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A new nonhydrolyzable reactive cGMP analogue, (Rp)-guanosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl)monophosphorothioate, which targets the cGMP binding site of human platelet PDE3A

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ABSTRACT

The amino acids involved in substrate (cAMP) binding to human platelet cGMP-inhibited cAMP phosphodiesterase (PDE3A) are identified. Less is known about the inhibitor (cGMP) binding site. We have now synthesized a nonhydrolyzable reactive cGMP analog, Rp-guanosine-3′,5′-cyclic-*S*-(4-bromo-2, 3-dioxobutyl)monophosphorothioate (Rp-cGMPS-BDB). Rp-cGMPS-BDB irreversibly inactivates PDE3A (K_i = 43.4 ± 7.2 μM and k_{cart} = 0.007 ± 0.0006 min^{-1}). The effectiveness of protectants in decreasing the rate of inactivation by Rp-cGMPS-BDB is: Rp-cGMPS (K_d = 72 μM) > Sp-cGMPS (124), Sp-cAMPS (182) > GMP (1517), Rp-cAMPS (3762), AMP (4370 μM). NAD^+, neither a substrate nor an inhibitor of PDE3A, does not protect. Nonhydrolyzable cGMP analogs exhibit greater affinity than the cAMP analogs. These results indicate that Rp-cGMPS-BDB targets favorably the cGMP binding site consistent with a docking model of PDE3A-Rp-cGMPS-BDB active site. We conclude that Rp-cGMPS-BDB is an effective active site-directed affinity label for PDE3A with potential for other cGMP-dependent enzymes.

1. Introduction

Affinity labeling is a powerful technique for identifying the amino acids which comprise the substrate or coenzyme binding sites of enzymes (as recently reviewed [1]). Although these sites can be inferred from the three-dimensional structure determined by X-ray or NMR analysis, less than 20% of human proteins have had such structures determined. Furthermore it is possible by affinity labeling to establish whether a specific amino acid is in the substrate binding site (affecting the K_m) or if it is involved in catalysis and/or a regulatory role (influencing the k_{cat}). Once this is determined, when a three dimensional structure or even a good homology model becomes available, measurements of the molecular distance from the reactive group of the affinity label to the amino acid identified by proteolytic degradation of the irreversibly modified enzyme can be used to validate the function of the modified amino acid in the enzyme. Subsequent studies in which the identified amino acid is subjected to site-directed mutagenesis and kinetic studies of the mutant enzyme will help to confirm the conclusions derived from affinity labeling.

PDEs exist as eleven gene families with greater than 100 isoenzyme variants derived from 21 genes by the use of alternative transcriptional start sites and alternative splicing of mRNA precursors [2]. PDEs share sequence homology and substrate specificity (cAMP and/or cGMP) but differ in kinetic behavior, inhibitor specificity, physical properties, regulator mechanisms and cell localization [3] Most tissues express 2–4 PDEs [4]. Crystal structures of 7 PDE families show a conserved catalytic region with about 300 amino acids arranged on 14 alpha-helices [5]. The C-terminal portion of the proteins contains ~250 amino acids which are well conserved with identities of 30–35% among and 60–65% within families and the highly variable N-terminal region contains regulatory domains which are sites for phosphorylation, cGMP binding and membrane insertion. The principal inhibitory regulator of platelet functions such as adhesion, shape change, aggregation and secretion is cAMP. cAMP is predominantly hydrolyzed by PDE3A, the most abundant PDE in platelets. cGMP is an important competitive inhibitor of PDE3A acting directly at the catalytic site. We previously used a human erythroleukemia cell line [6] and other investigators used human cardiac myocytes [7] to clone the gene for PDE3A. We also used the cloned catalytic domain of the PDE3A gene (amino acids 665–1141) to produce the functional protein in the baculovirus/Sf9 insect cell expression system [8].

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Site-directed mutagenesis in the active site of PDE3A and enzyme kinetic studies of PDE3A mutants led to the conclusion that amino acid residues N845, E866, E971, F972 and F1004 are involved in cAMP binding, while Y751, H836, H840, E866, D950 and F1004 participate in cGMP binding. E866 and F1004 are of special interest because they appear to be involved in both cAMP and cGMP binding [9]. The active site of PDE3A thus contains overlapping but distinguishable binding sites for cAMP and for cGMP. Each site contains distinct amino acids, with the common amino acids accounting for the competitive inhibition by cGMP of cAMP hydrolysis. We recently identified a unique substrate binding site for cAMP in the 44 amino acid insert of PDE3A, dramatizing the need for a specific cGMP affinity label [10]. We now report specific inactivation, protection and incorporation of PDE3A by a reactive inhibitor analog, Rp-cGMPS-BDB. This novel nonhydrolyzable cGMP reagent has proven to be an effective affinity label of the catalytic site of PDE3A.

2. Experimental procedures

2.1. Materials

1,4-Dibromobutanedione (DBBD) was purchased from Aldrich (Milwaukee, WI). The Rp isomer of guanosine 3′,5′-cyclic phosphorothioate (Rp-cGMPS), used for synthesis, was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Sf9 insect cell lines, Sf-900 II SFM medium, BaculoDirect Transfection and Expression System, the ProBound Resin, and Anti-HisG Antibody were purchased from Invitrogen, Carlsbad, CA. Protease inhibitor cocktail set III (PIC III) was purchased from EMD Biosciences, San Diego, CA. Microcon YM-30 centrifugal filter devices were purchased from Millipore (Bedford, MA). Coomassie Plus Protein Assay Reagent kit and GelCode Blue Stain Reagent were purchased from Pierce (Rockford, IL). Gentamicin sulfate, triethylamine, methanol, Sp-cGMPS, 5′-AMP, 5′-GMP, and NAD^+ were purchased from Sigma (St. Louis, MO). Sp-cAMPS and Rp-cAMPS used in the protection studies were also purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Adenosine 3′,5′-cyclic phosphate ammonium salt [2,8-^{3}H]-cAMP was purchased from Perkin Elmer Life Sciences (Boston, MA). Biodegradable counting cocktail, Bio-Safe II, was purchased from Research Products International Corporation (Mount Prospect, IL).

