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BINDING OF CYCLIC NUCLEOTIDES TO PHOSPHODIESTERASE 10A AND 11A GAF DOMAINS DOES NOT STIMULATE CATALYTIC ACTIVITY

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Short title: Cyclic nucleotide binding to PDE10A and 11A GAF domains

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To date, 11 human cyclic nucleotide phosphodiesterase (PDE) families have been identified. Of these, five families contain non-catalytic tandem GAF domains (GAFa and GAFb) in the N-terminal part of the enzyme. For PDE2A, PDE5A and PDE6, the GAF domains have been shown to bind cyclic GMP with high affinity. For PDE2A and PDE5A, the ligand binding has been shown to stimulate the catalytic activity of the enzyme. For the most recently described PDEs, PDE10A and PDE11A, the GAF domains have previously been suggested to bind cAMP and cGMP, respectively. We have developed scintillation proximity based assays for cyclic nucleotide binding to PDE2A, PDE10A and PDE11A GAF domains. We directly demonstrate binding of cyclic nucleotides to the PDE10A and PDE11A GAF domains and show that these non-catalytic sites bind cAMP and cGMP, respectively, with much higher affinity than has previously been suggested from indirect assessment of the interaction with cyclic nucleotides. The GAFb domain of PDE10A binds cAMP with a K_D of 48nM. For PDE11A, the GAFa domain binds cGMP with a K_D of 110nM. The effect of binding of ligand to the GAF domains on enzyme activity was investigated through the use of modified cyclic nucleotides. In contrast to the other GAF domain containing PDEs and to what has previously been predicted, ligand binding to the GAF domains of PDE10A and PDE11A does not stimulate catalytic activity.

Keywords: PDE10A, PDE11A, GAF domain, cAMP, cGMP, scintillation proximity assay

Abbreviations: PDE, 3',5'-cyclic nucleotide phosphodiesterase; GAF, cGMP-stimulated PDEs, adenylyl cyclase, FhlA; SPA, scintillation proximity assay; CPM, counts per minute

INTRODUCTION

The cyclic nucleotides cAMP and cGMP are ubiquitous intracellular second messengers that are involved in control of cell functions through several signal transduction pathways [1]. Therefore, intra- and subcellular concentrations of the cyclic nucleotides have to be strictly regulated. Their synthesis is catalyzed by adenylyl and guanylyl cyclases, which in turn are regulated by G protein-coupled receptors and nitric oxide, respectively [2,3]. The hydrolysis of cAMP and cGMP is catalyzed by cyclic nucleotide phosphodiesterases (PDEs)¹, restricting cyclic nucleotide signalling both spatially and temporally [4].

The human genome contains 21 PDE genes, phylogenetically divided into 11 PDE families, numbered 1-11 [5-7]. Though the encoded enzymes all catalyze the same reaction, they differ in affinities for substrate(s), physiological regulation, tissue distribution and subcellular distribution. Consequently, the biological effect of inhibition of individual PDE enzymes differ, and selective PDE inhibitors are being used or are under development for treatment of a variety of diseases [6,8,9]. PDE10A and PDE11A are both dual substrate PDEs. PDE10A has the highest affinity for cAMP, but with the highest V_{\max} for cGMP [10,11], whereas for PDE11A, the catalytic characteristics are nearly equal for both substrates [7]. PDE10A is highly expressed in all medium spiny neurons in the striatum [12], and is a potential target for treatment of schizophrenia [13]. Less is known about the biological importance for PDE11A, but there is some evidence for a role in spermatozoa physiology [14].

