Purification and Molecular Characterization of cGMP-dependent Protein Kinase from Apicomplexan Parasites

A NOVEL CHEMOTHERAPEUTIC TARGET*

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The trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidin-4-yl)-1H-pyrrol-3-yl]pyridine (Compound 1) inhibits the growth of Eimeria spp. both in vitro and in vivo. The molecular target of Compound 1 was identified as cGMP-dependent protein kinase (PKG) using a tritiated analogue to purify a ~120-kDa protein from lysates of Eimeria tenella. This represents the first example of a protozoal PKG. Cloning of PKG from several Apicomplexan parasites has identified a parasite signature sequence of nearly 300 amino acids that is not found in mammalian or Drosophila PKG and which contains an additional, third cGMP-binding site. Nucleotide cofactor regulation of parasite PKG is remarkably different from mammalian enzymes. The activity of both native and recombinant E. tenella PKG is stimulated 1000-fold by cGMP, with significant cooperativity. Two isoforms of the parasite enzyme are expressed from a single copy gene. NH₂-terminal sequence of the soluble isoform of PKG is consistent with alternative translation initiation within the open reading frame of the enzyme. A larger, membrane-associated isoform corresponds to the deduced full-length protein sequence. Compound 1 is a potent inhibitor of both soluble and membrane-associated isoforms of native PKGs, as well as recombinant enzyme, with an IC₅₀ of <1 nM.

Protozoan parasites of the genus Eimeria are the causative agents of the intestinal disease known as coccidiosis. Coccidiosis occurs in several domesticated and wild animal species, but of major economic importance is the impact that Eimeria spp. have on the poultry industry. During acute infections, these parasites cause significant morbidity and mortality in broiler breeds of chicken (reviewed in Ref. 1). Anticoccidial compounds have been and continue to be used prophylactically in the majority of poultry operations today. The most successful anticoccidials have been the polyether ionophores, a family of compounds that continues to be the industry standard since their introduction nearly 30 years ago (2). Not surprisingly, reports of resistance development due to the extended and constant chemotherapeutic pressure exerted by this class of compounds are not uncommon (3, 4). Since that time no novel anticoccidials with efficacy and economic features that approach the ionophore class have been introduced into the poultry industry. The need to identify and develop new drugs for the control of coccidiosis is critically important. In this report we describe the chemotherapeutic efficacy of a novel anticoccidial reagent. Data from biochemical purification and molecular cloning efforts predict that the therapeutic target of this class of compounds in Eimeria is a cGMP-dependent protein kinase (PKG).¹ PKG transfers the γ-phosphate of ATP in a cGMP-dependent reaction to serine and/or threonine residues of several cellular proteins (5). Cyclic GMP is a ubiquitous intracellular messenger that has a role in several aspects of signal transduction that potentially regulate a myriad of physiological processes (reviewed in Ref. 6). Cyclic GMP also modifies the activity of proteins other than PKG, including cGMP-gated ion channels and cGMP-regulated phosphodiesterases (6).

Cyclic nucleotide-dependent protein kinases from unicellular organisms such as Paramecium to humans have been biochemically characterized and/or cloned (6–9). Members of this group of kinases share sequence homology in both their regulatory and catalytic domains. The most striking feature that distinguishes cAMP-dependent (PKA) from cGMP-dependent protein kinases is that PKA exists as a heterotetramer in its inactive conformation, composed of two identical regulatory and two identical catalytic subunits, while PKG is in most cases a homodimeric enzyme (6, 10). The regulatory and catalytic subunits of PKA are distinct gene products. Activation of PKA by cAMP occurs as a result of a conformational change in the enzyme initiated by the binding of two molecules of the cyclic nucleotide to each regulatory subunit. The conformational
change releases the regulatory dimer from the inhibited complex, thereby activating the catalytic dimer (11, 12). Unlike PKA, the nucleotide-binding/regulatory and catalytic domains of PKG are part of the same protein. In the absence of cGMP, PKG assumes a conformation that is autoinhibited (13). Binding of cGMP to two nucleotide-binding domains within each subunit of the homodimer causes an intramolecular conformational change that activates kinase activity. While the two families of enzymes have significant structural differences, the mechanisms of autoinhibition and activation by the respective cyclic nucleotide cofactors are similar.