2.2. Synthesis of Rp-cGMPS-BDB

Rp-cGMPS-BDB was prepared, by analogy to the synthesis of AMPS-BDB and GMPS-BDB[11,12] by the reaction of Rp-cGMPS with DBBD (Fig. 1). The DBBD (183 mg, 750 μmoles) was dissolved in 0.5 ml of methanol contained in a 5 ml round bottom flask and cooled to 4 °C in an ice bath. Rp-cGMPS (9.6 mg, 25 μmoles) was dissolved in 0.5 ml of methanol by the addition of a sufficient amount of triethylamine. The pH of the solution was maintained between 5.3 and 5.6. This solution was added in two portions to the vigorously stirred solution of DBBD in methanol. The reaction was allowed to proceed for 20 min. After the completion of the reaction, the total volume of the reaction mixture was brought down to 0.5 ml by air through the solution at 4 °C. Cold diethyl ether (13 ml) was added and the solution was cooled in an ice bath for 15 min. The precipitated products were collected by centrifugation. The crude product was washed three times with 6 ml of cold diethyl ether to remove any traces of unreacted DBBD. The pale yellow product, Rp-cGMP-BDB, was dried in a jet of air, was dissolved in 20 mM 4-Morpholineethanesulfonic Acid (MES) buffer pH 5.0 and was stored in a freezer at −80 °C. The compound was stable for ∼6 months when stored this way, and was shown to give consistent enzymatic inactivation rates.

2.3. Characterization of Rp-cGMPS-BDB

The UV absorption spectrum of the synthesized Rp-cGMPS-BDB in a buffer containing 20 mM MES, pH 5.0, was recorded using a Beckman Coulter spectrophotometer Du 7400. The NMR spectra were obtained using a Bruker DrX400 spectrometer.

2.4. Construction, expression and purification of PDE3A

A PDE3A cDNA coding for the catalytic region (amino acid residues 665–1141) [8] was subcloned into a pENTER-TOPO vector (Invitrogen, Carlsbad, CA) to produce two sites for linear recombination. The DNA sequence was confirmed by nucleotide sequence analysis (Sidney Kimmel Nucleic Acid Facility, Thomas Jefferson University, Philadelphia, PA). Expression of the catalytic region (residue 665–1141) of PDE3A wildtype enzyme using a baculovirus/insect cell Sf9 system and protein purification using a ProBond Nickel resin column has been previously described [8].

2.5. Protein concentration determination

Protein concentration of the purified enzymes was determined using Coomassie Plus Protein Assay Reagent using BSA as standard. The absorbance at 595 nm was measured using a Bio-Tek automatic microplate reader equipped with a KC4 Module for data analysis (Bio-Tek Instruments, Inc., Winooski, VT).

2.6. Protein gel electrophoresis and Western blot analysis of PDE3A

PDE3A-containing protein solutions and protein standards were subjected to electrophoresis in 10% Bis–Tris Gel with 3-(*N*-morpholino) propane sulfonic acid (MOPS) running buffer using the NuPAGE Electrophoresis System. Gels were either stained with GelCode Blue Stain Reagent or transferred to a PVDF membrane using the Xcell II module at constant voltage of 25 V for 1 h at room temperature for Western blotting. Transferred membranes were processed using the Chromogenic Western Breeze System and

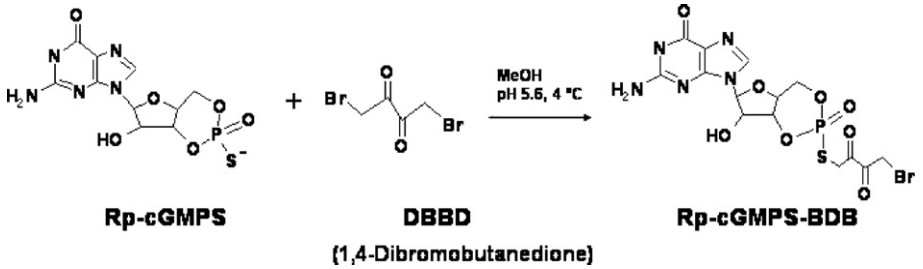

Fig. 1. Scheme for Synthesis of Rp-cGMPS-BDB. Rp-cGMPS-BDB was prepared by reaction of Sp-cAMPS with DBBD.

probed with Anti-HisG Ab to detect the HIS-tag PDE3A. As described previously, the PDE3A is a single band on protein gel electrophoresis and on a Western blot [8].

2.7. Enzyme activity assay

PDE3A activity was measured as previously described [10]. Enzyme containing solutions were added to a buffer containing 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, and 0.8 μM [^{3}H]cAMP (10,000 cpm/assay) to make a final volume of 100 μl. Reaction mixtures containing experimental samples or no enzyme were incubated at 30 °C for 15 min. Catalysis was terminated by serial addition of 0.2 ml of 0.2 M of $ZnSO_4$ and 0.2 ml of 0.2 M $Ba(OH)_2$. Samples were vortexed and centrifuged at 10,000g for 5 min. The pellets containing $BaSO_4$-precipitated [^{3}H]-5′-AMP were discarded. Aliquots of supernatants containing unreacted [^{3}H]cAMP were removed and counted in a Beckman Coulter liquid scintillation analyzer (Model LS6500, Fullerton, CA). Enzyme activity was measured by comparing the amount of cAMP hydrolyzed in PDE3A containing samples with that of controls without enzyme. These data were then used to calculate the enzyme specific activity in nmoles of cAMP hydrolyzed per mg of protein per minute [13].

