The Role of a Parasite-Specific Allosteric Site in the Distinctive Activation Behavior of *Eimeria tenella* cGMP-Dependent Protein Kinase

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Received August 9, 2001; Revised Manuscript Received January 4, 2002

ABSTRACT: A cGMP-dependent protein kinase (PKG) was recently identified as an anticoccidial target for the apicomplexan parasite *Eimeria tenella* [Gurnett, A., Liberator, P. A., Dulski, P., Salowe, S., Donald, R. G. K., Anderson, J., Wiltsie, J., Diaz, C., Harris, G., Chang, B., Darkin-Rattray, S. J., Nare, B., Crumley, T., Blum, P., Misura, A., Tamas, T., Sardana, M., Yuan, J., Biftu, T., and Schmatz, D. (2002) *J. Biol. Chem.* (in press)]. Unlike the PKGs of higher organisms that have two cGMP binding sites in their regulatory domain, the PKG from *Eimeria tenella* (Et-PKG) contains three putative cGMP binding sites and has distinctive activation properties, including a very large stimulation by cGMP (∼1000-fold) with significant cooperativity (Hill coefficient of 1.7). During our investigation of Et-PKG activation, we found that 8-substituted cGMP analogues are weak partial activators. For example, 8-NBD-cGMP provides a maximal stimulation of activity of only 20-fold with little evident cooperativity, although cGMP can synergize with the analogue to provide full activation. The results suggest that partial activation is a consequence of restricted binding of 8-NBD-cGMP to a subset of cGMP sites in the enzyme. Site-directed mutagenesis of conserved arginine and glutamate residues in the parasite-specific third cGMP site confirms that this site is an important functional participant in the allosteric regulation of the kinase and that it exhibits very high selectivity against 8-NBD-cGMP. Since the results are consistent with full activation of Et-PKG requiring cyclic nucleotide binding in all three allosteric sites, one role for the additional cGMP site may be to establish a stricter regulatory mechanism for the kinase activity than is present in the PKGs of higher organisms containing only two allosteric sites.

Among the large number of protein kinases that have been identified, some of the best characterized are the cyclic nucleotide-dependent protein kinases. The cAMP-dependent and cGMP-dependent protein kinases are homologous enzymes that respond to modulations in second messenger levels during the signal transduction events that are integral to a variety of physiological phenomena (*3, 4*). A cGMP-dependent protein kinase (PKG)[1] from the poultry parasite *Eimeria tenella* has recently been identified as the likely molecular target of a newly disclosed broad-spectrum coccidiostat (*2*). The same compound also inhibits the growth of *Toxoplasma gondii*, a related apicomplexan parasite and opportunistic human pathogen, and has proven efficacious in vivo (*5*). The results of molecular genetic experiments in *T. gondii* have shown that the PKG gene is essential, further validating the kinase as an antiparasitic target (*22*). While a role for cyclic nucleotide involvement in the developmental biology of apicomplexan parasites has been reported (*6*−*8*), the signaling pathways involved in these processes remain to be elucidated.

The PKGs from both *E. tenella* and *T. gondii* have been cloned and share clear similarities with each other as well as their counterparts in higher organisms; all are a single polypeptide containing an N-terminal regulatory region and a C-terminal kinase catalytic domain. In mammals and *Drosophila*, the regulatory region contains a dimerization domain, autophosphorylation sites, an autoinhibitory domain, and two tandem cGMP binding sites. The cyclic nucleotide binding sites in PKG, like those in PKA, are evolutionarily related to the CAP family of cyclic nucleotide binding proteins (*3*). PKG and PKA are distinct in this family in having two cyclic nucleotide binding domains, although the mechanistic significance of this is unclear. In contrast to the mammalian and invertebrate PKGs, the parasite PKGs have a significantly larger N-terminal domain which appears to contain a third cGMP site further downstream from two tandem sites (*2*) (Figure 1). Through the use of cGMP analogues and site-directed mutagenesis, we demonstrate in this paper that the putative "third" cGMP site in *E. tenella*

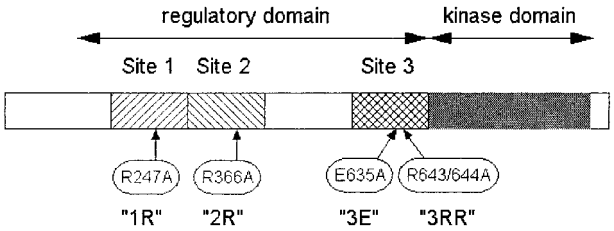

FIGURE 1: Schematic representation of the Et-PKG gene. Positions of mutations studied in this work are indicated as well as the nomenclature for the resultant proteins.

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[1] Abbreviations: PKG, protein kinase G; PKA, protein kinase A; Et-PKG, PKG from *E. tenella*.

10.1021/bi0156658 CCC: $22.00
Published on Web 03/09/2002

PKG (Et-PKG) is indeed functional and is an important participant in the distinctive activation behavior exhibited by this kinase.

