NEW INSIGHTS FROM THE STRUCTURE FUNCTION ANALYSIS OF THE
CATALYTIC REGION OF HUMAN PLATELET PDE3A: A ROLE FOR THE
UNIQUE 44 AMINO ACID INSERT
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Running title: Functional Role of the Unique Insert of PDE3A

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Human phosphodiesterase 3A (PDE3A) degrades cAMP, the major inhibitor of platelet function, thus
potentiating platelet function. Of the 11 human PDEs only PDE3A and 3B have 44 amino acid inserts in the catalytic domain.
Their function is not clear. Incubating Sp-adenosine-3',5'-cyclic-S-(4-bromo-2,3-
dioxobutyl) monophosphorothioate (Sp-cAMPS-BDB) with PDE3A irreversibly
inactivates the enzyme. HPLC analysis of a tryptic digest yielded an octapeptide within
the insert of PDE3A [(K)T806YNVTDDK813] suggesting a substrate binding site exists
within the insert. Since Sp-cAMPS-BDB reacts with nucleophilic residues, mutants
Y807A, D811A and D812A were produced. Sp-cAMPS-BDB inactivates D811A and
D812A but not Y807A. A docking model showed Y807 is 3.3 angstroms from the reactive carbon whereas D811 and D812 are
> 15 angstroms away from Sp-cAMPS-BDB. Y807A has an altered Km but no change in
kcat. Activity of wildtype but not Y807A is inhibited by an anti-insert antibody. These
data suggest that Y807 is modified by Sp-cAMPS-BDB and involved in substrate binding.
Because the homologous amino acid in PDE3B is C792, we prepared the mutant
Y807C and found that its Km and kcat were similar to the wildtype. Moreover, Sp-
cAMPS-BDB irreversibly inactivates Y807C with similar kinetics to wildtype suggesting
that the tyrosine may, like the cysteine, serve as a H donor. Kinetic analyses of 9 additional
insert mutants reveal that H782A, T810A, Y814A and C816S exhibit an altered kcat but
not Km, indicating catalysis is modulated. We document a new functional role of the insert
in which substrate binding may produce a conformational change. This change would
allow the substrate to bind to Y807 and other amino acids in the insert to interact with
residues important for catalysis in the active site cleft.

The antiplatelet drugs aspirin and clopidogrel have proven efficacy in secondary
prevention of stroke, myocardial infarction and peripheral vascular recoclusion (1,2). Aspirin
inhibits cyclooxygenase thereby decreasing synthesis of thromboxane A2 (TXA2).
Clopidogrel, a P2Y12 antagonist, blocks the ability of ADP to inhibit stimulated adenylate
cyclase. However, despite prophylaxis with these antiplatelet drugs, reoclusion of coronary
arteries occurs in 20-30% of patients after thrombolytic therapy or angioplasty probably
due to the inability of these drugs to inhibit thrombin induced platelet activation (3,4). At
low concentrations of thrombin, platelets are aggregated and activated by pathways
independent of both ADP and TXA2. At high concentrations of thrombin, platelets are
aggregated and activated by pathways independent of both ADP and TXA2. In
contrast, elevation of intracellular cAMP produces potent inhibition of all pathways of
platelet activation including increase in intracellular Ca++, shape change, aggregation,
secretion, and the effects of phospholipases A2 and C, as well as their responses of platelets to thrombin.

Cyclic nucleotide phosphodiesterase 3A (PDE3A) is the most abundant cAMP PDE in platelets. PDE3A hydrolyzes cAMP resulting in lowering the intracellular cAMP levels which in turn potentiates platelet activation. Drugs that inhibit PDE3A raise cAMP levels in platelets, thereby increasing the phosphorylation of proteins by cAMP- and cGMP-dependent protein kinases (PKA and PKG) (5). Currently two PDE3A competitive inhibitors cilostazol and milrinone have respectively been used for treating patients with intermittent claudication and acute congestive heart failure (6,7). Unfortunately cilostazol is contraindicated in patients with congestive heart failure and milrinone is associated with undesirable cardiac arrhythmias. Examination of the inhibitory mechanism of PDE3A is important to exploit other ways of inhibiting this enzyme to minimize side effects.

The available PDE family crystal structures known to date are those of the catalytic domains cAMP-PDE (PDE4B2B and PDE4D) (8,9), cGMP-PDE (PDE5A and PDE9A) (10,11) and dual cAMP/cGMP-PDE (PDE1B and PDE3B) (12,13). The overall crystal structures of the catalytic domains of these PDEs contain a compact structure consisting of 16 $\alpha$-helices. Each PDE has three subdomains with a deep hydrophobic pocket at the interface and two conserved metal binding sites within that pocket. The hydrogen bond network of the neighboring residues H948 and W1072 in PDE3B (H956 and W1085 in PDE3A) serve to orient the absolutely conserved residue Q988 (Q1001 in PDE3A) to accept or donate hydrogen bonds to the purine ring, thereby determining the nucleotide recognition specificity of the enzyme (13). In PDE3B, residues F991 (F1004 in PDE3A) and I955 (I967 in PDE3A) on each side of the purine ring and Y960 and P941 (Y973 and P954 in PDE3A) form the hydrophobic clamp (13). Residues H741, H821, D822, D937 (H756, H836, D837, D950 in PDE3A) and one water molecule in PDE3B are involved in the first metal Mg$^{2+}$ binding. The second Mg$^{2+}$ forms hydrogen bonds with D822 and five water molecules (13).

Water molecules coordinated to the metal ions may act as the nucleophile in the hydrolysis reaction to mediate catalysis.