2.8. Inactivation of PDE3A by Rp-cGMPS-BDB

Purified PDE3A was incubated at 25 °C with the affinity label, Rp-cGMPS-BDB at various concentrations (0–70 μM) in a reaction buffer containing 45 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2), 20 mM $MgCl_2$ and 4 mM MES. At timed intervals, aliquots of the reaction mixture was withdrawn, diluted in a buffer containing 47.5 mM HEPES (pH 7.04), 20 mM $MgCl_2$, and 4 mM MES, and then assayed in triplicate for residual PDE3A activity. Control samples were treated under identical conditions without the presence of the affinity label.

2.9. Protection by ligands against Rp-cGMPS-BDB

The effect of nucleotides (substrates, inhibitors or products) Sp-cAMPS, Rp-cAMPS, Sp-cGMPS, Rp-cGMPS, 5′-AMP and 5′-GMP on the rate of inactivation of PDE3A by the affinity label, Rp-cGMPS-BDB, was evaluated by incubation of the purified enzyme with each ligand individually for 2 min prior to the addition of the affinity label. Aliquots of each final reaction mixture were removed at timed intervals, diluted in a buffer containing 47.5 mM HEPES (pH 7.04), 20 mM $MgCl_2$ and 4 mM MES, and then assayed in triplicate for residual PDE3A activity. NAD^+ was used as control for the protection experiments since it is neither the substrate nor the inhibitor of PDE3A [10,13].

2.10. Measurement of the Incorporation of Rp-cGMPS-BDB into PDE3A

PDE3A was incubated with 30 μM Rp-cGMPS-BDB as a reaction mixture in a 50 mM HEPES buffer at pH 7.3 containing 4 mM MES, 10 mM $MgCl_2$ and 0.5 M NaCl. At each indicated time of incubation (0, 15, 30, 45, 60 80 and 100 min, respectively), aliquots were removed, and the residual enzyme activity of PDE3A was determined. 100 mM [^{3}H]$NaBH_4$ (dissolved in 20 mM NaOH) was added to the remaining reaction mixture to reach a final concentration of 2 mM and allowed to remain at 4 °C for a total of 1.5 h. [^{3}H]$NaBH_4$ reduces the diketo group from Rp-cGMPS-BDB to a [^{3}H]-diol group. The excess [^{3}H]$NaBH_4$ and the free Rp-cGMPS-BDB were removed by four consecutive centrifugations using Microcon centrifugal devices (Millipore, Billerica, MA) at 14,000g for 20 min. Aliquots were removed from the retentate to measure the protein concentration using the Coomassie Plus Protein Assay. The amount of the Rp-cGMPS-BDB incorporated into PDE3A from reduction of the affinity labeled enzyme by [^{3}H]$NaBH_4$ was calculated by measuring the radioactive tritium content using a Beckman Coulter liquid scintillation analyzer (Model LS6500). We used two moles of [^{3}H] per mole affinity label in calculating the incorporation. Control samples were tested using a similar procedure with the pretreatment of Rp-cGMPS-BDB with cold $NaBH_4$ prior to the addition of enzyme.

2.11. Molecular modeling

A homology model of PDE3A based on the crystal structure of PDE4B2B has been published [9]. However, the model did not contain the additional 44 amino acid insert found in PDE3A. We have recently refined the PDE3A model using the published PDE3B structures which contain the 44 amino acid insert unique to PDE3 [10]. Sybyl 6.91 FlexX docking module (Tripos) was then used to dock the affinity label Rp-cGMPS-BDB to PDE3A. Residues (Y751, H836, H840, E866, D950 and F1004) involved in cGMP binding were used as a defined cGMP binding pocket.

3. Results

3.1. Synthesis and characterization of Rp-cGMPS-BDB

Sp-cGMPS and Rp-cGMPS act as competitive inhibitors of PDE3A when tested using the substrate cAMP[13]. The K_i values for Sp-cGMPS and Rp-cGMPS are 305 ± 54 and 210 ± 33 μM (Table 1), respectively, indicating that the enzyme binds the Rp isomer better than it binds the Sp-cGMPS. Since Rp-cGMPS exhibited the strongest affinity for PDE3A, we coupled the nonhydrolyzable cGMP analog, Rp-cGMPS, with 1,4-dibromobutanedione (DBBD) to synthesize the affinity label, Rp-cGMPS-BDB. Fig. 1 shows the synthesis of Rp-cGMPS-BDB. The yield of Rp-cGMPS-BDB was 60% (5.8 mg). The purity of the product was tested by thin-layer chromatography (TLC), using cellulose F on plastic sheets, 0.1 mm thickness, (EMD Chemicals, Inc.) with isobutyric acid/concentrated NH_4OH/H_2O (66:1:33) as the solvent system. The Rp-cGMPS-BDB exhibits one spot on TLC with an Rf of 0.83, clearly distinguishable from its precursor, Rp-cGMPS, which has an Rf of 0.11. The ultraviolet (UV) absorption spectrum of the synthesized Rp-cGMPS-BDB in 20 mM MES, pH 5.0 buffer showed a single peak at 252 nm (ε = 13,500 $M^{-1}cm^{-1}$), comparable to GMP, GMPS and GMPS-BDB [11,12]. The UV spectrum demonstrates that the purine ring is not alkylated, since purine ring substitution of guanosine causes a shift in V_{max} [11]. The ^{1}H NMR spectrum of Rp-cGMPS-BDB (D_2O) exhibits chemical shifts at 8.04 ppm (s, H-8) and 6.05 ppm (d, H-1′), comparable to those of cGMPS and GMP. These results also indicate that the purine ring is not alkylated. The ^{31}P NMR spectrum of the product Rp-cGMPS-BDB (D_2O) has a charac-