All human PDEs share a similar structure with a catalytic domain in the C-terminal half and different N-terminal domains that appear to serve regulatory functions. The genes of five PDE families (PDEs 2, 5, 6, 10 and 11) have regions coding for tandem GAF domains (GAFa and GAFb) located in the N-terminal part of the enzyme (the GAF acronym is derived from the enzymes in which they were first described: cGMP-stimulated PDEs, *Anabaena* adenylyl cyclase and *Escherichia coli* transcription factor FhlA) [15,16]. In PDEs 2, 5 and 6, the GAF domains contain a non-catalytic binding site in GAFa (PDE5A and PDE6) or GAFb (PDE2A) with high binding affinity for cGMP [17-22]. cGMP binding to PDE2A and PDE5A GAF domains stimulates the catalytic activity of the enzymes [23-25], whereas for PDE6, cGMP binding is known to stimulate binding of its inhibitory subunit [26]. The properties of PDE10A and PDE11A GAF domains have only been assessed indirectly. Two recent papers have assessed their function and ligand interactions indirectly through measuring catalytic activity of a bacterial adenylyl cyclase catalytic domain fused to the human PDE GAF domains in chimeric constructs [27,28]. They found that adenylyl cyclase activity of a chimera with PDE10A GAF domains was stimulated by cAMP with an EC_{50} of 19.8 μ M, while the adenylyl cyclase activity of a chimera with PDE11A GAF domains was stimulated by cGMP with an EC_{50} of 72.5 μ M. However, the EC_{50} s observed in their assays seem to be too high to have any physiological relevance and the chimeras do not allow direct assessment of the impact of ligand binding on phosphodiesterase activity, though they suggest that phosphodiesterase activity may be regulated by the GAF domains upon ligand binding as for PDE2A and PDE5A.

In this study, we describe new cyclic nucleotide binding assays for the human PDE10A and PDE11A GAF domains. PDE2A is also included, primarily as a methodological control. We find that PDE10A and -11A contain high affinity binding sites for cAMP and cGMP, respectively. We have discovered modified cyclic nucleotides that bind much more strongly to the GAF domains than to the catalytic domains and we use these nucleotides to examine the impact of GAF ligand binding on catalytic function. Unlike for PDE2A and GAF-adenylyl cyclase chimeras, we find no evidence of regulation of PDE10A and PDE11A enzymatic activity upon binding of cyclic nucleotides to the GAF domains.

MATERIALS AND METHODS

Materials

cAMP and cGMP analogues were purchased from BioLog. AMP, GMP, cAMP and cGMP were purchased from Sigma. Tritium labelled nucleotides [5', 8-³H]-cAMP (1μCi/μl, 30-60Ci/mmol) and [8-³H]-cGMP (1μCi/μl, 5-25Ci/mmol) were purchased from Amersham Radiochemicals.

Cloning and expression of constructs

cDNA for human PDE2A3, PDE10A2, and PDE11A4 was used as templates for PCR reactions. All GAF constructs and PDE10A catalytic domain (cata) were cloned into the pET28a vector, which adds a His₆-tag and a T7-tag to the N-terminal of the expressed protein, and were then transformed into *Escherichia coli* TOP10 cells. DNA was purified from small-scale vector preparations using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol. The DNA segments subjected to cloning were sequenced to ensure the correctness of the sequence and the proper in-frame cloning.

The PDE10A catalytic domain and the different GAF domain constructs were expressed in the *E. coli* strain Rosetta TM2. Cells were grown in LB medium (GIBCO) with added chloramphenicol (34μg/ml) and kanamycin (50μg/ml) at 37°C until OD₆₀₀ was 0.4-0.6. The cells were then induced with isopropyl-β-D-thiogalactopyranoside (0.5mM) and incubated at room temperature over night. Cells were disrupted in lysis buffer (50mM Tris pH 8.0, 1mM MgCl₂, 1% Complete protease inhibitor (Roche)) by sonication. 0.1% Triton X-100 was added and the sample was then centrifuged at 5300x g for 15 min. at 4°C and the pellet discarded. Glycerol was added to the lysate to a final concentration of 44% and samples were then stored at -20°C.