MATERIALS AND METHODS

Molecular Biology, Protein Purification, and Protein Assay. The expression of recombinant Et-PKG with an N-terminal FLAG epitope tag in *T. gondii* has been described elsewhere (*2*, *22*). Details of the methods for site-directed mutagenesis and purification of the recombinant proteins have also been described (*22*). The protein assay was adapted from a literature procedure (*9*). Samples and sufficient water to make up a volume of 0.5 mL were added to 1.5 mL microfuge tubes followed by 50 μL of a 0.15% (w/v) solution of sodium deoxycholate. After standing 10 min at ambient temperature, 50 μL of a 72% (w/v) solution of trichloroacetic acid was added to each, and the samples were vortexed vigorously. The tubes were centrifuged at 15000*g* for 5 min, and the supernatants were discarded by aspiration. The protein pellets, which usually were not visible, were then resuspended in 200 μL of BCA working reagent from the Pierce Micro BCA Protein Assay Reagent Kit. The capped tubes were incubated in a 60 °C water bath for 1 h and then allowed to cool to room temperature. A 150 μL aliquot of each sample was transferred to a half-area microtiter plate and A_{562} read on a plate reader; bovine serum albumin was used to create the standard curve. Controls established that the FLAG peptide used for elution of proteins from the affinity resin does not give detectable signal with this protocol.

Enzyme Assay and Data Analysis. The peptide substrate biotinyl-ϵ-aminocaproyl-GRTGRRNSI-OH was synthesized in-house by standard methods. PET-cGMP (β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphate), 1-NH_2-cGMP (N^1-aminoguanosine-3′,5′-cyclic monophosphate), 8-APT-cGMP [8-(2-aminophenylthio)guanosine-3′,5′-cyclic monophosphate], and 8-NBD-cGMP (8-[[2-[(7-nitro-4-benzofurazanyl)amino]ethyl]thio]guanosine-3′,5′-cyclic monophosphate) were obtained from Biolog Life Science Institute (Bremen, FRG), while 8-Br-cGMP (8-bromoguanosine-3′,5′-cyclic monophosphate) came from Biomol Research Laboratories and 8-CPT-cGMP [8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphate] came from Calbiochem. Bovine PKG was obtained commercially; recombinant isoform Iα was purchased from Calbiochem, while native Iα enzyme was purchased from Promega. The kinase assay was performed in a 50 μL reaction volume containing 25 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 20 μM ATP, 20 μM peptide substrate, and 2.5 μCi of [γ-^{33}P]ATP (Amersham). Cyclic nucleotide was serially diluted in buffer before adding 5 μL of each concentration into 40 μL of the assay mix. The reaction was initiated with 5 μL of enzyme (or buffer for the background) and incubated for 30 min in a heating block at 30 °C. The assays were terminated by the addition of 25 μL of 8 M guanidine hydrochloride solution (Pierce) before spotting 15 μL onto a SAM^2 streptavidin membrane (Promega). The membrane was washed twice with 1 M NaCl and twice with 1 M NaCl + 1% H_3PO_4 on a rotating mixer for 20 min. The membrane was then rinsed successively with water and ethanol and dried under a heat lamp. The individual assays were then separated, placed in scintillation vials containing 2 mL of Ultima Gold cocktail (Packard), and counted in a Packard TriCarb 2500 liquid scintillation counter. The amount of enzyme was adjusted to give between 10 000 and 140 000 cpm when maximally activated; substrate turnover was less than 10% in all cases. The concentration of Et-PKG varied between 0.26 and 3.4 μg/mL for cGMP titrations and between 7 and 25 μg/mL for 8-NBD-cGMP titrations, depending on the activity of the enzyme form used. Assays with bovine PKG used 0.059 μg/mL recombinant or 0.034 μg/mL native enzyme with both activators. After subtracting the appropriate background for each assay point, titrations were fit to the following modified Hill equation using Kaleidagraph (Synergy Software):

$$V_A = V_0 + (V_{max} - V_0)/[1 + (K_A/[A])^h]$$

V_A is the observed velocity at concentration [A] of cyclic nucleotide, V_0 is the velocity in the absence of activator, V_{max} is the velocity of the maximally activated enzyme, K_A is the concentration for half-maximal activation, and h is the Hill coefficient. The activation parameters are reported with their standard errors from the curve fit.