Table 1
The Apparent K_d values of cAMP and cGMP analogs measured from protection against PDE 3A inactivation by Rp-cGMPS-BDB

cAMP and cGMP Analogs	K_i (μM)[a]	K_d (μM)
Sp-cAMPS	47.6 ± 6.2	202 ± 22
Rp-cAMPS	4400 ± 2,600	2115 ± 383
AMP	9600 ± 3,060	4370 ± 711
Sp-cGMPS	305 ± 54	124 ± 22
Rp-cGMPS	210 ± 33	72 ± 8
GMP	5600 ± 3,110	1606 ± 200

[a] The inhibition constants, K_i of each ligand were previously described [10,30]. The K_m of cAMP is 0.46 ± 0.20 μM. The values of K_d were calculated using the following equation: $k_{obs} = k_{-ligand}/(1+[ligand]/K_{ligand})$ where k_{obs} is the pseudo-first-order rate constant at given ligand concentration, $k_{-ligand}$ is the rate constant without the presence of ligand, and K_{ligand} is the apparent dissociation constant, K_d, for the ligand. Ligands are the nucleotide analogs.

teristic chemical shift of 20.3 ppm, and is readily distinguished from the 56.7 ppm found for the starting compound cGMPS. This change in chemical shift upon S-alkylation of a thiophosphate is typical of a purine monophosphorothioate, and is observed for AMPS-CH_3 (22.9 ppm), GMPS-BOP (21.7 ppm) and AMPS-BOP (19.2 ppm) [11,12,14–16].

3.2. Inactivation of PDE3A by Rp-cGMPS-BDB is time dependent and irreversible

To evaluate whether Rp-cGMPS-BDB inactivates PDE3A, we incubated the enzyme with various concentrations of Rp-cGMPS-BDB from 15 to 70 μM for 60 min in 45 mM Hepes buffer, pH 7.2, containing 20 mM $MgCl_2$ and 4 mM MES. The inactivation of PDE3A by Rp-cGMPS-BDB is a time-dependent, irreversible reaction (Fig. 2A). When incubated under the same conditions except for the absence of reagent, PDE3A exhibits constant activity over 60 min at 95% of the residual activity (Fig. 2A). The initial rate constant, k_{obs}, of inactivation was determined at each concentration of Rp-cGMPS-BDB tested using the equation of $[\ln(y_1) - \ln(y_2)] = k_{obs}(t_1 - t_2)$, where y_1 is the residual activity (E/E_o) measured at time interval t_1 and y_2 is the residual activity measured at time interval t_2. As seen in Fig. 2B the pseudo-first order rate constant exhibits a non-linear dependence on the concentration of Rp-cGMPS-BDB. This result indicates reversible binding followed by irreversible inactivation. The observed rate constant (k_{obs}) is defined by the irreversible inhibition rate equation from Kitz and Wilson [17]

$$k_{obs} = \frac{k_{max}[R]}{K_I + [R]}$$

where [R] is the reagent concentration, k_{max} is the maximum rate constant at saturating concentration of Rp-cGMPS-BDB, and $K_I = (k_{-1} + k_{max})/k_1$, the apparent dissociation constant of enzyme–reagent complex. The data shown in Fig. 2B were analyzed using SigmaPlot Enzyme Kinetics Module 1.1 (Systat Software, San Jose, CA). The calculated k_{max} is $0.0074 \pm 0.0004\ min^{-1}$ and K_I is 42.9 ± 5.2 μM. Rp-cGMPS-BDB irreversibly inactivates PDE3A, since no reactivation was observed upon dialysis following the inactivation reaction.

3.3. Protection by Inhibitors and substrates analogs Against Rp-cGMPS-BDB inactivation of PDE3A is concentration dependent

The ability of various nonhydrolyzable inhibitors and substrates analogs to decrease the rate of inactivation by 40 μM Rp-cGMPS-BDB was tested to ascertain whether the reaction occurred within the inhibitor binding site. Fig. 3A shows the results of varying concentrations of Sp-cGMPS added prior to the PDE3A inactivation by Rp-cGMPS-BDB. Sp-cGMPS (40, 80 and 120 μM) protect against the inactivation of PDE3A by Rp-cGMPS-BDB, causing 1.3-, 1.7- and 2.1-fold decreases in the pseudo first order rate constant (k_{obs} = 0.0078, 0.0059, 0.0047 min^{-1}, compared to 0.01 min^{-1} with no ligand present, Fig. 3E). Fig. 3B shows the results of adding various concentrations of Rp-cGMPS to protect against inactivation of PDE3A by Rp-cGMPS-BDB. Addition of 20, 40 or 80 μM of Rp-cGMPS exhibits decreases in k_{obs} (0.0079, 0.0065, and 0.0044 min^{-1}) of 1.3-, 1.5- and 2.3-fold when compared with the absence of ligand (Fig. 3F). Fig. 3C shows the results of 100, 200 and 300 μM of Sp-cAMPS in protecting against the inactivation of PDEA by Rp-cGMPS-BDB causing decreases in k_{obs} of 1.4-, 2.0- and 2.5-fold compared to no ligand present (Fig. 3G). In contrast, higher concentrations of Rp-cAMPS (0.2, 1 and 2.0 mM) cause 22, 32, and 46% decreases in k_{obs} (Fig. 3D and H).

The protective effects of Sp-cGMPS, Rp-cGMPS, Sp-cAMPS and Rp-cGMP on the rate of inactivation of PDE3A by Rp-cGMPS-BDB were concentration dependent (Fig. 3E–H). Complete protection against inactivation of PDE3A by Rp-cGMPS-BDB were observed at 220, 140, 475 μM of Sp-cGMPS, Rp-cGMPS and Sp-cAMPS, respectively (Fig. 3E–G). In contrast, Rp-cAMPS required 4.4 mM for complete protection against the inactivation of PDE3A by Rp-cGMPS-BDB (Fig. 3H).