The presence of a 44 amino acid insert within the catalytic domain is a unique feature of the PDE3 gene family. In the PDE3B crystal structure the 44 amino acid insertion (P758-C801, Figure 1) is located between helices 6 and 7 (13). This insertion lacks any clear secondary structure organization, and residues 767–781 are not visible in the electron density maps. This insert in human PDE3A is comprised of amino acid residues 773 to 816 (Figure 1). Within the insert there is 38.6% identity between PDE3A and PDE3B including conserved triplets at the N terminal, C terminal and the middle of the insert. Tang et al (14) showed that removal of this insert from PDE3A resulted in complete loss of activity. Double mutants, P773A/G774A and Y814A/G815A, from each of the N- and C-terminus of the PDE3A insert which are $\beta$ turns display markedly reduced activity (14). However, knowledge of the role of the 44 amino acid insert of PDE3A in the regulation of enzyme activity or interactions with substrate and/or inhibitor is incomplete.

Previously, we have synthesized a nonhydrolyzable, reactive substrate analog, Sp-cAMPS-BDB, which irreversibly inactivates PDE3A in a time-dependent fashion with $K_I = 10.1 \pm 1.7 \mu M$ and $k_{max} = 0.0116 \pm 0.0004 \text{ min}^{-1}$ (15). We have demonstrated that Sp-cAMPS-BDB targets both the cAMP and cGMP binding sites but favors the cAMP site. The protection studies indicate effectiveness of protectants in decreasing rate of inactivation by Sp-cAMPS-BDB is: Sp-cAMPS (Kd = 24 \mu M) > Rp-cGMPS (1360), Sp-cGMPS (1460) > GMP (4250), AMP (10600), Rp-cAMPS (22170 \mu M). Sp-cAMPS-BDB has proven to be an effective active site-directed affinity label for PDE3A.

In this paper, we describe specific incorporation of PDE3A by a reactive substrate analog, Sp-cAMPS-BDB, isolation of a peptide in the unique insert of PDE3A, and construction of mutant enzymes which identify the amino acid targeted by Sp-cAMPS-BDB. In addition, the role of the insert was further explored by kinetic analyses of 9 additional insert mutants. The results define a new functional mechanism...
by which binding of cAMP to the flexible loop of platelet PDE3A may induce a local conformational change which allows interaction with catalytic residues.

**EXPERIMENTAL PROCEDURES**

**Materials** – Adenosine 3',5'-cyclic phosphate ammonium salt [2,8-3H]-cAMP was purchased from Perkin Elmer Life Sciences, Boston, MA. The nonhydrolyzable, reactive cAMP analog, Sp-adenosine-3',5'-cyclic-S-(4-bromo-2,3-dioxobutyl)monophosphoro-thioate (Sp-cAMPS-BDB) was synthesized as previously described (15). 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. Coomassie Plus Protein Assay Reagent Kit was purchased from Pierce, Rockford, IL. Gentamicin sulfate, cAMP, and N-ethylmaleimide were purchased from Sigma, St. Louis, MO. HPLC grade acetonitrile was obtained from Fisher Scientific, Pittsburgh, PA.

**Measurement of the incorporation of Sp-cAMPS-BDB into PDE3A** – PDE3A was incubated with 100 µM Sp-cAMPS-BDB in a 50 mM Hepes buffer at pH 7.3 containing 20 mM MES, 10 mM MgCl₂ and 0.5 M NaCl. At various times of incubation (0, 20, 30, 40, 60 and 80 minutes, respectively), aliquots were removed, and the residual enzyme activity of PDE3A was determined to correlate with the incorporation. At each time interval, 100 mM [3H]NaBH₄ (dissolved in 20 mM NaOH) was added consecutively to reach a final concentration of 2 mM at 4°C for a total of 1.5 hours. [3H]NaBH₄ reduces the two oxygens of the diketo group from Sp-cAMPS-BDB to two [3H] hydroxyl groups. The excess [3H]NaBH₄ and the free Sp-cAMPS-BDB were removed by four consecutive centrifugations using Microcon centrifugal devices (Millipore, Billerica, MA) at 14,000 xg for 20 minutes. Aliquots were removed from the retentate to measure the protein concentration using the Coomassie Plus Protein Assay. The amount of the Sp-cAMPS-BDB incorporated into PDE3A from reduction of the affinity labeled enzyme by [3H]NaBH₄ was calculated by measuring the radioactive (³H) content by using a Beckman Coulter liquid scintillation analyzer (Model LS6500, Fullerton, CA). Control samples were tested using a similar procedure with the pretreatment of cold NaBH₄ with Sp-cAMPS-BDB prior to the addition of enzyme.

**Trypsin digestion of the Sp-cAMPS-BDB-modified enzyme** – PDE3A (0.8 mg) was incubated with 100 µM Sp-cAMPS-BDB at 25°C for 3 hours (~10% residual activity remained). The incubated mixture was treated twice with 100 mM [3H]NaBH₄ for a total of 1.5 hours (final concentration 2 mM), followed by a carboxylation of free SH groups with 10 mM N-ethylmaleimide for 10 minutes. After removal of the excess reagents by centrifugation using Microcon centrifugal devices, the modified enzyme was digested at 37°C by 2 consecutive additions of 5% (w/w) TPCK-treated bovine pancreatic trypsin for a total of 2 hours.

**Purification and determination of the sequence of modified peptide** – The radioactive tryptic digest was lyophilized, redissolved in 250 µL of 0.1% trifluoroacetic acid (TFA), and applied to an HPLC system using a reverse phase Vydac (Hesperia, CA) C18 column (0.46 X 25 cm). Separation was conducted at the elution rate of 1 mL/min using solvent A (0.1% TFA in water) for the first 10 minutes, followed by a linear gradient from solvent A to 45% Solvent B (0.1% TFA in acetonitrile) for 220 minutes, a linear gradient from 45% solvent B to 100% Solvent B for 20 minutes, and solvent B for 10 minutes, successively. The eluent was monitored at 220 nm. Fractions of 1 mL were collected, from which 400 µL was counted for radioactivity. The amino acid sequence of isolated radioactive peptides was determined using an automated gas phase peptide sequence analyzer from Applied Biosystems (Model 470A, Foster City, CA) equipped with an on-line PTH analyzer (Model 120) and Computer (Model 900A). The sequencing results were used to identify the location of the modified peptide in the active site of the catalytic region of PDE3A. This process was repeated twice with identical results.
Construction and purification of PDE3A mutants – A deletion mutant of PDE3A cDNA coding for the amino acid residues 665-1141 (16) was subcloned into a pENTER-TOPO vector (Invitrogen, Carlsbad, CA) to produce two sites for linear recombination. PDE3A insert mutants H782A, H796A, H798A, S804A, K805A, Y807A, Y807C, T810A, D811A, D812A, Y814A, G815A, and C816S were constructed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutants were confirmed by nucleotide sequence analysis (Sidney Kimmel Nucleic Acid Facility, Thomas Jefferson University, Philadelphia, PA). Recombinant mutant baculoviruses were produced by linear combination using BaculoDirect Transfection kit (Invitrogen). Expression of the catalytic region (residue 665-1141) of PDE3A wildtype and mutant enzymes using a baculovirus/insect cell Sf9 system and protein purification using a ProBond Nickel resin column has been previously described (17,18).