In this report we demonstrate that Apicomplexan parasite PKG has several features that distinguish it from PKG homologues in other organisms. Unlike mammalian and invertebrate PKG enzymes which are typically homodimers, the *E. tenella* enzyme is a monomeric protein. The *E. tenella* enzyme has remarkably different cGMP activation kinetics, characterized by a minimal basal kinase activity in the absence of nucleotide cofactor which is induced by as much as 1000-fold upon addition of cGMP. Moreover, the activation profile shows strong cooperativity, a feature that is not as striking in mammalian PKG. The size of the parasite enzyme, demonstrated biologically and confirmed by cDNA cloning of five Apicomplexan PKGs, is 30–40% larger than most PKG enzymes that have been described. Consistent with the architecture of other PKGs, the nucleotide-binding/regulatory domain of the parasite enzymes is located toward the amino-terminal end of the protein and the catalytic domain is positioned C-terminal. A conserved parasite signature sequence of nearly 300 amino acids resides between the regulatory and catalytic domains of the protein. Within this region we have identified a third nucleotide-binding site, yet another feature that is unique to, but shared among, the collection of Apicomplexan parasite enzymes.

**EXPERIMENTAL PROCEDURES**

**Parasite Preparation and in Vico Studies—**Chickens were infected orally with 7.5 × 10^6 *Eimeria tenella* L518 sporulated oocytes. The unsporulated oocytes were harvested from the ceca 7 days post-infection and purified according to the method of Schmatz et al. (14), and then sporulated by continual agitation for 36 h at 29°C. For the *in vivo* studies drug was administered in feed at 50 ppm on day 1. Birds were infected with 3.5 × 10^6 *E. tenella* sporulated oocytes on day 2 and 3 × 10^6 *Eimeria acervulina* sporulated oocytes on day 3. Medicated feed was continuously available for the duration of the experiment. The experiment was terminated on day 8 and efficacy was estimated by performing oocyst counts.

**Determination of in Vitro Antiprotozoal Activity—**Conditions for the *in vitro* culture of parasites and determination of IC₅₀ and or minimal inhibitory concentrations (defined as the lowest concentration (ng/ml) at which parasite growth was fully inhibited) for compounds were conducted according to previously described methods for *E. tenella* (14), *Toxoplasma gondii* (15), and Besnoitia jellisoni (16). The Neospora caninum cell based assay (17) was adapted for use by increasing length of assay from 5 days to 7 days. Twenty tryptic peptide sequences generated from the purified ligand-binding protein were used to design several degenerate ligand-binding oligonucleotides. Restriction and modifying enzymes were purchased either from Invitrogen or New England Biolabs. Electroporation competent bacterial cells were from Invitrogen.

Seven tryptic peptide sequences generated from the purified *E. tenella* ligand-binding protein were used to design several degenerate oligonucleotides. These were used in coupled reverse transcription-PCR reactions with mRNA from *E. tenella* sporozoites to yield PCR products of interest were excised and sequenced. A pooled cDNA clone generated by RT-PCR was used as a hybridization probe to isolate full-length cDNA clones from an *E. tenella* unsporulated oocyst cDNA library (19). Clones coding for *T. gondii* PKG were isolated from a cDNA library obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD, catalog number 1986). Clones were identified by heterologous screening at a reduced hybridization stringency using a

either filtered through Whatman GF/F glass fiber filters (presoaked in 0.6% polyethyleneimine for 1 h at 25°C), or filtered through prepackaged gel filtration columns (800 μl, Edge Biosystems). The filters were washed with 100 nm NaCl, 10 mM Tris, pH 7.4, dried, and radioactivity determined by scintillation counting using Ready-SAFE scintillation fluid (PerkinElmer Life Science) which had been equilibrated in Buffer A with 100 nm NaCl. The column was washed with 100 nm NaCl in Buffer A and then eluted with a linear salt gradient (1 M NaCl) which was started once the unbound proteins had been eluted. The Compound 1 binding activity, which did not bind to the column in 100 nm NaCl, was dialyzed against Buffer A and re-applied to the same column. After unbound proteins had been eluted in the absence of NaCl and the absorbance had returned to baseline, column bound proteins were eluted with a NaCl gradient to 1 M (Fig. 2B) in Buffer A. Fractions were collected and aliquots were assayed for binding activity and also for purity on polyacrylamide gels. In some fractions with ligand binding activity were then applied to a Superdex 200 column in Buffer A and analyzed as described above. SDS-polyacrylamide gel electrophoresis was performed on gradient gels (Novagen). Blotting onto nitrocellulose and immunoreactive proteins were detected using the ECL procedure (Amersham Bioscience). Protein sequence analysis of the purified protein was performed on Coomassie Blue-stained gel slices. The slices were digested with trypsin, peptides separated by high performance liquid chromatography on C18 reverse phase chromatography, and sequence performed on isolated peptides using Edman degradation.