RESULTS

It has previously been shown that the activity of Et-PKG increases enormously in the presence of micromolar levels of cGMP but only weakly in the presence of cAMP (*2*). The activity of the protein in the absence of cyclic nucleotide activator is extremely low and difficult to measure with great precision; consequently, the fold activation, which represents the quotient of the maximal rate and the unactivated rate, can vary widely between experiments, although it usually falls between 500 and 1000 (data not shown). We have hypothesized that the parasite-specific "third" cGMP site might have an important role in defining the activation characteristics of this enzyme, including the significant cooperativity. To explore the individual roles of the three putative cGMP sites, a recombinant expression system was established. Initial attempts to express the enzyme in more conventional systems such as *E. coli* or baculovirus-infected insect cells were unsuccessful. Et-PKG can, however, be expressed in another apicomplexan organism, *Toxoplasma gondii*, which unlike *Eimeria*, can be cultured and manipulated genetically (*22*). An N-terminal FLAG tag was appended to facilitate affinity purification. The activation properties of recombinant wild-type Et-PKG expressed in *T. gondii* were found to be identical to the native enzyme purified from parasite oocysts (*2*).

Analogues of cGMP have been used in the past to probe the functions of the cyclic nucleotide binding sites in mammalian PKG enzymes (*10*, *11*). As part of our characterization of the mechanism of activation of Et-PKG, a series of commercially available cGMP analogues were examined. The structures of the analogues are presented in Figure 2, and Table 1 summarizes their experimentally determined activation parameters with Et-PKG including the activation constant, K_A, and Hill coefficient, h. While the two analogues modified on the pyrimidine portion of the purine, PET-cGMP and 1-NH_2-cGMP, are capable of maximally activating the enzyme to at least 70% of the level obtained with cGMP, the remaining four analogues substituted at the 8-position

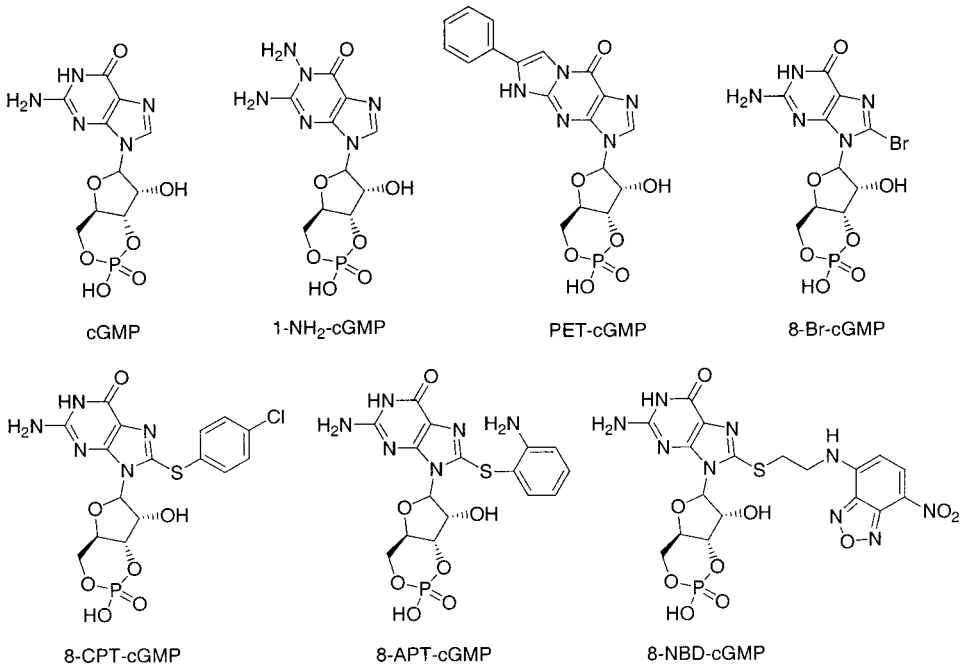

FIGURE 2: Structures of cGMP and analogues cited in the text.

Table 1: Activation Parameters for cGMP Analogues with Et-PKG

cyclic nucleotide	% max activation	K_A (μM)	*h*
cGMP	(100)	2.2 ± 0.1	1.7 ± 0.1
1-NH_2-cGMP	79	3.70 ± 0.17	1.7 ± 0.1
PET-cGMP	72	0.67 ± 0.02	1.4 ± 0.1
8-Br-cGMP	18	1.00 ± 0.08	1.3 ± 0.1
8-CPT-cGMP	6	0.23 ± 0.02	1.1 ± 0.1
8-APT-cGMP	3	0.90 ± 0.06	1.0 ± 0.1
8-NBD-cGMP	2	0.17 ± 0.01	1.1 ± 0.1

Table 2: Activation Parameters for Bovine Iα PKG

cyclic nucleotide	max sp act.[a]	K_A (μM)	*h*
native			
cGMP	480 ± 15	0.17 ± 0.02	1.2 ± 0.1
8-NBD-cGMP	460 ± 10	0.11 ± 0.01	1.6 ± 0.2
recombinant			
cGMP	260 ± 10	0.047 ± 0.008	1.0 ± 0.1
8-NBD-cGMP	250 ± 3	0.035 ± 0.002	1.3 ± 0.1

[a] Units are nmol min^{-1} mg^{-1}. Protein concentration supplied by vendor was used for the calculation.

of the purine are seriously compromised in their activation ability. It may also be seen that cooperativity, as measured by *h*, for the analogues decreases along with their maximal activation level. These results are quite distinct from the behavior of these analogues with mammalian PKG that is reported in the literature. Direct comparisons of bovine PKG, either native enzyme purified from lung tissue or recombinant protein expressed in insect cells, with Et-PKG confirmed a number of dramatic differences in activation constant, fold activation, cooperativity, and response to analogues between the kinases of mammalian and parasite origins. As seen in Table 2 and Figure 3, the K_A for bovine PKG is >10-fold lower than for Et-PKG, and the maximal fold activation is >40-fold lower. Furthermore, whereas cGMP activation of Et-PKG is strongly cooperative with *h* approaching 2, the mammalian enzyme displays little or no cooperativity with *h* close to 1.