The apparent dissociation constant, K_d, was calculated using the following equation, $k_{obs} = k_{-ligand}/(1+([ligand]/(K_{ligand}))$ where k_{obs} is the pseudo-first-order rate constant at given ligand concentration, $k_{-ligand}$ is the rate constant without the presence of ligand, and K_{ligand} is the apparent dissociation constant, K_d, for the ligand. Table 1 indicates that Rp-cGMPS has a smaller K_d value (72 ± 8 μM) compared to that of Sp-cGMPS, Sp-cMAPS, and Rp-cAMPS (124 ± 22, 202 ± 22, and 2115 ± 383 μM, respectively). Rp-cAMPS, which does not inhibit the enzyme, has a K_d value that is 30-fold greater than that of Rp-cGMPS. Rp-cGMPS, therefore, is the most effective ligand in protecting PDE3A against inactivation by Rp-cGMPS-BDB.

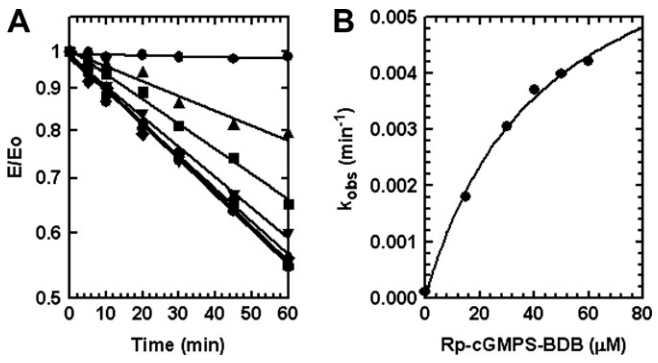

Fig. 2. Time course of the inactivation of PDE3A by Rp-cGMPS-BDB. The enzyme was incubated at 30 °C with Rp-cGMPS-BDB in 50 mM Hepes buffer, pH 7.5 and 5 mM $MgCl_2$. At timed intervals, aliquots were removed and assayed in duplicate for PDE3A catalytic activity. Plot (A) depicts the average of three experiments utilizing 0–70 μM of Rp-cGMPS-BDB (○ 0; ▲, 15; ■, 30 ▼, 40; ♦, 50; ⬣, 60; ●, 70 μM). Plot (B) depicts the pseudo first order rate constant, k_{obs}, for inactivation of PDE3A by Rp-cGMPS-BDB at concentrations ranging from 0 to 70 μM.

3.4. Protection of PDE3A against Inactivation by Rp-cGMPS-BDB requires higher concentrations of products

The protective effect of 5′-AMP and 5′-GMP on the rate of inactivation of PDE3A by Rp-cGMPS-BDB were also concentration dependent (Fig. 4A–D). However, higher concentrations of 5′-AMP and 5′-GMP were required to protect against the inactivation of PDE3A by Rp-cGMSP-BDB. Millimolar concentrations of 5′-AMP (1.0, 3.5 and 5 mM) were required to decrease k_{obs} of 1.2-, 1.7- and 2.4-fold when compared to no ligand added (Figs. 4A and C). 5′-GMP of 1.0, 2.0 and 3.0 mM decreased k_{obs} 1.6, 2.2 and 3.2-fold compared to the absence of ligand (Figs. 4B and D). Complete protection against inactivation of PDE3A by Rp-cGMPS-BDB was observed at 8.4 and 4.7 mM, respectively, for 5′-AMP and 5′-GMP (Figs. 4C and D). 5′-AMP and 5′GMP have a higher K_d value (4370 ± 711, 1606 ± 200 μM) as compared to that of Sp-cGMPS, Rp-cGMPS, and Sp-cAMPS (124 ± 22, 72 ± 8 and 202 ± 22 μM, respectively, Table 1). The higher K_d values of 5′-AMP and 5′-GMP is consistent with their higher K_i values (Table 1) indicating that Rp-cGMPS-BDB does not target either the 5′-AMP or 5′-GMP site. NAD^+, a nucleotide which is not a substrate, does not affect the rate of inactivation of PDE3A by Rp-cGMPS-BDB (Fig. 5).

3.5. Incorporation of Rp-cGMPS-BDB into PDE3A is time dependent

To quantify the amount of the affinity label Rp-cGMPS-BDB incorporated into PDE3A, the enzyme was incubated with 30 μM