**Ligand Binding Assay—**Soluble extracts for binding studies were prepared by vortexing 2 × 10^9 *E. tenella* unsporulated oocytes with an equal volume of buffer (10 mM HEPES pH 7.4, 1 mM sodium orthovanadate, 20% glycerol, 0.1 mg/ml Bactin-tracin, and 0.5% Sigma protease inhibitor mixture P8340), and an equal volume of 4-mM glass beads for 20 min. The resulting homogenate was centrifuged (100,000 × g, 1 h) and the supernatant (S100) used directly. *E. tenella* protein (10–25 μl) was assayed in 100 μl at a final concentration of 75 nm Tris, pH 7.5, 12.5 mM MgCl₂, 1.5 mM EDTA, and 2 μM [³H]Compounds 1 (60 Ci/mmol). Nonspecific counts were estimated using a 1000-fold molar excess of unlabeled Compound 1. Samples were incubated for 1 h at 25°C, and

**SUMMARY**

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B. Nare, unpublished observations

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2 B. Nare, unpublished observations.
portion of the *E. tenella* cDNA as probe. A *Cryptosporidium parvum* expressed sequence tag containing a fragment of a PKG homologue was identified in a preliminary survey sequence analysis of the *Cryptosporidium parvum* genome (expressed sequence tag AAS083827 (20)). To obtain the complete *C. parvum* PKG gene, a PCR fragment derived from this expressed sequence tag was used as a probe in a *C. parvum* genomic library (number 1644, NIH AIDS Research and Reference Reagent Program). A PKG open reading frame spanning two overlapping EcoRI genomic clones was subsequently identified by DNA sequence analysis.

An identical un-annotated open reading frame (Contig number 1655) was also located in a BLAST search of the partial *C. parvum* genome sequence database that was accessible through the NTI by the *Plasmodium Minnesota* *C. parvum* sequencing project (www.ncbi.nlm.nih.gov/Genome/Bin/Entrez/genome_table_cgi (21)). An alternative strategy was employed to isolate PKG cDNA clones from both *Eimeria maxima* and *Plasmodium falciparum* parasites. The first step involved a coupled RT-PCR to generate partial PKG cDNAs for each parasite. Using an alignment of the deduced amino acid sequence from cDNA clones coding for *E. tenella* and *T. gondii* PKG proteins, areas of sequence identity within the presumptive cGMP-binding (peptides a, VKFFEM; and b, GEFGERAL) and catalytic domains (peptides c, RDLKPEPN; and d, HYMAPEV) were identified. Total RNA purified from *E. maxima* sporulated oocysts and a trophozoite-enriched preparation of *P. falciparum* was first converted into cDNA using reverse transcriptase. The respective cDNA libraries of cDNA products were then used as templates in PCR reactions using degenerate oligonucleotide primers from peptides a and d. Primary PCR reaction products were then used as templates in secondary nested PCR reactions using degenerate primers from peptides b and/or c. PCR reaction products produced in the secondary nested reactions were subcloned, DNA sequence confirmed, and then used as hybridization probes to screen *E. maxima* and *P. falciparum* cDNA libraries to isolate full-length cDNA clones.

PKG cDNA and deduced protein sequence for *E. tenella*, *E. maxima*, *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Plasmodium falciparum* have been deposited in GenBank™ with accession numbers AF411961, AF465543, AF435130, AF435171, and AF465544. Sequences were aligned using the modified ClustalW algorithm MultiClustal 22). The resulting alignment was shaded using Genedoc (available at www.cris.com/–/ketchapp/genedoc.shtml) and Adobe Illustrator.

Parasite cDNAs encoding PKG open reading frames were modified prior to subcloning by appending an NH2-terminal or COOH-terminal FLAG epitope (Kodak) via PCR amplification (Phu polymerase, Stratagene). DNA fragments encoding FLAG epitope-tagged PKGs were placed in an expression vector under the control of a *Toxoplasma* *α-tubulin* promoter (25). Following transfection, transgenic *Toxoplasma* lines were selected with chloramphenicol (24, 25) and clones expressing recombinant PKG were identified by immunofluorescence analysis with FLAG M2 antisera (Sigma). Recombinant FLAG-tagged *Eimeria* PKG was purified from parasite lysates by FLAG immunoprecipitation as described for the recombinant Torpedo PKG 2.

Production of Polyclonal Antisera—Antisera were raised in rabbits to the following peptides based on amino acid sequence of the *EtpKG* full-length clone: EDITQAEDARLGLHKEKRT (TR3) and EED-EGIELEDEYEWDKEPD (TR6). Both peptides were conjugated to Keyhole Limpet hemocyanin prior to immunization. Antibody production was performed at Covance Research Laboratories, Denver, PA.