A particularly striking example of the significantly different response of mammalian and parasite PKGs to cGMP analogues is seen with 8-NBD-cGMP. Bovine PKG is activated by this compound with a nearly identical K_A and somewhat higher *h* than the corresponding parameters for cGMP (Table 2 and Figure 3A,B). In contrast, the K_A for 8-NBD-cGMP with Et-PKG is more than 10-fold lower than that for cGMP although the analogue activates the enzyme by only 20-fold compared to ~1000-fold for cGMP (Table 1 and Figure 3C,D). Furthermore, the activation by 8-NBD-cGMP also occurs with reduced cooperativity. In fact, the activations of Et-PKG and bovine PKG by this compound are remarkably similar with respect to K_A, fold activation, and *h*. The similarity of these activation profiles suggested to us that Et-PKG was only partially activated by 8-NBD-cGMP, and that this partial activation was occurring through restricted binding of the cyclic nucleotide to only the tandem sites 1 and 2 of the regulatory domain, analogous to the tandem sites of bovine PKG.

If this partial activation hypothesis is correct, then cGMP binding in site 3 should synergize with the analogue in sites 1 and 2. Figure 4 illustrates that when cGMP was titrated in the presence of 2 μM 8-NBD-cGMP, a concentration expected to fill sites 1 and 2, the enzyme could be fully activated to the same level as cGMP alone. Consistent with the hypothesis that a single site was being filled in this titration, *h* was equal to 1. Although the definitive assignment of the site(s) bound by each cyclic nucleotide and the actual order in which those sites are normally filled cannot be determined from these experiments, the results are consistent with a model in which a partial 20−30-fold activation occurs when sites 1 and 2 are filled and an additional 30−50-fold activation occurs when site 3 is subsequently occupied. Cyclic nucleotide binding to sites 1 and 2 alone does not manifest much cooperativity, in contrast to the significant cooperativity seen when all three sites are available.

To probe directly the functional role of the various cGMP binding sites, we wished to create mutant enzymes with