Protein concentration determination - Protein concentration of the purified enzymes and purified anti-insert antibody were 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 KC4 Module for data analysis (Bio-Tek Instruments, Inc., Winooski, VT).

Western blot analysis – The PDE3A wildtype and mutants were separated on 10% Bis-Tris gel electrophoresis purchased from Invitrogen. The proteins were transferred to a PVDF membrane using the Xcell II module at a constant voltage of 30 volts for one hour at room temperature for Western blotting. The membranes were processed using the Chromogenic WesternBreeze System and probed with Anti-insert PDE3A antibody (see effects of anti-insert antibody) to detect the presence of PDE3A.

Enzyme activity assay – PDE3A activity was measured by the amount of cAMP hydrolyzed as previously described (19). Enzyme was added to a buffer containing 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, and 0.8 µM [3H]cAMP. Reaction mixtures both with and without enzymes were incubated at 30°C for 15 minutes. Catalysis was terminated by serial addition of 0.2 M of ZnSO4 and 0.2 M Ba(OH)2, which precipitates AMP but not cAMP. Samples were vortexed and centrifuged at 10,000 xg for 5 minutes. The BaSO4 pellets containing the [3H]-5'-AMP precipitant were discarded. Aliquots of supernatants containing unreacted [3H]-cAMP were removed and counted in a Beckman Coulter liquid scintillation analyzer. Enzyme activity was measured by comparing the amount of cAMP hydrolyzed in PDE3A containing samples to no enzyme controls. This data was then used to calculate enzyme specific activity in nmoles of cAMP hydrolyzed per mg of protein per minute.

Kinetic constants determination – The rates (n mole/sec) of cAMP hydrolysis for the PDE3A wildtype and mutant enzymes were determined using various concentrations of substrate cAMP from 0.02 µM to 14 µM. The values of Km and Vmax for each of the enzymes were determined by Michaelis-Menten equation as calculated by Enzyme Kinetics Module 1.1 Software (Systat Software, Point Richmond, CA). The kcat (sec-1) was obtained by dividing Vmax (nmole/sec) by the molar enzyme concentration (nmole).

Reaction of Sp-cAMPS-BDB with mutant enzymes – Purified PDE3A mutant enzyme (Y807A, Y807C, D811A or D812A) was incubated at 25°C with various concentrations of Sp-cAMPS-BDB in a 50 mM Hepes buffer at pH 7.3 containing 20 mM MES, 10 mM MgCl2 and 0.5 M NaCl. At timed intervals (0, 5, 10, 20, 30 45 and 60 minutes) aliquots of the reaction mixture were withdrawn, diluted in a buffer containing 47.5 mM Hepes, pH 7.04, 20 mM MgCl2, 4 mM MES, and assayed in triplicate for residual PDE3A activity. Control samples were performed under identical conditions without the presence of affinity label Sp-cAMPS-BDB.

Effect of anti-insert antibody on enzyme activity – A rabbit polyclonal antibody against the synthetic peptide V802FKTYNVTDDKYGC816, the C-terminal 15 amino acids of the PDE3A insert (Figure 1) which also contains the octapeptide, was prepared by Sigma Genosys, St. Louis, MO and designated as an anti-insert antibody. PDE3A and mutants Y807A and Y807C were incubated respectively with various concentrations of the anti-insert antibody to a enzyme to antibody ratio of 1.3, 2.0, 4.0 for 1
hour at 37 °C. After incubation, enzyme activity was determined according to the “Enzyme activity assay” procedure. The activity of PDE3A wildtype, Y807A and Y807C without antibody was set as 100% activity. The pre-immune IgG was used as a control to compare the activity of wildtype, Y807A and Y807C. All experiments were performed in triplicate.

Molecular modeling – A homology model of PDE3A based on the crystal structure of PDE4B2B has been published (8). However, the model did not contain the additional 44 amino acid insert found in PDE3A. We have now refined the PDE3A model using the recently published PDE3B structures (13) which contain the 44 amino acid insert unique to PDE3. Sybyl 6.91 FlexX docking module (Tripos) was then used to dock the affinity label Sp-cAMPS-BDB to PDE3A. Since mutant Y807A affected the Km, Y807 was included in the defined cAMP binding pocket to construct the model. Residues involved in cAMP binding (17,18) were used as a defined cAMP binding pocket (Y807, N845, E866, E971 F972 and F1004). This docking model was utilized to illustrate and further evaluate the kinetic results obtained from the mutants of insert amino acids of PDE3A.

RESULTS

Incorporation of Sp-cAMPS-BDB into PDE3A is time dependent – To quantify the amount of the affinity label Sp-cAMPS-BDB incorporated into PDE3A, the enzyme (0.38 mg/mL) was incubated with 100 µM Sp-cAMPS-BDB at pH 7.3, as described in Materials and Methods. Figure 2A shows that the incorporation of PDE3A by Sp-cAMPS-BDB is linear as a function of time. The addition of [3H]NaBH4 to an incubation mixture of enzyme and Sp-cAMPS-BDB stops the reaction by reducing the diketo group of Sp-cAMPS-BDB to a [3H]-diol group. Figure 2B shows that the residual enzymatic activity is inversely proportional to the incorporation. At 80 min, 0.86 moles of Sp-cAMPS-BDB was incorporated for each mole of enzyme which corresponded 19% of residual enzymatic activity or 81% inactivation. Thus, 1.08 moles of Sp-cAMPS-BDB was required to inactivate each mole of enzyme indicating a stoichiometry close to 1.0 of the affinity label and the enzyme.