PKG Catalytic Assay—Kinase activity was detected using a peptide substrate and [γ-32P]ATP. An aliquot containing enzyme (1 μl) was mixed with a reaction mixture (10 μl) whose composition is as follows: 25 mM HEPES pH 7.4, 10 mM MgCl2, 20 mM β-glycerophosphate, 5 mM β-mercaptoethanol, 10 μM cGMP, 1 mg/ml bovine serum albumin, 400 μM Kemptide, or 9 μM myelin basic protein, 2 μM [γ-32P]ATP (0.1 mCi/ml). The reaction was allowed to proceed for 1 h at room temperature and then terminated with the addition of phosphoric acid to a final concentration of 25 mM. Labeled peptide was captured on PS1 filters or on Millipore 96-well plates (MAB-NOH). In both cases filters were washed with 75 mM phosphoric acid, dried, and 32P-labeled phosphopeptide was detected by liquid scintillation counting.

Kinetic Characterization of *E. tenella* PKG—AMP-PCP and guanethidine (1-(2-guanidinoethyl)octahydroazocine) were obtained from Biochemical and Molecular Characterization of cGMP-dependent Protein Kinase

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3 Donald, R. G. K., Alloco, J., Nare, B., Singh, S. B., Salowe, S. P., Wiltsie, J., and Liberato, P. (2002) Eukaryotic Cell, in press.
peak of binding activity from MonoQ are visualized by silver staining in Fig. 2C. Two proteins with apparent molecular weights of 120,000 and 150,000 are coincident with the peak of binding activity. Subsequent gel filtration chromatography of a pool of fractions 34 and 35 was able to enrich for the 120- and 150-kDa pair, but was not able to resolve the two proteins (data not shown).

**Compound 1-binding Protein Is a cGMP-dependent Protein Kinase—**The structural similarity between Compound 1 and protein kinase inhibitors that have been described in the literature (29, 30) prompted us to assay for and detect kinase activity (using the peptide substrate Kemptide) in the ligand binding fractions during the purification protocol. However, protein kinase activity was marginal (data not shown), even in the final fractions from the MonoQ column pictured in Fig. 2C.

Following electrophoretic resolution of the column purified ligand binding activity, both the 120- and 150-kDa proteins were submitted for sequence analysis. Seven tryptic peptide sequences were generated from the 120-kDa protein (Table I), but no sequence information was retrieved from the larger protein. Each of the peptides was used as a query to search *in silico* translations of nucleotide data bases as well as protein data bases, but no significant matches were found. Several degenerate oligonucleotides were designed based on the first four peptides and used in various combinations in coupled RT-PCR reactions with *E. tenella* sporozoite mRNA as template. One primer set consisting of oligonucleotides from peptides 3 and 4 generated a PCR product that was successfully nested in a secondary reaction with a second oligonucleotide from peptide 3 along with the original peptide 4 oligonucleotide. The primary PCR product from this series was cloned and its 452-nucleotide insert has an open reading frame that is 31% identical to both *Drosophila melanogaster* PKG (PID:g140293) and the regulatory subunit of *Saccharomyces cerevisiae* PKA (PID:g172689).

Based on the prediction that the 120-kDa ligand-binding protein is a cyclic nucleotide-dependent protein kinase, enzyme activity in the purified fractions was assayed in the presence of cyclic nucleotides. While cAMP only provided a 2–3-fold stimulation (data not shown), cGMP stimulated kinase activity by ~500–1000-fold (Table II). Moreover, Compound 1 inhibited the cGMP-dependent activity with an IC50 of ~0.6 nM. These data are consistent with the conclusion that the antiparasitic activity of Compound 1 is due, at least in part, to inhibition of PKG.
An assay of nucleotide-dependent kinase activities in fractions of crude *E. tenella* S100 resolved by HiLoadQ chromatography is shown in Fig. 3. As noted earlier using the ligand binding assay, there was a small amount of nucleotide-dependent kinase activity that was not retained by this matrix. There was only a single peak of PKG activity in this initial purification step, and this activity was inhibited by Compound 1 (IC$_{50}$ of 0.6 nM). The peak of ligand binding activity co-eluted with the PKG activity (data not shown). In addition, there were two areas of PKA activity in this chromatographic profile. Both of these were resolved from the cGMP dependent activity and the cAMP dependent activity in these fractions was considerably less sensitive to Compound 1 (IC$_{50}$ of > 600 nM).