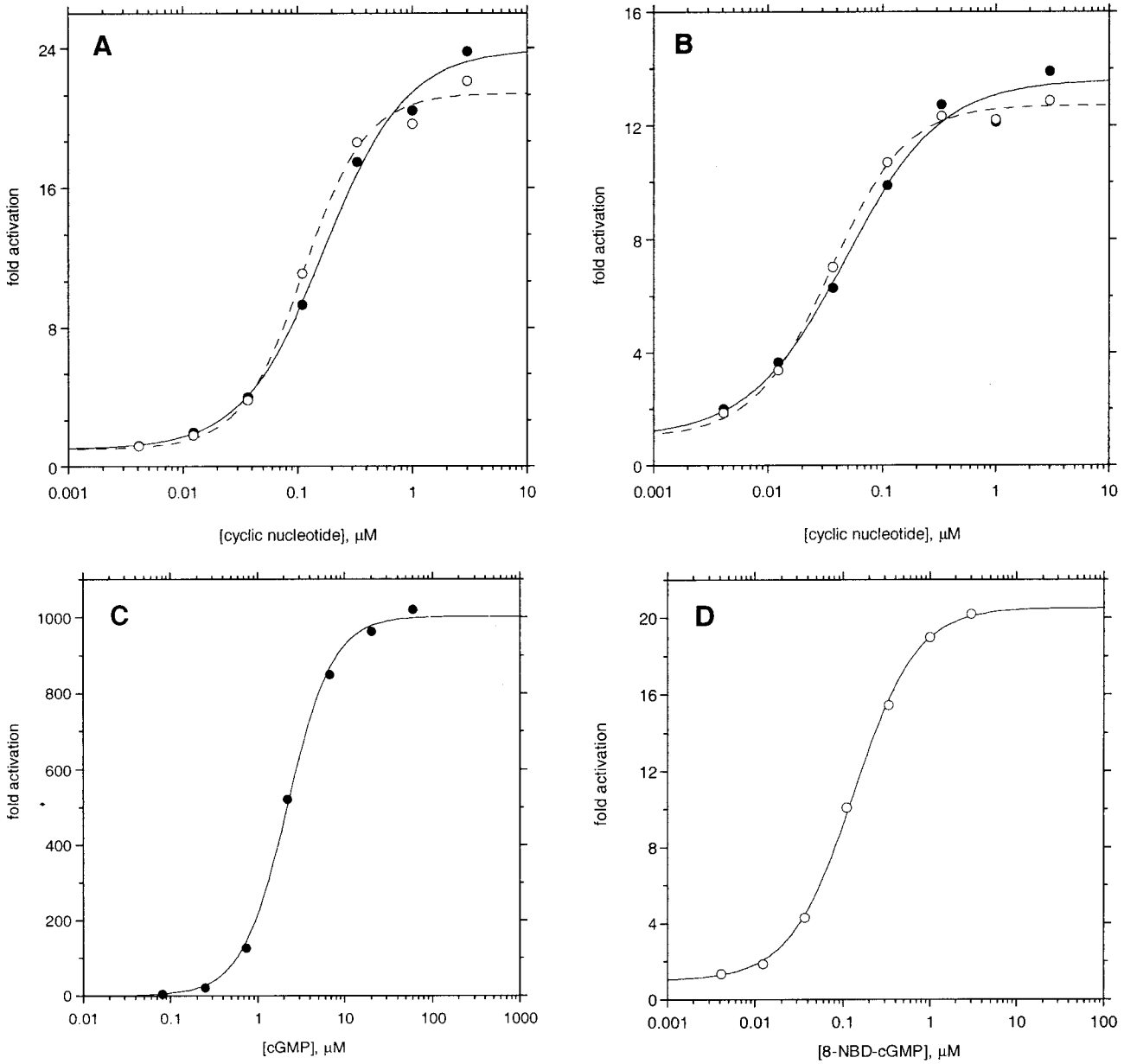

FIGURE 3: Activation of PKGs by cGMP (closed circles) and 8-NBD-cGMP (open circles): (A) native bovine PKG; (B) recombinant bovine PKG; (C and D) Et-PKG.

deactivating point mutations in the regulatory domain. We were guided by precedent with extensive work on cAMP-dependent protein kinase (PKA). The X-ray crystal structure of the PKA regulatory domain shows an arginine residue that interacts with the phosphodiester of the cyclic nucleotide and a glutamate residue that interacts with the ribose 2′-hydroxyl (*12*). Alignment of protein sequences revealed candidate residues within the presumptive cGMP binding sites of Et-PKG that may fulfill similar functions (Figure 5). Mutant proteins were first created by replacing each putative key arginine residue with an alanine. In the case of site 3, two adjacent arginine residues were replaced since it was not certain which would be the more important. The mutated positions and nomenclature for the resultant proteins are given in Figure 1. The FLAG-tagged recombinant proteins were expressed in and purified from *T. gondii* in a manner similar to the wild-type enzyme. Comparative titrations with cGMP were performed for each construct and are presented in Figure 6A with parameters obtained in Table 3. Although the K_As increase and cooperativity is lost when the arginine side chains in either sites 1 and 2 (1R/2R) or site 3 alone (3RR) are truncated, these proteins can still be fully activated. Even a protein with mutations in all three sites (1R/2R/3RR) can still be activated to 50% of the wild-type level with millimolar amounts of cGMP.

From these data, it is apparent that site 3 of Et-PKG is indeed functional. Since activation appears to be only impaired, but not eliminated, by the R→A substitutions, mutation of the conserved glutamate residue in site 3 to alanine, singly (3E) and in combination with the arginine mutations (3RR/3E), was investigated. The 3E enzyme had reduced cooperativity, had an increased K_A, and could only be activated to 23% of the wild-type level. Even more impaired was the 3RR/3E enzyme which was activated with no, or even slightly negative, cooperativity. The maximal activation to 6% of the wild type-level for this mutant enzyme is only a 60-fold increase over the unactivated basal rate. This likely represents the extent of activation by sites

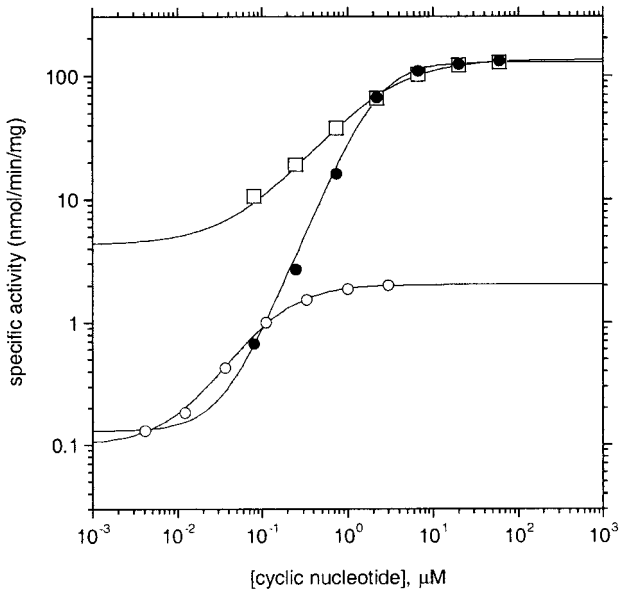

FIGURE 4: Activation of Et-PKG by cGMP and 8-NBD-cGMP singly and in combination. The cyclic nucleotide titrated is cGMP (closed circles), 8-NBD-cGMP (open circles), and cGMP in the presence of fixed 2 μM 8-NBD-cGMP (open squares).