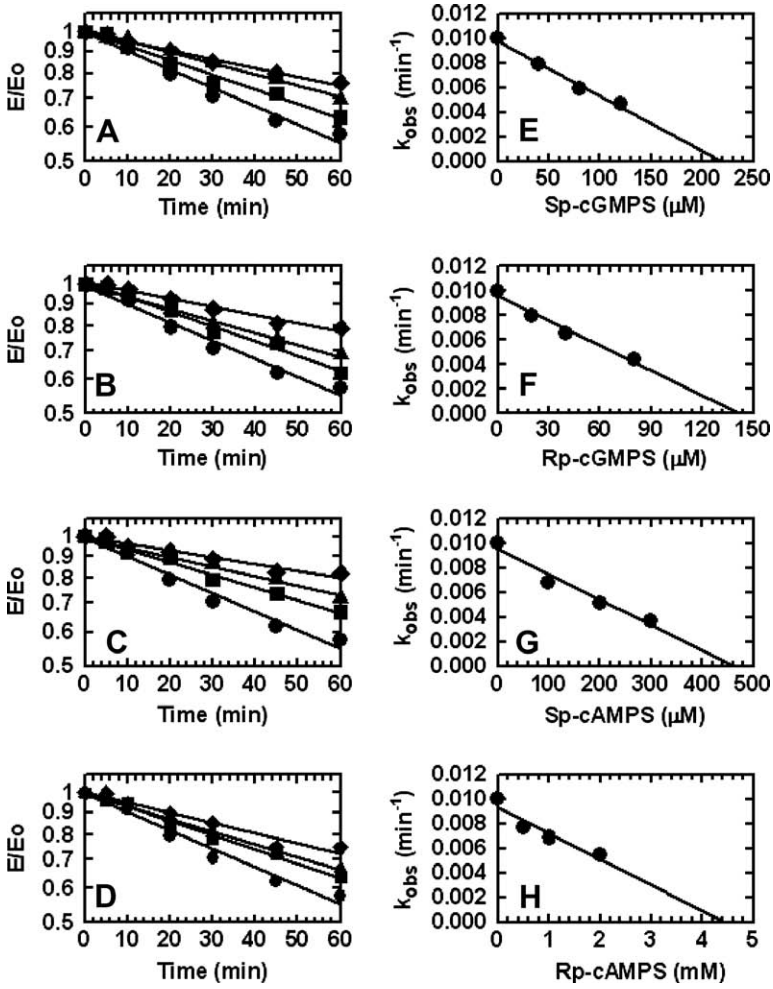

Fig. 3. Effect of the cGMP and cAMP analogs, Rp-cGMPS, Sp-cGMPS, Rp-cGMPS and Sp-cAMPS on the inactivation of PDE3A by Rp-cGMPS-BDB. PDE3A was preincubated with various concentrations of cGMP and cAMP analogs, Rp-cGMPS, Sp-cGMPS, Rp-cGMPS or Sp-cAMPS for 2 min in 50 mM HEPES buffer at pH 7.3. At this point, 30 μM Rp-cGMPS-BDB was added to reaction mixture, aliquots were removed at indicated timed intervals, and assays for PDE3A catalytic activity were performed. The apparent K_d values of cAMP and cGMP analogs were calculated as described in the text and listed in Table 1. (A) Protection effect of the various concentrations [(●) 0, (■) 40, (▲) 80, and (♦) 120 μM] of Sp-cGMPS on the inactivation of PDE3A by Rp-cGMPS-BDB. (B) Protection effect of the various concentrations [(●) 0, (■) 20, (▲) 40, and (♦) 80 μM].of Rp-cGMPS on the inactivation of PDE3A by Rp-cGMPS-BDB. (C) Protection effect of the various concentrations [(●) 0, (■) 100, (▲) 200, and (♦) 300 μM] of Sp-cAMPS on the inactivation of PDE3A by Rp-cGMPS-BDB. (D) Protection effect of the various concentrations [(●) 0, (■) 0.5, (▲) 1.0, and (♦) 2.0 mM] of Rp-cAMPS on the inactivation of PDE3A by Rp-cGMPS-BDB. (E) Effect of Sp-cGMPS on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. (F) Effect of Rp-cGMPS on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. (G) Effect of Sp-cAMPS on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. (H) Effect of Rp-cAMPS on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. These data are the mean of three independent experiments. Each experiment was performed in triplicate. The SEM was not shown because of the multiple lines which would make graphic representation difficult. However, the coefficients of the variance range are less than 20%.

Rp-cGMPS-BDB at pH 7.3, as described in Section 2. Fig. 6A shows that the incorporation of PDE3A by Rp-cGMPS-BDB is linear as a function of time. The addition of [^{3}H]$NaBH_4$ to an incubation mixture of enzyme and Rp-cGMPS-BDB stops the reaction by reducing the diketo group of Rp-cGMPS-BDB to a [^{3}H]-diol group. Fig. 6B shows that the residual enzymatic activity is inversely proportional to the incorporation. At 100 min, 0.80 moles of Rp-cGMPS-BDB was incorporated for each mole of enzyme which corresponded to 26% of residual enzymatic activity or 74% inactivation. Thus, 1.1 moles of Rp-cGMPS-BDB was required to inactivate each mole of enzyme indicating a stoichiometry close to 1.0 of the affinity label and the enzyme.

3.6. Docking model shows that Rp-cGMP-BDB is within the active site PDE 3A

The catalytic domain of PDE3A, including the unique 44 amino acid "insert", was previously modeled using Sybyl Composer based on the crystal structure of PDE3B (1SO2 and 1SOJ) [7]. FlexX docking module (Sybyl 6.91) is now used to dock Rp-cGMPS-BDB into the PDE3A model with a defined active site pocket of Y751, H836, H840, E866, D950 and F1004 (Fig. 7). Based on the docking model of Rp-cGMPS-BDB into the PDE3A model, the affinity label is within the active site of PDE3A (Fig. 7). These results further support the inactivation, protection and incorporation data that the affinity label Rp-cGMPS-BDB targets at the cGMP binding site.

4. Discussion

Reactive purine nucleotide analogs have been used as affinity labels to probe nucleotide binding sites [18–22]. Peptides containing cysteine [19], histidine [23–25] tyrosine [10,26], glutamate [27], aspartate [27,28] and arginine [29] modified by BDB-nucleotides have actually been isolated and characterized. We have described the use of the cAMP affinity analog 8-[(4-bromo-2, 3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate (8-BDB-