To further demonstrate that the purified 120-kDa ligand-binding protein is parasite PKG, in-gel kinase assays were performed. The MonoQ purified fractions pictured in Fig. 2C were separated in this gel system. The autoradiographs shown in Fig. 4A demonstrate that kinase activity was detected in the area of 120 kDa in the presence of cGMP. No activity was detected in the absence of cGMP. Nucleotide-dependent in-gel kinase activity was inhibited by Compound 1 (data not shown). No activity was detected in the area corresponding to the 150-kDa protein that co-purified with *E. tenella* PKG through the 4-column protocol. The peak of Compound 1 binding activity following MonoQ ion exchange chromatographic fractionation as shown in Fig. 2, was further characterized using cGMP-agarose affinity chromatography. Elution of PKG activity is coincident with ligand binding activity (Fig. 4B). A silver-stained gel of the cGMP eluate highlights an enrichment of the 120-kDa protein (Fig. 4C). Taken together, these data strongly support the conclusion that the purified 120-kDa ligand-binding protein from *E. tenella* is a PKG.

**Kinetic Characterization of *E. tenella* PKG**—A detailed analysis of the kinetic mechanism of purified native *E. tenella* PKG (ETPKG) was undertaken using a biotinylated PKI-derived peptide substrate. Systematic variation of both substrate concentrations yielded an intersecting double reciprocal plot (not shown) with $K_m$ values of 12 ± 2 μM for ATP and 19 ± 1 μM for the peptide (Table II). These results establish that ETPKG employs, in common with all protein kinases, a sequential kinetic mechanism wherein both substrates are bound to the enzyme prior to the release of product. Inhibition patterns with dead-end inhibitors were used to further elucidate the binding events. As indicated in Table III, the non-hydrolyzable ATP analog AMP-PCP was a competitive inhibitor versus ATP and noncompetitive with respect to peptide substrate. Conversely, guanethidine (a peptide competitive inhibitor) was competitive with the peptide substrate and noncompetitive versus ATP. These symmetrical inhibition patterns are consistent with the random addition of substrates to the enzyme. Compound 1 was also established to be a very potent ATP-competitive and peptide-noncompetitive inhibitor by a similar analysis.

**Cloning and Recombinant Expression of Parasite PKG cDNAs**—The partial cDNA clone generated as a RT-PCR product was used as a hybridization probe to screen an *E. tenella* unpurified oocyst cDNA library and several clones were carried to plaque purity. The largest cDNA clone in this group (PKG7) is 4283 nucleotides in length with a deduced open reading frame of 1003 amino acids, capable of coding for a protein of nearly 113 kDa. Clone PKG7 has a 614-nucleotide 5'-untranslated region and a 657-nucleotide 3'-untranslated region. Six additional cDNA clones were also full-length, each capable of coding for the same 1003-amino acid open reading frame. Each of the seven tryptic peptide sequences from the purified 120-kDa protein are contained within this open reading frame. The deduced amino acid sequence of the full-length purified 120-kDa protein are contained within this open reading frame. The deduced amino acid sequence of the full-length protein still most closely resembles Drosophila PKG; 51% identity to the cGMP-binding domains in the amino-terminal half of the protein and 45% identity to the catalytic domain at the carboxyl-terminal end of the kinase. PKG clones from *E. maxima*, *T. gondii*, *C. parvum*, and *P. falciparum* have also been isolated and the deduced amino acid sequences of the parasite proteins are aligned in Fig. 5A. The parasite PKG proteins are similar in size and, except at the extreme amino-terminal end, they share considerable sequence identity across their entire length.

The parasite proteins are also aligned in Fig. 5A with a human homologue, cGKII. It is immediately apparent that the parasite PKG proteins are considerably larger than the human enzyme and all other PKGs that have been described in the literature (generally 75–85 kDa). One notable exception is a splice variant of Drosophila PKG (31) that is predicted from cDNA clones to code for a 120-kDa protein. A schematic comparison of parasite and human PKG in Fig. 5B calls attention to a length of nearly 300 amino acids between the nucleotide-binding and catalytic domains that is found in the parasite PKG sequences, but is noticeably absent from human cGKII. The 300-amino acid signature sequence in the parasite proteins calls attention to the bulk of the increase in size relative to other
PKGs. Closer inspection of the amino acid sequence in this region has tentatively identified a third cGMP-binding site, which is absent in the human enzyme.