PKA RIα site A	198	FG**E**LALIYGTP**R**AATV
PKA RIα site B	322	FG**E**IALLMNRP**R**AATV
PKG Iα site A	165	FG**E**LAILYNCT**R**TATV
PKG Iα site B	289	FG**E**KALQGEDV**R**TANV
Et-PKG site 1	236	FG**E**ISLIHNSA**R**TATI
Et-PKG site 2	355	FG**E**RALLYDEP**R**SATI
Et-PKG site 3	633	FG**E**RALLHDER**R**SATV

FIGURE 5: Partial sequence alignments of the cGMP binding sites of PKA (*12*), bovine PKG (*21*), and Et-PKG (*2*).

1 and 2 alone, and is similar in magnitude to the activation of wild-type enzyme by 8-substituted cGMP analogues.

Comparative titrations for the various constructs with 8-NBD-cGMP and the parameters obtained are presented in Figure 6B and Table 4, respectively. The mutant enzymes

Table 3: Activation Parameters for Et-PKG Proteins with cGMP[a]

enzyme form	% of WT max	K_A (μM)	h
WT	(100)	2.2 ± 0.1	1.7 ± 0.1
1R/2R	125	70 ± 20	1.0 ± 0.3
3RR	155	120 ± 10	0.9 ± 0.04
1R/2R/3RR	51	1050 ± 50	2.3 ± 0.3
3E	23	24 ± 5	1.0 ± 0.2
3RR/3E	6	550 ± 380	0.7 ± 0.2

[a] Parameters are derived from curve fits to the titrations shown in Figure 6A. These are representative of experiments that were repeated at least twice.

Table 4: Activation Parameters for Et-PKG Proteins with 8-NBD-cGMP[a]

enzyme form	% of WT max	K_A (μM)	h
WT	(100)	0.13 ± 0.01	1.2 ± 0.1
1R/2R	140	20 ± 5	1.6 ± 0.6
3RR	58	0.066 ± 0.004	1.1 ± 0.1
1R/2R/3RR	33	56 ± 8	2.1 ± 0.5
3E	56	0.30 ± 0.11	1.0 ± 0.2
3RR/3E	46	0.064 ± 0.004	1.4 ± 0.1

[a] Parameters are derived from curve fits to the titrations shown in Figure 6B. These are representative of experiments that were repeated at least twice

could be maximally activated to 33−140% of the level for wild-type enzyme. Inspection of the data clearly reveals that the protein forms with mutations in sites 1 and 2 (1R/2R and 1R/2R/3RR) show substantially increased K_As. In contrast, the mutations in site 3 of the enzyme have little effect; while K_A increases very slightly for 3E, it actually decreases for 3RR and 3RR/3E. The striking behavior of the 3RR/3E enzyme, whose activation is drastically impaired with cGMP but is nearly identical to wild type with 8-NBD-cGMP, strongly supports the earlier inference that 8-NBD-cGMP, and likely other 8-substituted cGMP analogues, binds selectively to the tandem sites 1 and 2 of the regulatory domain of Et-PKG.