The catalytic domain from PDE2A and PDE11A and all full length constructs were cloned into pFastBac-HT vectors and were expressed in Sf9 cells using the Bac-to-Bac® Baculovirus Expression System (Gibco). Sf9 cells were grown at 27°C in Sf-900 II serum free medium containing 50units/ml penicillin and 50μg/ml streptomycin and were infected with 1ml of virus for 25ml of media. At 72h, cells were harvested and disrupted in lysis buffer for 15 min. on ice and then centrifuged at 20,000x g for 20 min. Glycerol was added to the lysate to a final concentration of 44% and samples were then stored at -20°C.

The integrity of recombinant proteins was assessed by denaturing SDS/PAGE on 4-12% Bis-Tris gel (Invitrogen) and subsequent Western blotting on polyvinylidene difluoride membranes (Millipore). Rabbit polyclonal anti-T7 antibody (QED Bioscience Inc.) was used as primary antibody - the T7 epitope tag is encoded by the pET28a vector. Swine anti-rabbit conjugated with horseradish peroxidase (DAKO) was used as secondary antibody. The blot was developed using the SuperSignal West Dura kit (Pierce).

GAF domain binding assays

Recombinant GAF domains were mixed with copper-coated Scintillation Proximity Assay (SPA) beads (Amersham Bioscience) and [³H]-labelled cyclic nucleotides. The His₆-tags attach the proteins to the beads through binding to Cu²⁺ and binding of tritiated ligands to the GAF domains consequently induce measurable light from the scintillant in the beads. The measurements were compared to the background assessed by either competition with high concentrations of unlabeled cyclic nucleotide or naked beads mixed with the [³H]-labelled cyclic nucleotides.

Assays were conducted in 96-well plates with a final volume of 64µl per well in a buffer containing 50mM HEPES pH 7.6, 10mM MgCl₂ and 0.2mg/ml bovine serum albumin (BSA). 7µl Polyvinyltoluene (PVT) Copper His-Tag SPA beads (20mg/ml) and lysate containing PDE2A, PDE10A or PDE11A GAF-domains was first added to each well. For binding assays, [5', 8-³H]-cAMP or [8-³H]-cGMP was then added in the various concentrations indicated in the figures. For competition binding assays, the respective unlabeled nucleotides were added first followed by the radioactively labelled ligands in final concentrations of 14nM [³H]-cAMP (PDE10A) or 60nM [³H]-cGMP (PDE2A and PDE11A). After 1 hour at room temperature the plates were counted in a liquid scintillation counter (Wallac Trilux).

For binding assays, data was fitted to a hyperbolic equation (one site binding) using GraphPad Prism. For competition binding assays, data was fitted to a sigmoidal dose-response curve with variable slope. IC₅₀ values was converted to K_i values using the equation

$$K_i = \frac{IC_{50}}{1 + \frac{[({}^3\text{H})\text{-cNMP}]}{K_D}}$$

Results represent the mean ± SEM of two to four experiments for each analogue.

PDE activity assays

PDE activity assays were based on binding of the products of phosphodiesterase enzyme reactions - [³H]-AMP or [³H]-GMP - to Yttrium silicate (YSi) SPA beads (Amersham Bioscience). These beads bind the products but not the substrates of the enzyme reactions leading to light emittance from the scintillant in the beads, which is quantified.

Assays were conducted in 96-well plates with a final volume of 64µl in a buffer containing 40mM Hepes pH 7.2, 140mM KCl, 10mM NaCl, 1.5mM MgCl₂, 0.05% Tween, 0.04mg/ml BSA. Competing cold nucleotides and radioactively labelled ligands ([5', 8-³H]-cAMP for PDE2A and PDE11A and [8-³H]-cGMP for PDE10A) were added to buffer containing lysates containing the relevant recombinant phosphodiesterase enzymes. Enzyme amounts were kept so that less than 30% of the substrate in each reaction was hydrolyzed during the reaction time. The reaction was allowed to run for one hour at room temperature before 15µl of SPA beads were added (8mg/ml). The beads contain Zn²⁺ that terminates the reaction by inactivating the enzymes. After another hour at room temperature to allow binding of the enzyme products to the beads, plates were counted for two min. in a liquid scintillation counter (Wallac Trilux). IC₅₀ values were obtained by fitting the data to a sigmoidal dose-response curve with variable slope and subsequently converted to K_i using the equation

$$K_i = \frac{IC_{50}}{1 + \frac{[({}^3\text{H})\text{-cNMP}]}{K_M}}$$

Results represent the mean ± SEM of two or three experiments for each analogue.