The isolated Sp-cAMPS-BDB modified peptide in PDE3A is located in the unique 44 amino acid insert – PDE3A (11 nmoles) was incubated with 100 µM Sp-cAMPS-BDB for 3 hours and treated with [3H]NaBH4 as described in Materials and Methods. The modified enzyme was digested by trypsin for 2 hours as described in Materials and Methods. Figure 3 (solid line) shows that on the reverse phase HPLC separation of the trypsin digest, most of the peptides elute between 0 to 160 minutes (0% and 30 % solvent B). Two major radioactive peaks were observed as shown in Figure 3 (dashed line) labeled I and II.

The amino acid sequence of the purified peptides (Figure 3, peaks I and II) was determined by Edman degradation using an automated gas-phase sequencer. Peak I contains small peptides (data not shown). The amino acid sequence of the peptide from peak II exhibits a single octapeptide, assigned as T806YNVTDDK813 within the unique 44 amino acid insert of PDE3A (Figure 1). This peptide results from enzyme cleavage after K805 and K813, consistent with the specificity of the trypsin recognition sites. The yield of each PTH-derivative was recorded and ranged from 40 – 20 pmoles (data not shown). As expected, the yield decreases as the cycle number increases. Peptide 806-813 is located C-terminal of the first metal binding motif, H752NRIH756.

Residue Y807 in PDE3A is the amino acid modified by the affinity label Sp-cAMPS-BDB – Sp-cAMPS-BDB reacts with nucleophilic amino acids. Thus Y807, D811 and D812 from peptide 806-813 (determined from the tryptic cleavage study) are candidates for interacting with the affinity label. K813 was not considered since this is the trypsin cleavage site and cleavage would not have occurred if that lysine were modified. To identify which amino acid is being modified by Sp-cAMPS-BDB, mutant enzymes Y807A, D811A and D812A were constructed, expressed and purified.

To evaluate the effect of mutations on the reaction with Sp-cAMPS-BDB, the mutant enzymes were incubated with the affinity label and their activity was tested as a function of time. Figure 4A, 4B, 4C and 4D show the
results of reaction of wildtype and mutant enzymes, Y807A, D811A and D812A, respectively with Sp-cAMPS-BDB. Sp-cAMPS-BDB irreversibly inactivates both mutants D811A and D812A exhibiting saturation kinetics (Figure 4C, 4H, 4D and 4I). The $k_{\text{max}}$ values for D811A and D812A are 0.005 ± 0.0002 and 0.003 ± 0.0001 min$^{-1}$ and the $K_\text{I}$ values are 29.9 ± 2.9 and 24.9 ± 2.5 µM, respectively. The $K_\text{m}$ values of both D811A and D812A is 2.5- to 3-fold larger than that of wildtype ($K_\text{m} = 10.1 \pm 1.7$ µM, Figure 4F). The $k_{\text{max}}$ of D811A and D812A is one half and one third respectively to that of wildtype ($k_{\text{max}} = 0.0116 \pm 0.0004$ min$^{-1}$, Figure 4F). These relatively minor changes in kinetics indicate that residues D811 and D812 are not the modified amino acid of the wildtype enzyme that reacts with Sp-cAMPS-BDB. In contrast, Y807A is not inactivated by Sp-cAMPS-BDB (50 to 400 µM, Figure 4B and 4G), identifying Y807 as the amino acid modified by Sp-cAMPS-BDB.

Docking model of Sp-cAMPS-BDB into PDE3A supports Y807 as the amino acid modified by the affinity label – The catalytic domain of PDE3A, including the unique 44 amino acid “insert”, was modeled using Sybyl Composer based on the crystal structure of PDE3B (1SO2 and 1SOJ) (13). FlexX docking module (Sybyl 6.91) was then used to dock Sp-cAMPS-BDB into the PDE3A model with a defined active site pocket of Y807, N845, E866, E971, F972 and F1004. Molecular modeling of the “insert” region, based on the crystal structure of PDE3B, suggests that this region is a flexible loop (Figure 5). Based on the docking model of Sp-cAMPS-BDB into the PDE3A model, Y807 (green) is most likely to be the amino acid modified by Sp-cAMPS-BDB, since the reactive carbon $C_9$ of the affinity label is 3.3 Å from the hydroxyl oxygen of Y807 whereas the carboxyl oxygens of D811 and D812 are more than 15 Å away from the reactive carbon of the affinity label (Figure 5). These results further support the inactivation data that Y807 is the amino acid modified by Sp-cAMPS-BDB.

Residue Y807 in PDE3A is involved in substrate cAMP binding – Table 1 shows the kinetic characteristics of the mutant enzymes D811A and D812A. The $K_\text{m}$ values for both D811A and D812A are similar to that of the wildtype. The $k_{\text{cat}}$ values of D811A and D812A are similar to that of wildtype and suggest that single alanine mutation of the residues, D811 and D812 does not affect the enzyme catalytic activity. We further studied the mutant Y807A. The $K_\text{m}$ of Y807A is 6.79 ± 0.83 µM which is 30-fold greater than that of the wildtype PDE3A. This indicates that Y807 is involved in cAMP binding. The $k_{\text{cat}}$ value of the mutants Y807A was similar to the wildtype (Table 1).