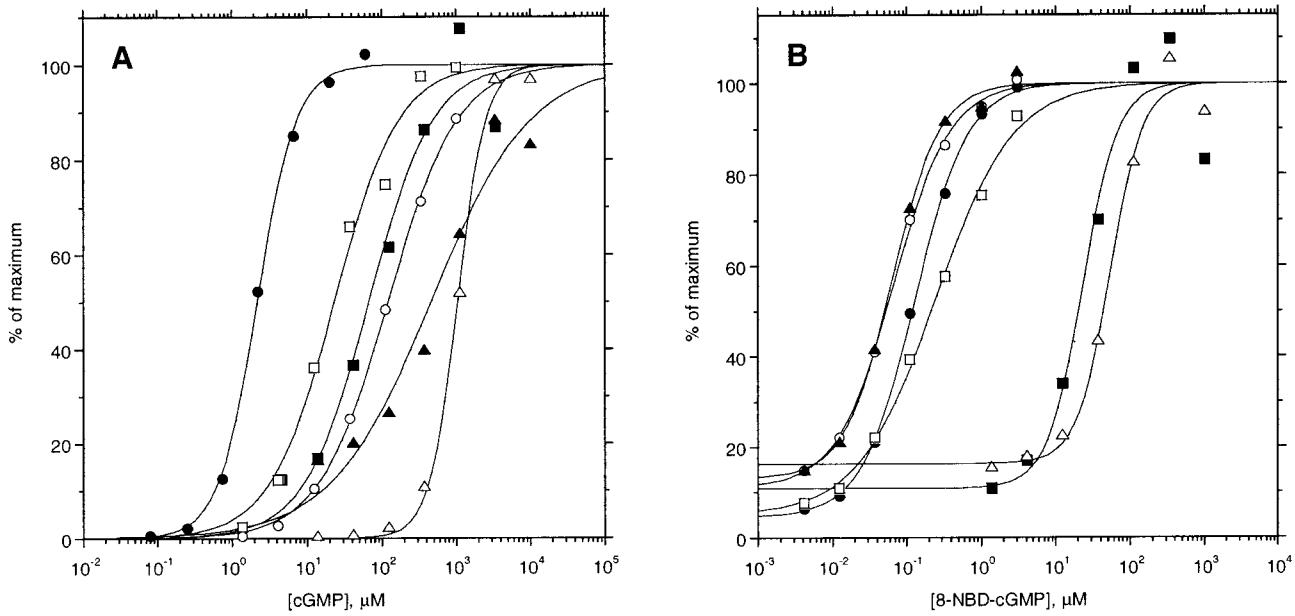

FIGURE 6: Activation by cGMP of wild-type and mutant Et-PKGs by cGMP (A) or 8-NBD-cGMP (B). The titrations are normalized so that the maximal activation level for each protein is 100%. Wild type (closed circles), 1R/2R (closed squares), 3RR (open circles), 1R/2R/3RR (open triangles), 3E (open squares), and 3RR/3E (closed triangles).

DISCUSSION

Among the novel biochemical properties of the PKG from *E. tenella*, perhaps the most intriguing is its allosteric mechanism for regulating activity. Since all known PKG regulatory domains of higher organisms contain two consensus cGMP binding sites, the discovery of a putative third cGMP binding site in the primary sequence of Et-PKG was unexpected. Consequently, we wondered whether this site was indeed functional and could account for the unusual activation properties of the enzyme. The large magnitude of cyclic nucleotide stimulation, as well as the steep sensitivity to cGMP level and micromolar activation constant, all deviate significantly from the well-studied bovine Iα isoform of PKG. For example, under our conditions, we found this mammalian PKG to be maximally activated, depending on source, 14−24-fold by cGMP with little cooperativity (h = 1.0−1.2) at submicromolar concentrations (K_A < 200 nM). While the fold activation and activation constants are consistent with literature, Hill coefficients have not been routinely reported and are more difficult to compare. Although there is one early report of significant cooperativity ($h = 1.57 \pm 0.09$) (*13*), the activation curves for both native and recombinant Iα kinase in the figures of more recent publications (*14*, *15*) appear to support the lower Hill coefficient measured in this report. The DG1 PKG from *Drosophila* also appears quite comparable to Iα kinase, exhibiting 20-fold activation with a K_A of 0.19 μM and an h of 1.28 ± 0.1 (*16*). Among other PKGs that have been at least partially characterized, a recombinant human Iβ isoform has been reported to have a K_A of 0.57 μM and an h of 1.41 ± 0.24 (*17*), while a recombinant mammalian type II PKG activates 16-fold with a K_A of 0.2 μM and an undetermined h (*1*).

The occupation of both cGMP sites in mammalian PKGs is required for full activation (*11*). Binding studies with bovine Iα enzyme have characterized one cGMP site as fast dissociating/low affinity and the other site as slow dissociating/high affinity (*13*). The sites are also distinguishable on the basis of their selectivity for cGMP analogues; analogues substituted at N-1 of the purine ring are less well tolerated at the slow site, while analogues with substitutions at C-8 of the purine ring are disfavored at the fast site (*11*). The maximal selectivity for binding does not exceed 20-fold for the analogues that have been tested with this PKG isoform, and despite varying selectivity, all are apparently capable of complete activation (*11*).

We found quite different behavior for activation of Et-PKG by cGMP analogues. While pyrimidine-modified analogues (PET-cGMP and 1-NH_2-cGMP) were capable of near full activation, the imidazole-substituted analogues were significantly impaired (Table 1). Upon close inspection, we found that the analogues with the poorest fold activation also showed little cooperativity. In fact, it may be seen in Table 1 that the magnitude of stimulation correlates with cooperativity as measured by the Hill coefficient. This was the first indication that not all of the cGMP sites are available to the 8-substituted cGMP analogues. We subsequently examined 8-NBD-cGMP in greater detail with both Et-PKG and bovine Iα PKG. This analogue has previously been shown to bind the mammalian PKG with high affinity (*18*), although kinase activation data were not presented in the paper. We found 8-NBD-cGMP to be a full activator of Iα kinase with slightly higher h than cGMP (Table 2 and Figure 3A,B). Despite having the lowest activation constant among the analogues tested against Et-PKG, 8-NBD-cGMP was quite anemic in stimulating parasite kinase activity compared to cGMP. However, the activation parameters were fairly similar to those for mammalian PKG. This suggested to us that the partial activation of Et-PKG is analogous to the full activation of Iα kinase in using only the tandem cGMP sites. This model requires exclusion of the analogue from site 3 of Et-PKG. In support of this hypothesis, cGMP could additionally activate Et-PKG even when 8-NBD-cGMP was present at a concentration 10-fold higher than its activation constant (Figure 4). The Hill coefficient of 1 supports the idea that it is a single site, presumably site 3, that is being titrated in this experiment to give maximal activation. This degree of selectivity for a cGMP analogue between sites in a PKG is unprecedented in the literature, although the RII subunit of PKA discriminates against some 8-substituted cAMP analogues by factors exceeding 1000 (*19*).