For all SPA assays the amount of lysate was adjusted to obtain a maximum scintillation output of approximately 1000 counts per minute (CPM).

RESULTS

The GAF domains of PDE10A bind cAMP and the GAF domains of PDE11A bind cGMP with high affinity

In this study, a new GAF domain binding assay based on scintillation proximity assay (SPA) beads was developed for PDE2A3, -10A2 and -11A4 recombinant GAF domains. Residue boundaries are indicated in Figure 1A, Western blots of the PDE GAF domain constructs are

shown in Figure 1B. Unlike previously described GAF binding assays, our assay is a homogenous equilibrium assay without capture and washing steps. As shown in Figure 2, binding assays with [³H]-cAMP gave an approximately 15-fold window for binding to PDE10A GAF domains, while [³H]-cGMP gave similar windows for binding to PDE2A and PDE11A GAF domains. Essentially no window was observed for binding of [³H]-cGMP to PDE10A GAFab and [³H]-cAMP to PDE2A GAFab and PDE11A GAFab. The binding curve corrected for background gave an apparent K_D of 48nM for PDE10A GAFab with [³H]-cAMP. For PDE2A and PDE11A GAF domains with [³H]-cGMP, the dissociation constants were 66nM and 110nM, respectively. [³H]-cAMP was therefore chosen as radioactive ligand for the competition binding assays with PDE10A GAF domains and [³H]-cGMP was chosen for PDE2A GAF domains and PDE11A GAF domains.

Figure 3 shows competition binding assays for the GAF domains from PDE2A, -10A and -11A with unlabeled cyclic nucleotides. For PDE10A GAFab, cAMP exhibited a K_i of $0.030 \pm 0.004\mu\text{M}$ when used as competitive substrate whereas cGMP gave a K_i of $1.6 \pm 0.12\mu\text{M}$. For PDE2A and PDE11A GAFab, the reverse preference was observed, as expected from the binding assays. Binding of cGMP to the GAF domains of PDE2A and PDE11A showed the highest affinities with K_i s of $0.031 \pm 0.004\mu\text{M}$ and $0.035 \pm 0.006\mu\text{M}$, respectively, while cAMP gave K_i s of $4.3 \pm 0.55\mu\text{M}$ and $41 \pm 5.8\mu\text{M}$, respectively. Thus, cAMP is a high affinity ligand for binding in the non-catalytic binding site in PDE10A and cGMP for PDE11A.

Localization of the non-catalytic binding site

For all known human PDE enzymes containing GAF domains, the respective gene encodes two GAF domains arranged in tandem (a and b). However, for PDE2, -5 and -6, only one of the GAF domains has been found to be involved in ligand binding [24,25,29]. To determine whether both or which of the GAF domains contain a binding site, the N-terminal most (GAFa) and the C-terminal most (GAFb) of the GAF domains of PDE10A and PDE11A were expressed separately and tested in SPA-based binding assays (residue boundaries are indicated in Figure 1A). Figure 4 shows that only one of each tandem repeats binds cyclic nucleotides. PDE10 GAFb exhibited strong binding to [³H]-cAMP, while PDE11A GAFa bound [³H]-cGMP. Inhibition constants for the single domains are indicated in Table 1.