**Mutant Y807C mimics the wildtype PDE3A** – The amino acid corresponding to Y807 in the second member of the PDE3 gene family PDE3B is C792 (20). We hypothesized that the cysteine 792 might serve as a hydrogen donor similar to tyrosine Y807. Therefore, we produced the mutant Y807C in PDE3A. Both the $K_\text{m}$ (0.16 ± 0.01 µM) and $k_{\text{cat}}$ (105 s$^{-1}$) of PDE3A mutant Y807C were similar to the wildtype. To test the hypothesis that the thiol group mimics the phenolic group, we performed the inactivation studies of the mutant Y807C using Sp-cAMPS-BDB. This mutant, Y807C is irreversibly inactivated by the affinity label Sp-cAMPS-BDB in a time dependent manner exhibiting a $K_\text{I}$ of 18.0 ± 2.7 µM and $k_{\text{max}}$ of 0.004 ± 0.0002 min$^{-1}$ (Figure 4E and 4J). These values of Y807C are very close to the wildtype.

**Mutant Y807A is not inhibited by the anti-insert antibody** The anti-insert antibody was raised against the fifteen amino acids (V802FSKTYNVTDDKYG816) located at the C-terminal end of the insert within which the octapeptide 806-813 is identified by the affinity label. When the anti-insert antibody was added prior to the PDE3A activity assay, the wildtype decreased in activity to 84%, 55% and 32% proportional to antibody concentration (Figure 6A). Under the same conditions, PDE3B, the other gene product of the PDE3 gene family does not decrease in enzymatic activity when the anti-insert antibody is added (data not shown) indicating the specificity of the neutralization by antibody to PDE3A.

Two mutants were made at residue 807 position to compare with the wildtype tyrosine, Y807A eliminates the phenolic group and Y807C substitutes a thiol group mimicking the corresponding residue C792 of PDE3B. When
The anti-insert antibody was preincubated with the enzyme prior to the activity assay, the mutant Y807A did not decrease in activity at any of the antibody concentrations (Figure 6B). Similarly, when the Y807C was preincubated with varying concentrations of anti-insert antibody, the enzyme activity did not decrease as a function of antibody concentrations (Figure 6C). The failure of the anti-insert antibody to inhibit both the inactive mutant Y807A and the active mutant Y807C indicates that tyrosine residue is a critical part of the epitope of the antibody. Aromatic amino acids are frequently highly antigenic. The results also indicates that the concentration of the antibody did not block the active site.

The $K_m$ of the wildtype enzyme was similar in the presence or absence of the anti-insert antibody. ($K_m = 0.203$ vs $0.197 \mu M$). The $k_{cat}$ is decreased 2.2-fold ($k_{cat} = 158$ vs $70.6 \text{ s}^{-1}$ respectively) which is consistent with the decrease of 3.6-fold in enzymatic activity in the presence of $0.053 \mu M$ anti-insert IgG (Figure 6A). The preimmune IgG which does not contain the epitope to interact with the enzyme did not inhibit the activity of wildtype, Y807A and Y807C (data not shown). These data support the finding that residue Y807 is the amino acid modified by the affinity label Sp-cAMPS-BDB and is consistent with the markedly increased $K_m$ of the mutant Y807A.

Four out of the nine additional insert mutants are involved in catalytic activity – Based on the conservation of the amino acid sequence (Figure 1) and molecular model of PDE3A (Figure 5), eight additional amino acid residues H782, H796, H798, S804, K805, T810, Y814 and G815 at the unique 44 amino acid insert were chosen to mutate to alanine. In addition, C816 was mutated to a serine, since as previously reported mutant C816A completely abrogated the enzyme activity (21). Each of the mutants exhibited a single band on SDS gel electrophoresis (data not shown). Table 1 shows that mutants H796A, H798A, S804A, K805A, T810, Y814 and G815A at the unique 44 amino acid insert were chosen to mutate to alanine. In addition, C816 was mutated to a serine, since as previously reported mutant C816A completely abrogated the enzyme activity (21). Each of the mutants exhibited a single band on SDS gel electrophoresis (data not shown). Table 1 shows that mutants H796A, H798A, S804A, K805A and G815A have no marked changes in kinetic constants compared to wildtype PDE3A. In contrast, mutants H782A, T810A, Y814A and C816S show a significant 5- to 29-fold decrease in $k_{cat}$ but no major changes in $K_m$ as wildtype PDE3A. Since these four amino acid residues are distant from those involved in catalysis in the enzyme, the insert amino acid residues H782, T810, Y814 and C816 are important for PDE3A catalytic activity only after substrate binding.

The functional role of the insert is supported by the conformational similarity of mutants and wildtype – It is possible that mutation of a single amino acid could extensively alter the protein conformation and that this change would result in the loss of enzymatic function. Therefore the immunoreactivity of all the mutants was analyzed to ensure that the conformation was similar between the wildtype and mutants. Each mutant was detected as a single band in Western blot with the polyclonal anti-insert antibody (Figure 7). In addition, the twelve mutants had insignificant changes of $K_m$, with exception of Y807A (Table 1). The CD spectra of both PDE3A wildtype and mutant Y807A were measured to test whether the secondary structures were similar. The CD spectra of both wildtype and mutant Y807A are virtually superimposable and display a negative trough at 209 nm ($[\theta]_W T = -5040$ and $[\theta]_Y807A = -4890 \text{ deg cm}^2 \text{ dmol}^{-1}$, respectively (Data not shown). The overall similarity in the far-UV CD spectra of the mutant Y807A and wildtype PDE3A indicates that the mutant Y807A maintains a native secondary structure. The Western blot results and the CD spectral data suggest that all mutants have overall conformation similar to that of the wildtype PDE3A.

DISCUSSION

Reactive purine nucleotide analogs have been used as affinity labels to probe nucleotide binding sites (22, 23,24). We have described the use of the cAMP affinity analog 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3',5'-cyclic monophosphate (8-BDB-TcAMP) in studies to identify important amino acids within the active site of PDEs. 8-BDB-TcAMP irreversibly inactivated PDE2A (25), PDE3A (26) and PDE4A (27). 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 inactivates
PDEs only at millimolar concentrations, because of continuous hydrolysis to the 5'-AMP derivative by the enzymes under investigation. We reported the synthesis of a 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 (15). 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.