Stable heterologous expression of the E. tenella PKG cDNA clone with an amino-terminal FLAG-epitope tag (\textit{FLAG}EtiPKG) has been accomplished in the related Apicomplexan parasite \textit{T. gondii}.\textsuperscript{3,4} Native and recombinant EtPKG enzyme preparations are indistinguishable in so far as \( K_m \) for ATP and peptide substrates (summarized in Table II). Inhibition of native and recombinant enzyme activity by Compound 1 is also equivalent. The activity of both enzymes in the absence of cyclic nucleotide activator was extremely low and difficult to measure. The fold activation, which represents the quotient of the maximal rate and the unactivated rate, thus cannot be determined with great precision. This is unlike bovine PKG,\textsuperscript{4} which has detectable enzyme activity in the absence of nucleotide cofactor. The cGMP nucleotide cofactor activates both native EtPKG and recombinant \textit{FLAG}EtiPKG dramatically and to a similar extent, typically by 500–1000-fold (Table II). Analysis of activity in a cGMP titration revealed a strongly cooperative activation of both native and recombinant enzymes, with Hill coefficients of 2.3 and 1.8, respectively (Table II). The cyclic nucleotide-dependent activation kinetics of native and recombinant EtPKG are indistinguishable and yet are considerably different from mammalian PKG enzymes (33, 34).\textsuperscript{4}

\textit{Membrane-associated and Soluble Isoforms of Parasite PKG}—Polyclonal antisera were raised against two peptides derived from the deduced \textit{E. tenella} PKG sequence. In an S100 fraction prepared from \textit{E. tenella}, each antisera detects two immunoreactive proteins and one of these co-migrates with the purified 120-kDa protein (Fig. 6A). The antisera also cross-reacted with a 120-kDa protein in the peak binding fractions throughout the purification protocol (Fig. 6B). Upon final purification following MonoQ column chromatography, a second faster migrating immunoreactive band is evident. As this protein is not present in the S100 or in earlier fractions during purification, it is thought to represent a breakdown product. The 150-kDa protein that co-purifies with the 120-kDa protein does not react with either antibody.

An \textit{E. tenella} P100 fraction was also analyzed by Western blot analysis. Following extensive washes of the P100 fraction with buffer lacking detergent (see “Experimental Procedures”), only a single immunoreactive band was detected. This protein co-migrates with the slower migrating bands in the S100 fraction (Fig. 6C). In a Triton X-114 phase separation experiment, the slower migrating band partitioned into the detergent-enriched layer, while the smaller protein was found in the detergent-depleted layer (Fig. 6D). Both of these experiments suggest that the less mobile immunoreactive protein is membrane

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\begin{table}[h]
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Inhibitor & Varied substrate & Inhibition\textsuperscript{a} & \( K_m \) & \( K_u \) \\
\hline
AMP-PCP & ATP & C & 39 ± 2 & \text{\textmu}M \\
AMP-PCP & Peptide & NC & 86 ± 13 & 54 ± 6 \\
Guanethidine & ATP & NC & 4800 ± 900 & 5300 ± 500 \\
Guanethidine & Peptide & C & 2800 ± 300 & \\
Compound 1 & ATP & C & 0.00021 ± 0.00003 & \\
Compound 1 & Peptide & NC & 0.00047 ± 0.00008 & 0.00062 ± 0.00011 \\
\hline
\end{tabular}
\caption{Analysis of the kinetic mechanism of PKG}
\end{table}

\textsuperscript{a} C, competitive; NC, noncompetitive.

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\textsuperscript{4} Salowe, S. P., Wiltsie, J., Liberator, P., and Donald, R. G. K. (2002) \textit{Biochemistry}, in press.
associated. Since each of the proteins binds to and is specifically eluted from a cGMP-agarose affinity matrix (data not shown), it appears that they represent two isoforms of *E. tenella* PKG.

The soluble *E. tenella* PKG isoform has been purified in sufficient quantity and subjected to amino-terminal sequence analysis. The NH₂-terminal sequence begins with serine 49 within the open reading frame deduced from the cDNA clone. While this could represent a proteolytic degradation product, Ser⁴⁹ is adjacent to Met⁴⁸ which in turn is in a good translation initiation context. Accordingly, the soluble PKG isoform might be the product of an internal translation initiation event. The membrane-associated PKG isoform, which is believed to be acylated at the amino terminus (35), has not been sequenced. The membrane-associated and soluble isoforms of native PKG are equally sensitive to inhibition by Compound 1, with IC₅₀...
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values of 0.86 and 0.6 nM, respectively. Likewise, FLAGEtPKG is inhibited by Compound 1 with an IC50 of 0.6 nM.

E. tenella PKG Is a Monomeric Protein—While most PKG enzymes are homodimeric, dimerization is not required for catalytic activity (36). Dimer formation is dictated by a leucine zipper motif that is located at the amino-terminal end of PKG proteins (37, 38). However, no such motif can be detected within the parasite PKG sequence. Native EtPKG purified as a soluble protein from S100 and analyzed by gel filtration chromatography appears monomeric, with an apparent molecular weight between 113,000 and 125,000 (data not shown). Since the soluble native protein lacks the initial 48 amino acids predicted from the cDNA clone, we cannot dismiss a potential role for these sequences in dimerization. However, the full-length recombinant protein FLAGEtPKG also behaves as a monomer (data not shown). This demonstrates that the presence of the initial 48 amino acid residues of the open reading frame is not sufficient to cause dimerization. Experiments designed to estimate the molecular weight of the membrane-associated isoform of PKG are hampered by the association of the proteins with detergent micelles and are not reported here.