Binding of ligands to the non-catalytic binding site of PDE2A, -10A and -11A

In order to test the structural requirements for binding to the GAF domains and potentially to find compounds with increased preference for the non-catalytic binding site compared to the catalytic site, several cyclic nucleotide analogues were tested in GAF competition binding assays. The resulting K_i -values for the displacement of [³H]-cAMP from PDE10A GAF domains and [³H]-cGMP from PDE2A and PDE11A GAF domains by the cyclic nucleotide analogues are shown in Table 2.

The cyclic nucleotide analogues studied are divided into five groups depending on the sites altered relative to cAMP or cGMP (Figure 5 and Table 2). As expected, PDE10A GAF domains generally exhibit highest affinity for the analogues with the highest resemblance to cAMP while PDE2A and PDE11A GAF domains exhibit highest affinity for the cGMP analogues.

Group I consists of cyclic nucleotides with substitutions in the pyrimidine region, which is the moiety where the natural ligands cAMP and cGMP differ. Most substitutions in this region of the ligand lower the affinity for the non-catalytic binding site, but with a single exception all retain measurable affinity for the GAF domains. A few of the cyclic nucleotides from Group I exhibit affinities that are similar to the natural analogues. For PDE2A, the affinity for 5,6-DM-cBIMP (No. 10) is approximately the same as for cGMP, despite the substitution of the purine with a highly lipophilic benzimidazole and the different substituents. For PDE10A, substitution of the amino-group on C2 (for labelling of the

individual atoms, see structure of cAMP in Figure 5) with chlorine (No. 2) does not change the affinity for the GAF binding site substantially.

7-CH-cAMP is the only analogue tested that has a higher affinity than the presumed natural ligands. It belongs to the second group (II, Figure 5) with analogues substituted in the imidazole region. In 7-CH-cAMP (No. 12) the ring nitrogen in position 7 is replaced with a carbon atom. This substitution results in an about 3-fold higher affinity for PDE10A than cAMP, while lowering the already low affinity for PDE2A and PDE11A GAF domains.

Analogues with substitutions in the ribose region are assembled in Group III. Reduction of the C2' hydroxyl group (No. 15/16) reduces affinity 3 to 50 fold, while methylation of the same position (No. 17/18) results in more marked decreases of affinity for PDE2A and PDE11A and no measurable affinity for PDE10A.

The members of Group IV each have one of the two exocyclic oxygen atoms of the cyclic phosphate moiety substituted with sulphur, and they therefore contain a chiral centre. For both isomers, the substitution of one of the exocyclic oxygens makes the binding 40-200 fold weaker.

Group V consists of non-cyclic AMP (No. 24) and GMP (No. 25). The K_i for both is above the level of detection.

No activation of the catalytic activity is seen for PDE10A and PDE11A upon ligand binding to the GAF domains

cGMP and cGMP-derivatives can stimulate catalytic activity of PDE2A upon binding to the non-catalytic binding site [23], but the effect of cyclic nucleotide binding to PDE10A and PDE11A GAF domains are unknown. Since the ligand for the non-catalytic binding site also has high affinity for the catalytic site in these enzymes, inhibition at the catalytic site may conceal stimulating effects of binding to the GAF domains. Some of the cyclic nucleotide analogues might have better selectivity for the non-catalytic sites and thus be better tools for investigating regulation of enzymatic activity.

To find useful compounds, the cyclic nucleotide analogues with highest affinity for the non-catalytic binding sites were tested for their inhibition of phosphodiesterase activity in assays with recombinant catalytic domains of PDE2A, PDE10A and PDE11A (see Figure 1A for outline of constructs). The results are summarized in Table 3. All tested analogues displayed lower affinity for the catalytic sites compared to the respective non-catalytic binding sites in the regulatory GAF domains. For PDE2A, the ratio between the affinity for the catalytic and the non-catalytic binding site varies between almost 8000-fold for 5,6-DM-cBIMP and down to approximately 250-fold for 2'-dcGMP. For PDE10A, the ratios are smaller with an interval ranging from 247 for 1-NO-cAMP down to 8.7 for cAMP. For PDE11A, the highest ratio is more than 200 (Rp-cGMPS) and the lowest ratio for a ligand included in the table (2'-dcGMP) is 6.5.