We here demonstrate that Sp-cAMPS-BDB acts as an affinity label of PDE3A. The Sp-cAMPS-BDB is a substrate analog of cAMP with the reactive bromodioxobutyl group at the phosphorothioate ester. An octapeptide (T806YNVTDDK813) in PDE3A has been identified by tryptic digest, peptide isolation and N-terminal amino acid sequencing. Three nucleophilic amino acid residues in the octapeptide were selected to produce mutants (Y807A, D811A and D812A) to identify the target residue reacting with Sp-cAMPS-BDB. Both mutants D811A and D812A were inactivated by Sp-cAMPS-BDB, while Y807A is not inactivated by the affinity label. Furthermore, we showed that Y807 exhibits a large change in $K_m$ and that this amino acid is close (3.3 Å) to the reactive carbon of the affinity label in a docking model based on the crystal structure of PDE3B. Y807, although it is present in the 44 amino acid insert, is functionally part of the cAMP binding site. The other two amino acids D811A and D812A in the octapeptide capable of reacting with the affinity label are more than 15 Å from the reactive carbon of Sp-cAMPS-BDB and have similar $k_{cat}/K_m$ to wildtype. Thus, Y807 is the amino acid modified by the affinity label rather than D811 or D812.

Since the function of the unique 44 amino acid insert in PDE3A has not been fully understood, we have produced nine additional insert mutants to explore its function based on the affinity labeling data and alignment analysis of the PDE3 gene family. Four mutants (H796A, H798A, S804A, K805A and G815A) have similar $k_{cat}/K_m$ to wildtype. These results indicate that the flexible 44 amino acid insert of human platelet PDE3A predominately regulates the catalysis of the substrate cAMP.

To further explore the function of the 44 amino acid insert, we produced an antibody to a peptide containing the 15 amino acids at the C-terminal end of the insert. In the presence of the anti-insert antibody, the ability of PDE3A and mutant H798A to hydrolyze cAMP was reduced while the apparent $K_m$ remains unchanged. These results support the conclusion that one of the important functions of this insert is to regulate the enzyme catalytic activity. The exception to this is Y807 which is the closest amino acid to the docked affinity label Sp-cAMPS-BDB. Not only is this amino acid close enough to bind the affinity label but also appears to be the only residue of the 12 mutants tested to affect the $K_m$. Therefore Y807 is a participant in the substrate binding site. Because the $K_m$ is increased 30-fold compared to the wildtype, it was especially important to test whether a major conformational change had occurred in Y807A. The CD spectra of both wildtype and mutant Y807A are almost superimposable, indicating that the mutant Y807A maintains a native conformation (data not shown).

When we assessed Y807A with the anti-insert antibodies we found no inhibition at any of the concentrations tested in contrast to the behavior of the wildtype enzyme. These results indicate that although Y807 does not affect the $k_{cat}$ it directly regulates the catalytic activity, presumably due to conformational change upon substrate binding, and directly by influencing the substrate binding. However, this is not related to the loss of activity since the active mutant Y807C is also not inhibited by the antibody. These results suggest that Y807 is a critical part of the epitope of antibody but other epitopes exist in the loops.

We speculate that the C792 in PDE3B might play the same role as Y807 in PDE3A. The corresponding PDE3A mutant and its functional group reacts similarly to Y. The kinetic constants $K_m$ and $k_{cat}$ of Y807C were similar to that of wildtype. Furthermore, Y807C is irreversibly inactivated by the affinity label.
Sp-cAMPS-BDB in a time-dependent manner to a similar extent when compared to the wildtype. It is likely that the phenolic group of PDE3A and the thiol group of PDE3B both function as hydrogen donors in the interaction with substrate cAMP.

The 44 amino acid insert shown in the molecular model constitutes a flexible loop exposed on the surface of the enzyme (Figure 5). Although the unique insert lies in the first conserved metal binding motif H752NRH756(X)24-26E825 in the active site cleft from the primary structure of the enzyme. The homology model based on crystalline PDE3B indicates that the flexible loop of the insert is distant from the active site cleft in the model. These kinetic analyses and molecular modeling data imply that upon substrate binding this surface flexible insert may undergo substantial local conformational change. We hypothesize that the flexible insert flips into the active site cleft to regulate the substrate binding and catalytic activity. Further studies are underway to document any conformational change associated with substrate binding.

A precedent for local conformational change of a loop has been shown in the reaction of trypsin with α1 antitrypsin (28,29). When trypsin cleaves the reactive center loop (RCL) of α1 antitrypsin, the cleaved RCL undergoes a large local conformational change, and zips into a groove of β-sheet of the molecule with the translocation of trypsin to the other pole of α1 antitrypsin.

In the case of aspartic peptidases, the variation in flap conformations observed in X-ray studies of free and inhibitor bound enzymes indicates that the flaps in the free enzyme are flexible in solution. For example, binding of pepstatin to cathepsin D induced small structural changes in the flap region that contains the β-hairpin structure from residues 72-87 (30). Residues 79 and 80 at the tip of the flap moved in toward the inhibitor by about 1.7 Å and the flexibility of this β-bend decreases due to electrostatic interaction of H77 of the flap with the C-terminus of the inhibitor. Similar changes in conformation upon inhibitor binding have also been shown in the pepstatin-bound form of both rhizopuspepsin and penicillopepsin (31) (32). The closing of the flap over the inhibitor-substrate serves to remove the peptide bond of the substrate from effective contact with solvent.

In conclusion, use of the nonhydrolyzable affinity label Sp-cAMPS-BDB and structural analysis have allowed us to identify a new cAMP binding amino acid (Y807) in the 44 amino acid insert which forms a flexible loop unique for the PDE3 gene family. These results challenged us to produce nine additional insert mutants which defined the role of H782, T810, Y814 and C816 as amino acids interacting with the residues involved in catalysis and/or metal binding. The identical behavior of the mutant Y807C to the wildtype suggests that this tyrosine residue may be functioning as a H donor. The presence of a similar loop in PDE3B with a cysteine instead of tyrosine in PDE3A at the homologous position suggests a similar mechanism may be involved with PDE3B substrate binding. The affinity labeling results and the kinetic data from the mutants suggest a functional role of the insert and provide a new strategy for structural-based inhibitor design to develop new specific inhibitors for PDE3A.