DISCUSSION

The results presented in this article clearly demonstrate the antiparasitic activity of Compound 1, both in cell based assays and animal models (Fig. 1). Compound 1 is structurally similar to known protein kinase inhibitors (29, 30, 39, 40) and we show here that this compound does inhibit a kinase in the parasite, namely PKG. Like other protein kinase inhibitors, Compound 1 is competitive with respect to ATP. Structural conservation among protein kinases at the ATP-binding site predicts that inhibitor selectivity might represent a substantial obstacle. Purification of Compound 1 binding activity suggests otherwise. Only a single peak of ligand binding activity was detected throughout the purification scheme. While secondary binding sites with lower affinity or rapid off rates may not be detected using the assay conditions described here, we can conclude that PKG does represent the highest affinity and primary Compound 1-binding site in E. tenella.

The ability of Compound 1 to selectively inhibit parasite enzyme activity is a critical requirement for development as an antiparasitic compound. The area of greatest conservation between parasite and human PKG corresponds to the catalytic domain, where there is 44% identity (Fig. 5B). Modeling of the catalytic domain of mammalian PKG using the coordinates for the catalytic subunit of PKA, has called attention to several residues critical for ATP binding and catalytic activity (41). Each of these residues is conserved in the parasite PKG clones described here. Despite the amino acid identity of residues critical for ATP binding and catalytic activity, Compound 1 is a selective inhibitor of parasite enzyme activity. Chicken lung PKG, affinity purified by cGMP-agarose, and ion exchange chromatographies, is poorly inhibited by Compound 1(IC50 of 9.3 μM).5

In the absence of cyclic nucleotide, PKG resides in an auto-inhibited conformation (13, 42) in which the amino-terminal end of the protein interacts with the catalytic domain. Even with this type of structural regulation, mammalian PKG activity can be detected without nucleotide cofactor. Addition of cGMP to the bovine cGKI isoform activates basal kinase activity up to 24-fold with little cooperativity.4 In contrast, both native and recombinant preparations of E. tenella PKG have extremely low kinase activity in the absence of nucleotide cofactor. Upon addition of cGMP, the magnitude of stimulation of parasite kinase activity is up to 1000-fold and the Hill coefficient of ~2 predicts that cyclic nucleotide activation is a highly cooperative event (Table II).

The deduced amino acid sequence of PKG from multiple Apicomplexan parasite cDNA clones (Fig. 5, A and B) contains a block of nearly 300 amino acids that does not align with mammalian or invertebrate PKGs. Located in this region of the parasite proteins is a putative third cGMP-binding site, a feature that distinguishes the parasite enzymes from all other PKGs. The consensus for nucleotide binding is not striking (e.g. PKG site I, Glu235(1)-Arg236(2)-Thr238(3), PKG site II, Glu357-X358(1)-Arg359-Ser360(2), PKG site III, Glu637-X638-Arg644-Ser645(3) and only two residues are absolutely conserved for both cAMP and

5 P. Dulska and J. Patel, unpublished data.
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The x-ray crystal structure of the PKA regulatory domain (11) calls attention to a glutamic acid residue that hydrogen bonds with the 2'-OH of the nucleotide ribose. This is followed nine residues later by a conserved arginine that interacts with the phosphodiester of the cyclic nucleotide. The following amino acid, either a serine or threonine in PKG but not PKA, forms a hydrogen bond with the C-2 amino group of guanine in cGMP. Site-directed mutagenesis of mammalian kinases to replace conserved T/S residue, had marked effects on the relative cGMP versus cAMP binding affinities of PKG and PKA (45). Using a series of nucleotide analogs along with site-directed mutations engineered at each of the three parasite cGMP-binding sites, we now know that each site functionally contributes to the unique activation kinetics of the parasite kinases (35).4

Biochemical analysis demonstrates that there are at least two isoforms of parasite PKG (Fig. 6), a characteristic feature of mammalian cGMP-dependent kinases. However, unlike mammals, analysis of E. tenella6 and T. gondii genomic DNA3 indicates that PKG is coded for by a single gene in these organisms. The two genes typically found in animals code for related but distinct PKG isoforms. In some cases a primary transcript is also differentially spliced to generate further isoform heterogeneity (42). Exhaustive screening of E. tenella and T. gondii cDNA libraries never identified differentially spliced clones that might explain the presence of the two parasite isoforms. Expression of the full-length EtPKG and TgPKG cDNA clones each produces two proteins with electrophoretic mobilities that align with the native isoforms (35). Amino-terminal sequence of the soluble native isoform of E. tenella PKG has identified Ser49 as the first amino acid. This residue is adjacent to Met48, which in turn is in a good translational initiation context; a counterpart exists at Met103 in TgPKG.

