of convergence, the affinity of the competitive inhibitor, IBMX or Pb^{2+} , for the enzyme appears to be increased by the prior binding of Mg^{2+} or cyclic GMP, respectively, to the enzyme [21].

The findings from this study are summarized in Figure 6. The PDE binds Mg^{2+} or cyclic GMP in a random order with the indicated dissociation constants. The next co-reactant then binds to ultimately form the ternary complex prior to hydrolysis of cyclic GMP.

The results of this study have elucidated the mechanism of the Mg^{2+} -dependent hydrolysis of cyclic GMP. The potential regulation of PDE activity *in vivo* either directly by Mg^{2+} or by other factors remains to be determined. Future experiments will determine whether there are light-dependent changes in the intracellular concentration of free Mg^{2+} in the photoreceptor and

how these potential changes may affect components of the phototransduction cascade.

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