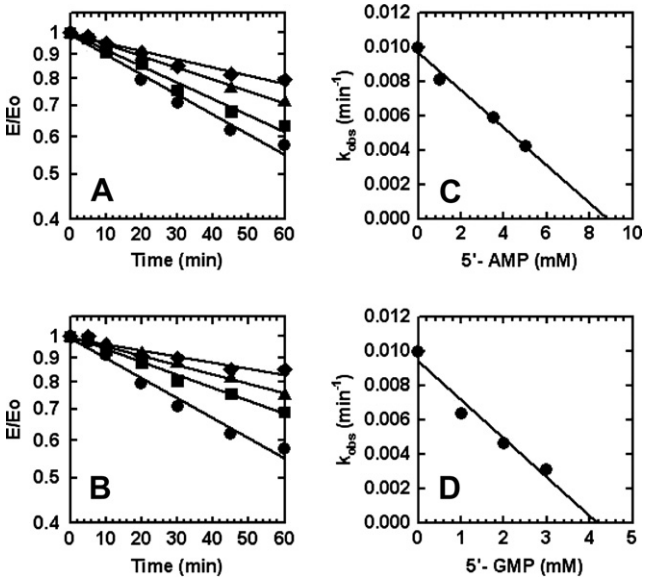

Fig. 4. Effect of the products, 5′-AMP and 5′-GMP on the inactivation of PDE3A by Rp-cGMPS-BDB. PDE3A was preincubated with various concentrations of 5′-AMP or 5′-GMP for 2 min in 50 mM Hepes buffer at pH 7.3, and 30 μM Rp-cGMPS-BDB was added to reaction mixture. Aliquots were removed at indicated timed intervals, and assays for PDE3A catalytic activity. The apparent K_d values of 5′-AMP and 5′-GMP were calculated as described in the text and listed in Table 1. (A) Protection effect of the various concentrations [(●) 0, (■) 1.0, (▲) 3.0, and (♦) 5.0 mM] of 5′-AMP on the inactivation of PDE3A by Rp-cGMPS-BDB. (B) Protection effect of the various concentrations [(●) 0, (■) 1.0, (▲) 2.0, and (♦) 3.0 mM] of 5′-GMP on the inactivation of PDE3A by Rp-cGMPS-BDB. (C) Effect of 5′-AMP on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. (D) Effect of 5′-GMP on the pseudo first order rate constant, $k_{obs,}$ of inactivation of PDE3A by Rp-cGMPS-BDB. These data are the mean of three independent experiments. Each experiment was performed in triplicate. The SEM was not shown because of the multiple lines which would make graphic representation difficult. However, the coefficients of the variance range are within 20%.

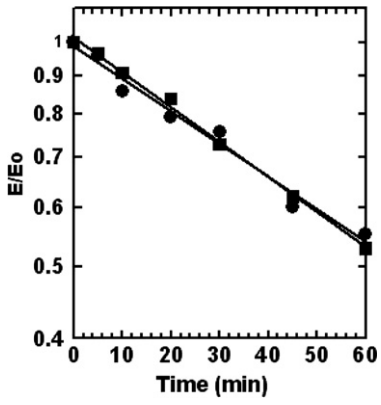

Fig. 5. Effect of NAD^+ on the inactivation of PDE3A by Rp-cGMPS-BDB. PDE3A was preincubated with NAD^+ for 2 min before 50 mM Rp-cGMPS-BDB was added to the reaction mixture. At timed intervals, aliquots were removed and assayed for PDE3A catalytic activity. The experimental points are: (●) no NAD^+ and (■) 10 mM NAD^+.

TcAMP) in studies to identify important amino acids within the active site of PDEs. 8-BDB-TcAMP irreversibly inactivated PDE2A [17], PDE3A [18] and PDE4A [22]. In the case of PDE4A, a peptide containing the residue modified by 8-BDB-TcAMP was isolated and the amino acid sequence identified. However, the utility of 8-BDB-TcAMP was limited since it only inactivates PDEs at millimolar concentrations because of continuous hydrolysis to the 5′-AMP derivative by the enzymes under investigation. We previously

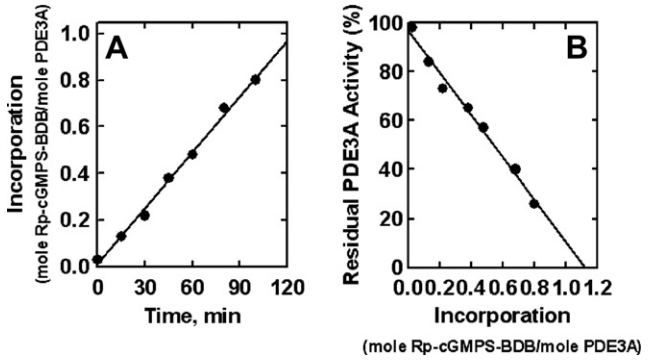

Fig. 6. Incorporation of Rp-cGMPS-BDB into PDE3A. (A) Shows the time-dependent incorporation of Rp-cGMPS-BDB into PDE3A. The enzyme was incubated in 50 mM Hepes buffer, pH 7.3 at 25 °C. At the indicated times (0, 15, 30, 45, 60 80 and 100 min), the incorporation was stopped by two additions of [^{3}H]$NaBH_4$ (2 mM). The excess reagent was removed by four consecutive centrifugations using Microcon centrifugal devices. Aliquots were removed from the retentate to measure the protein concentration and radioactivity. (B) Shows the relationship between inactivation and incorporation of Rp-cGMPS-BDB into PDE3A. The residual activity of the unmodified enzyme was determined at 0, 15, 30, 45, 60 80 and 100 min. Enzymes treated with the same procedure but without Rp-cGMPS-BDB in the incubation mixture were used as controls for activity. The data were fitted to linear regression equation and plotted. The results are the mean of three independent experiments.

reported synthesis of the new nonhydrolyzable reactive cAMP derivative (Sp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl) phosphorothioate (Sp-cAMPS-BDB), which contains both reactive bromoketo and dioxo groups [13]. The bromoketo group can form covalent bonds with the nucleophilic side chains of many amino acids including cysteine, aspartate, glutamate, histidine, tyrosine and lysine, while the dioxo provides the ability to react with arginine residues [29]. Using this affinity reagent we identified the substrate binding amino acid Y807 in the 44 amino acid insert region which induced a conformational change in a flexible loop which allowed interaction of the loop with the amino acids in the active site cleft of PDE3A [10].