REFERENCES

FOOTNOTES

We thank our students Miss Penelope Francis for producing the Y807A, D811A and D812A mutant plasmid DNA and baculoviruses, Miss Karine D. Barnes preparation of the wildtype, Y807A, D811A and D812A mutant enzymes; Mr. Christopher M. Matsko, Miss Rachel Noone, Miss Heather Scull Miss Lateeka D. Williams and Miss Jing Tao for performing kinetic studies of PDE3A wildtype and mutants. We thank Dr. Anna P. Tretiakova for using the molecular modeling to design the 9 additional insert mutants. We thank Dr. Patrick J. Loll from Department of Biochemistry, Drexel University for use of the Circular Dichroism spectropolarimeter. We thank Mr. Frank Cunliffe for help with manuscript preparation. This work was supported by grants from the Pennsylvania/Delaware Affiliated American Heart Association (026439U) (S.H.H.), The National Institute of Health (grant HL-64943) (R.W.C.) and the National Science Foundation (grant MCB97-28202) (R.F.C.).

FIGURE LEGENDS

Figure 1. Amino acid sequence alignment of the 44 amino acid insert in PDE3 gene family. The sequences of the 44 amino acid inserts from human PDE3A and PDE3B genes are presented. Numbers indicate residue positioning relative to each protein sequence. The bold underlined letters indicate the PDE3A insert mutants produced in this report. The line indicates the epitope of the antiinsert antibody.

Figure 2. Incorporation of Sp-cAMPS-BDB into PDE3A. Panel A shows the time-dependent incorporation of Sp-cAMPS-BDB into PDE3A. The enzyme was incubated in 50 mM Hepes buffer, pH 7.3 at 25°C. At the indicated times, the incorporation was stopped by two additions of [3H]NaBH4 (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. Panel B shows the relationship between inactivation and incorporation of Sp-cAMPS-BDB into PDE3A. The residual activity of the unmodified enzyme was determined at 20, 30, 40, 60 and 80 minutes. Enzymes treated with the same procedure but without Sp-cAMPS-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.

Figure 3. Isolation of trypsin digested peptides by HPLC. The affinity labeled PDE3A was digested with trypsin. The trypsin digested peptides were separated by a reverse phase HPLC C18 column under the conditions described in Materials and Methods. The curve with solid line shows the A220 nm HPLC profile. The gradient of acetonitrile of the tryptic digested peptides was shown in solid line. The curve with dotted line shows the radioactivity pattern of the same chromatograph. The two radioactive peaks are labeled I and II. This experiment was performed twice with the results in close agreement. This figure represents one of the two experiments.

Figure 4. Sp-cAMPS-BDB reaction plots (panels A, B, C, D and E) and pseudo first order rate plots (panels F, G, H, I and J) of PDE3A wildtype, Y807A, D811A, D812A and Y807C. The enzyme was incubated with Sp-cAMPS-BDB in 50 mM Hepes buffer at pH 7.3. At the indicated time intervals aliquots were removed, diluted, and assayed in duplicate for catalytic activity. Panel A: wildtype enzyme is incubated with (●) 0, (■) 12.5, (▲) 25, (◆) 50, (▼) 75, and (○) 100 µM of Sp-cAMPS-BDB. Panel B: Y807A is incubated with (●) 0, (■) 50, (▲) 100, (◆) 200, and (▼) 400 µM of Sp-cAMPS-BDB. Panel C: D811A is incubated with (●) 0, (■) 12.5, (▲) 25, (◆) 50, (▼) 75, (○) 100, and (○) 125 µM
of Sp-cAMPS-BDB. Panel D: D812A is incubated with (●) 0, (■) 25, (▲) 50, (♦) 100, (▼) 125, (●) 150 µM of Sp-cAMPS-BDB. Panel E: Y807C is incubated with (●) 0, (■) 12.5, (▲) 25, (♦) 50, (●) 100, and (▼) 200 µM of Sp-cAMPS-BDB. Panel F: The pseudo first order rate constant ($k_{obs}$) plot of wildtype incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 100 µM. Panel G: The $k_{obs}$ plot of Y807A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 400 µM. Panel H: The $k_{obs}$ plot of D811A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 125 µM. Panel I: The $k_{obs}$ plot of D812A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 150 µM. Panel J: The $k_{obs}$ plot of Y807C incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 200 µM. 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%.

Figure 5. Molecular model of PDE3A. PDE3A was modeled using Sybyl Composer with 16 chains of PDE3B (1SO2 and 1SOJ) shown in quintuple red lines. The 44 amino acid insert is depicted in solid red ribbon. FlexX (Sybyl) was then used to dock Sp-cAMPS-BDB into the PDE3A model with a defined cAMP binding pocket of Y807 (green), N845, E866, E971, F972 and F1004 labeled in black. Mutants in the insert that affect the $k_{cat}$ are labeled in blue.

Figure 6. Enzyme activity of the wildtype, Y807A and Y807C in the presence of the anti-insert antibody. The anti-insert antibody contains the epitope of the fifteen amino acids at the C-terminal end of the insert (V802FSKTYNVTDKDYGC816). The enzyme activities of the wildtype, Y807A and Y807C in the absence of the anti-insert antibody were set at 100%, respectively. The final concentration of wildtype and Y807C were 0.064 and 0.576 µM, respectively. Because of the low catalytic activity of Y807A the final concentration was 1.75 µM. The antibody concentrations used were calculated to yield same ratios of enzyme: antibody for wildtype, Y807A and Y807C. Panel A shows the activity of wildtype (●) were measured in the presence of 0.017, 0.034, and 0.051 µM the anti-insert antibody. Panel B shows the activity of Y807A (■) were measured in the presence of 0.43, 0.87 and 1.30 µM of the anti-insert antibody IgG. Panel C shows the activity of Y807C (▲) were measured in the presence of 0.017, 0.034, and 0.051 µM the anti-insert antibody. These data are the mean ± SEM of three independent experiments. Each experiment was performed in triplicate. Where the error bars are not seen it is because they are too small to be visualized.