While our analogue studies appeared to reveal functional differences within the cGMP binding sites of Et-PKG, assigning definitive roles to each site required site-directed mutagenesis. Our goals were to unambiguously determine whether site 3 of Et-PKG is functional in the allosteric regulation of kinase activity and, if possible, to dissect the individual role of this site in the activation mechanism. Mutagenesis in mammalian Type I PKGs has been restricted to conserved threonine residues that are critical for defining the cyclic nucleotide selectivity for cGMP versus cAMP (*14*, *17*, *20*). These are not highly deactivating mutations, however, as the K_A for cGMP is increased less than 39-fold. We referred instead to the X-ray crystal structure of the regulatory domain of PKA (*12*). Alignment of the regulatory domain protein sequences for Et-PKG, bovine Iα PKG, and PKA enabled us to select arginine and glutamate residues with potentially important roles in cyclic nucleotide binding (Figures 1 and 5).[2] Truncation (by mutation to alanine) of the arginine side chains, which putatively interact with the phosphodiester of cGMP, in either sites 1 and 2 or site 3 alone of Et-PKG increased K_As significantly while reducing h (Figure 6A and Table 3). While weakened, binding was apparently not eliminated since both proteins could still be fully activated.

While these results confirmed that site 3 does contribute to the overall activation of Et-PKG by cGMP, we sought a more complete loss-of-function mutation by deleting the conserved glutamate, singly and in combination with the deletion of conserved arginines. By itself, the site 3 glutamate mutant was significantly impaired in its ability to activate and had a 10-fold elevated activation constant with no cooperativity (Table 3). When combined with arginine mutations in site 3, the enzyme was further crippled; the mere 60-fold activation over the basal rate (as compared to 1000-fold for wild type) likely represents the contribution of sites 1 and 2 alone. The magnitude of this activation is comparable to that achieved with the 8-substituted cGMP analogues on wild-type enzyme, and is consistent with the selective binding

[2] While this manuscript was in preparation, a report appeared which showed that the mutation of conserved glycines to glutamates in the cGMP sites of Type II PKG is fully deactivating (*1*).

model for these analogues. The titrations of 8-NBD-cGMP against the various mutants (Figure 6B and Table 4) provide further support for the hypothesis. In contrast to the substantial effects seen upon activation with cGMP, the site 3 mutations had no impact upon activation by 8-NBD-cGMP. Only the mutations in sites 1 and 2 perturbed the activation by the analogue (>100-fold increase in K_A). Taken together, the results of the site-directed mutagenesis experiments confirm the initial conclusions of the cGMP analogue studies, namely, partial activation of Et-PKG by 8-substituted analogues occurs because of restricted binding to sites 1 and 2 alone, while full activation requires cyclic nucleotide binding in all three allosteric sites. Preliminary experiments have also shown that each of the mutant enzymes studied here has reduced binding of cGMP compared to wild-type enzyme (R. Donald, unpublished data).

The working model for cGMP activation of PKG is similar to that for cAMP activation of PKA (*3*). In the absence of cyclic nucleotide, the enzymes are held in a catalytically inactive state by the interaction between the autoinhibitory segment of the regulatory domain and the catalytic domain. Binding of cyclic nucleotides induces conformational changes which displace the autoinhibition domain and free up the kinase active site; the activation mechanism might thus better be described as "derepression". We found under our experimental conditions that the specific activity of fully activated bovine Iα PKG (250−460 nmol min^{-1} mg^{-1}; Table 2) is approximately 2−3.6-fold greater than that for maximally activated Et-PKG (130 nmol min^{-1} mg^{-1}). Adjusting for the molecular mass differences of these proteins (110 vs 75 kDa), the specific activity difference translates to only a 1.4−2.5-fold difference in k_{cat}. Since the catalytic turnover numbers of the "free" catalytic domains are nearly equivalent, the large difference in maximal stimulation for the PKGs must arise from a substantially lower basal activity of Et-PKG. It may thus be postulated that one role of the presumptive additional cGMP site in Et-PKG is to establish a more tightly repressed state for the unactivated enzyme. Until the physiological role of PKG in apicomplexan parasites is known, it will likely remain unclear why such strict regulation of the kinase activity is necessary.

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BI0156658