These results suggest that the truncated soluble PKG isoform could result from a secondary translational initiation event at an internal methionine codon. This conclusion is supported by mutagenesis studies (35) which demonstrate that differential isoform expression can be modulated by amino acid substitutions that alter potential initiator methionine codons M1, M48 (EtPKG), and M103 (TgPKG).

The NH2-terminal amino acid sequence of EtPKG and TgPKG deduced from the respective cDNA clones share a dual acylation signal (MGAC/S/ISK). Site-directed mutagenesis experiments conclusively demonstrate that both of these enzymes, when expressed as recombinants, are myristoylated at G-2 and palmitoylated at C-4 (35). Transfection studies have allowed us to conclude that acylation is capable of directing membrane association of the larger PKG isoform. Similarly, myristoylation of mammalian cGKII is required for membrane partitioning of this PKG isoform. Membrane association of this isoform is critically important, enabling it to regulate CPT, a reversibly phosphorylated integral membrane Cl− channel (46).

The two forms of E. tenella PKG that can be detected immunologically (Fig. 6) have different properties presumably dictated in part by their acylation status. Triton X-114 phase separation experiments (Fig. 6D) demonstrate that the higher molecular weight isoform is hydrophobic and the lower molecular weight isoform is hydrophilic. Although it might be predicted that the larger isoform would preferentially remain with the P100 fraction, we have not found this to be the case (Fig. 6C). The S100 fraction always contains an amount of the hydrophobic isoform. Extended washes of the P100 fraction in the absence of detergent are able to remove all traces of the soluble isoform under conditions where at least some of the hydrophobic isoform remains membrane associated. Consistent with these observations, proteins that are acylated, often only weakly associate with membranes (reviewed in Ref. 47) and have been found in soluble biochemical fractions (46).

When the E. tenella S100 fraction is applied to an anion-exchange column, Western blot analysis confirms that the hydrophobic isoform is found in the flow-through (Fig. 6B). If the initial parasite extract is prepared in and subjected to ion exchange chromatography in detergent, the two isoforms bind and co-elute in this fractionation step. The most obvious explanation for this result is that the hydrophobic isoform behaves anomalously in the absence of detergent. It fails to bind to the anion exchange matrix and is likely to be responsible for the ligand binding activity identified in the column flow-through.

While the amino-terminal end of PKGs are generally not very conserved, molecular analysis has demonstrated that this portion of the protein has several functional domains. The leucine zipper motif (37) found in most PKGs is responsible for homodimerization of this family of proteins. This motif is notably absent from each of the parasite PKGs described here. While the functional significance of a dimeric PKG remains unclear, dimerization has been proposed to play a role in the association of PKG with cellular targeting proteins (48). However, it is clear that monomeric PKG, generated by NH2-terminal proteolysis or by mutagenesis, is catalytically active in response to cGMP (14). We report data in this article which demonstrate that native soluble E. tenella PKG behaves as a fully functional monomeric enzyme. Since native soluble EtPKG lacks the NH2-terminal 48 amino acid residues of the full-length protein, it could be argued that the full-length protein is a homodimer. Because of its hydrophobic character and association with detergent micelles, full-length acylated PKG is technically difficult to characterize by gel filtration chromatography. The recombinant cF Eagle [T] PKG is a full-length protein that is not acylated but does contain the first 48 amino acids of the translational open reading frame. This recombinant EtPKG co-migrates in gel filtration experiments with the native soluble PKG, suggesting that the initial 48 amino acids of EtPKG do not contain a dimerization motif. Based on these data we conclude that both parasite PKG isoforms are monomeric proteins.

The list of potential substrates for cGMP-dependent protein kinases in higher eukaryotes has grown considerably long in the past several years (32, 49). A role for PKG has been implicated in multiple distinct metabolic processes including the control of blood pressure, intestinal fluid secretion, and even erectile function (26). Clearly, several of these physiologic processes do not have counterparts in the parasite. The biochemical role of PKG in the parasite is unknown. It is tempting to speculate that information on kinase substrates could lead to the identification of new or synergistic parasitic chemotherapeutic targets.

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Purification and Molecular Characterization of cGMP-dependent Protein Kinase from Apicomplexan Parasites: A NOVEL CHEMOTHERAPEUTIC TARGET
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