Figure 7. Western blot of insert mutants shows the Western blot of nine additional loop mutants and wildtype PDE3A. The purified wildtype and mutant enzymes were separated on SDS-PAGE gel, transferred to PVDF membrane. According to the manufacturer’s instruction, the transferred PVDF membrane was processed using the Chromogenic WesternBreeze System (Invitrogen) and probed with Anti-insert antibody to detect the presence of PDE3A.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ $\mu M$</th>
<th>$k_{cat}$ (x100) $s^{-1}$</th>
<th>$k_{cat}/K_m$ $\mu M^{-1} s^{-1}$</th>
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</thead>
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<tr>
<td>PDE3A</td>
<td>0.23 ± 0.15</td>
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<td>H782A</td>
<td>0.45 ± 0.10</td>
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<td><strong>0.50</strong></td>
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<td>0.20 ± 0.03</td>
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<td>H798A</td>
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<td>101.3</td>
<td>4.14</td>
</tr>
<tr>
<td>S804A</td>
<td>0.28 ± 0.01</td>
<td>69.0</td>
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<tr>
<td>K805A</td>
<td>0.27 ± 0.05</td>
<td>76.3</td>
<td>2.88</td>
</tr>
<tr>
<td>Y807A</td>
<td>6.79 ± 0.83</td>
<td>84.0</td>
<td><strong>0.12</strong></td>
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<tr>
<td>Y807C</td>
<td>0.16 ± 0.01</td>
<td>105.0</td>
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<tr>
<td>T810A</td>
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<td><strong>36.9</strong></td>
<td><strong>0.69</strong></td>
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<td>D811A</td>
<td>0.17 ± 0.03</td>
<td>136.0</td>
<td>8.02</td>
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<tr>
<td>D812A</td>
<td>0.20 ± 0.01</td>
<td>50.0</td>
<td>2.50</td>
</tr>
<tr>
<td>Y814A</td>
<td>0.65 ± 0.11</td>
<td><strong>23.1</strong></td>
<td><strong>0.36</strong></td>
</tr>
<tr>
<td>G815A</td>
<td>0.26 ± 0.01</td>
<td>86.7</td>
<td>3.40</td>
</tr>
<tr>
<td>C816S</td>
<td>0.34 ± 0.12</td>
<td><strong>8.5</strong></td>
<td><strong>0.25</strong></td>
</tr>
</tbody>
</table>

Mutants are listed in the order of their position in the sequence. Results are the mean of three sets of experiments and for $K_m$ values the standard error of the mean (SEM) is indicated. The data shown in bold indicate statistically significant changes (Student’s t test, $p<0.01$) in kinetic parameters of the mutant enzyme when compared that with the wildtype.
Figure 1

PDE3A

<table>
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<tr>
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<th>Sequence</th>
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<td>PGLSIVINDHGSDSDSDSDFTHGMYVFSKTVNDYKGC 816</td>
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<tr>
<td>Pig</td>
<td>PGLSIVINDHGSDSDSDSDFTHGMYVFSKMYVPDDKYGC 664</td>
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<tr>
<td>Rat</td>
<td>PGLSIVINDHGSDSDSDSDFTHGMYVFSKAYHVDDKYGC 816</td>
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<tr>
<td>Mouse</td>
<td>PGLSIVINDHGSDSDSDSDFTHGMYVFSKMYHVDDKYGC 816</td>
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PDE3B

<table>
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<tbody>
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<td>Human</td>
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</tr>
<tr>
<td>Rat</td>
<td>PGLOQILHNNHETKADSARLSGQIAYISSKCSGPDKSYGC 783</td>
</tr>
<tr>
<td>Mouse</td>
<td>PGLOQIHNHCYDSDGRLGQIAYISSKCSIPDMSYGC 777</td>
</tr>
</tbody>
</table>

Conserved: PGL---DSD---G---Y---SK---D---YGC

Figure 2

![Graphs showing incorporation and residual activity of PDE3A.](http://www.jbc.org/)

Incorporation (mole Sp-cAMPS-BDB/mole PDE3A) vs Time, min

Residual PDE3A Activity (%) vs Incorporation (mole of Sp-cAMPS-BDB/mole of PDE3A)
Figure 3
Figure 4

A

\[
\frac{E}{E_0} \quad \text{WT}
\]

B

\[
\frac{E}{E_0} \quad \text{Y807A}
\]

C

\[
\frac{E}{E_0} \quad \text{D811A}
\]

D

\[
\frac{E}{E_0} \quad \text{D812A}
\]

E

\[
\frac{E}{E_0} \quad \text{Y807C}
\]

F

\[
k_{\text{obs, min}^{-1}} \quad \text{WT}
\]

G

\[
k_{\text{obs, min}^{-1}} \quad \text{Y807A}
\]

H

\[
k_{\text{obs, min}^{-1}} \quad \text{D811A}
\]

I

\[
k_{\text{obs, min}^{-1}} \quad \text{D812A}
\]

J

\[
k_{\text{obs, min}^{-1}} \quad \text{Y807C}
\]
Figure 5
Figure 6

(A) WT

(B) Y807A

(C) Y807C

Graphs showing % Activity vs. IgG concentration for WT, Y807A, and Y807C.
Figure 7
New insights from the structure function analysis of the catalytic region of human platelet PDE3A: a role for the unique 44 amino acid insert
Su-Hwi Hung, Wei Zhang, Robin A. Pixley, Bradford A. Jameson, Yu Chu Huang, Roberta F. Colman and Robert W. Colman

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