To have the potential to identify new unique cGMP binding sites in cGMP dependent enzymes, we here demonstrate that Rp-cGMPS-BDB acts as an affinity label of PDE3A. Rp-cGMPS-BDB is an analog of a competitive inhibitor, cGMP, with the reactive bromodioxobutyl group at the phosphorothioate ester. We demonstrated that Rp-cGMPS-BDB inactivates PDE3A specifically and irreversibly in a time-dependent manner. Protection by nonhydrolyzable analogs of both the substrate cAMP and the inhibitor cGMP indicates the inactivation of PDE3A by Rp-cAMPS-BDB is a consequence of reaction at the overlap of both the cAMP and cGMP binding sites. In addition, Rp-cGMPS has the smallest apparent K_d, and is therefore the most effective ligand in protecting PDE3A against this new affinity label. The specific incorporation of the PDE3A and the affinity label demonstrates the stoichiometry of Rp-cGMPS-BDB and PDE3A to be 1.1 moles. These results indicate that Rp-cAMPS-BDB may modify a single amino acid common to both cAMP and cGMP sites or alternately modify a single amino acid at the cGMP site.

The Sp and Rp diastereomers of cGMP, Sp-cGMPS and Rp-cGMPS were developed as inhibitors of cGMP-dependent protein kinase [30]. Sp-cGMPS and Rp-cGMPS are both competitive inhibitors of PDE3A [13]. The K_d for both Sp-cGMPS and Rp-cGMPS are similar, indicating that there is no sterospecificity when PDE3A binds to either isomer of cGMPS.

Both Sp-cAMPS and Rp-cAMPS are potent, membrane-permeable activators of the cAMP dependent protein kinases I and II which mimic the effects of cAMP as a second messenger [31]. We have shown that Rp-cAMPS is a weak inhibitor of PDE3A similar

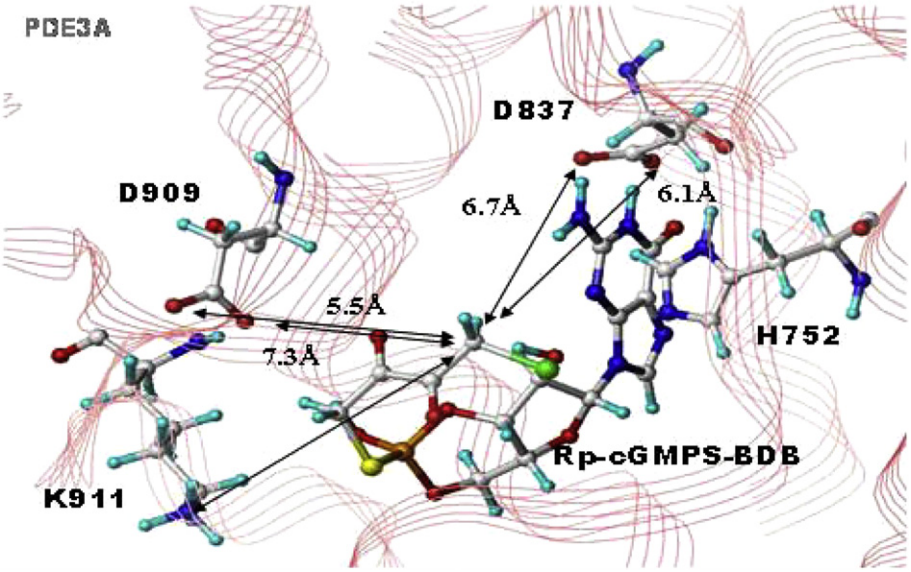

Fig. 7. Docking Model of PDE3A with Rp-cGMPS-BDB. PDE3A was modeled using Sybyl Composer with 16 chains of PDE3B (1SO2 and 1SOJ) shown in quintuple red lines. FlexX (Sybyl) was then used to dock Rp-cGMPS-BDB into the PDE3A model with a defined cGMP binding pocket of Y751, H836, H840, E866, D950 and F1004. Distances in Å from the reactive center of the affinity label to the nearest reactive amino acid residues are indicated.

to 5′-AMP and 5′-GMP, while Sp-cAMPS is a potent competitive inhibitor (Table 1) [13]. The K_d value of Rp-cAMPS for PDE3A is 10-fold larger than that of Sp-cAMPS. These results indicate the existence of sterospecificity for PDE3A when binding to cAMPS derivatives. This is consistent with the previous findings that the difference in the diastereoisomeric center of Sp-cAMPS and Rp-cAMPS has led to a marked change in their relative orientations in the active site [13]. This difference has positioned Rp-cAMPS out of the hydrophobic interaction range of F1004. In a homology model of PDE3A based on the X-ray structure of PDE4B2B, we have established that both clinically used PDE3A inhibitors, milrinone and cilostazol, mimic cGMP, but not cAMP. Either inhibitor occupies a subsite of PDE3A identical to the cGMP site [32]. The docking model allows the calculation of the distance between the reactive carbon of the affinity label and the nucleophilic side chain of amino acids. Residues Asp-837 and Asp-909 are the likely candidate to be labeled by Rp-cGMPS-BDB because the reactive carboxyl groups of these two aspartates are 6.1 and 5.5 Å, respectively, from the reactive carbon of the affinity label, Rp-cGMPS-BDB (Fig. 7). On the contrary, the cysteine residues are unlikely candidates for the affinity labeling since the distance of the reactive thiol groups are from 17.7 to 31.5 Å away from the reactive carbon of Rp-cGMPS-BDB. Further studies with this unique cGMP affinity label reagent should allow additional insights into the role of cGMP in the regulation of PDE3A.

Acknowledgments

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