Next, these ligands were tested for their effect on the activity of recombinant full-length enzymes comprised of both the catalytic subunit and the GAF domains. [^3H]-cAMP was used as substrate for PDE2A and PDE11A, while [^3H]-cGMP was used as substrate for PDE10A to eliminate binding of the tritiated substrate to the GAF domains. In agreement with previous reports [19,23], GAF ligands could stimulate full-length PDE2A3 activity approximately 4-fold compared to the basal activity at intermediate concentrations, while enzymatic activity is lowered again at higher ligand concentration – presumably due to inhibition at the catalytic site (Figure 6). The ligand concentration range in which activation is observed as well as the maximal observed activation correlates with the ratio between the affinity for the isolated PDE2A GAF domains and the potential for inhibition of the isolated PDE2A catalytic domain. However, no activation of full-length PDE10A2 and PDE11A4 enzymatic activity was observed in response to their respective GAF ligands, even though the selectivity ratios were similar to PDE2A ligands that gave effect (Figure 6). These experiments were repeated

with several different buffer conditions as well as with partially purified PDE10A from rat striatum instead of recombinant protein, but stimulation of enzymatic activity in response to GAF ligands was not observed under any of these conditions (results not shown). Thus, even for ligands with high apparent selectivity for GAF relative to the catalytic site, binding of ligand to the non-catalytic binding site does not stimulate the enzymatic activity for PDE10A2 and PDE11A4 under the conditions tested.

DISCUSSION

In this study, we developed new binding assays to assess GAF domain interactions. We show that cAMP is a high-affinity ligand for PDE10A GAFb and cGMP for PDE11A GAFa. We probed the structural requirements for cyclic nucleotide binding to the GAF domains and tested high affinity analogues for their interaction with the PDE catalytic sites. Analogues with high specificity for the non-catalytic site were used to assess the impact of cyclic nucleotide binding to the non-catalytic site on enzyme activity. The enzymatic activities of PDE10A2 and PDE11A4 were not stimulated by ligand binding to the GAF domains. This contrasts with earlier findings for PDE2A [23], confirmed in our study. Also, our findings for PDE10A and PDE11A are at variance with predictions based on experiments with chimeras of adenylyl cyclase and GAF domains from either PDE10A or PDE11A [27,28].

Previously described GAF-domain binding assays have mostly been based on capture of complexes on nitrocellulose membranes. Our scintillation proximity based assay has the advantage of being a homogenous equilibrium assay and is therefore not dependent on stability of complexes during washing. Comparative data exist only for PDE2A GAF domains. The K_i observed in the present assay – 31nM – is comparable to the IC_{50} observed previously with a nitrocellulose-based assay – 26nM [24].

We find that cAMP is a high affinity ligand for PDE10A GAF domains, while cGMP is a high affinity ligand for PDE11A GAF domains. Qualitatively similar results were found previously by measuring adenylyl cyclase activity from chimeras of PDE GAF domains and CyaB1 adenylyl cyclase [27]. In those experiments, adenylyl cyclase activity of a PDE10A GAF chimera was activated by cAMP, while a PDE11A GAF chimera was activated by cGMP. However, this activation was observed at three orders of magnitude higher concentrations. We observed a K_i of 30nM for cAMP in the PDE10A GAF domain binding assay compared to a reported EC_{50} of 19.8 μ M in the adenylyl cyclase activation assay and a K_i of 35nM for cGMP in the PDE11A GAF domain binding assay compared to an EC_{50} of 72.5 μ M in the adenylyl cyclase activation assay [27,28]. The nanomolar affinities that we report here are comparable to those reported in binding assays for other cyclic nucleotide binding GAF domains [24,30-32], while the EC_{50} s in the chimeric assays are very high and possibly too high to be physiologically relevant [1]. Furthermore, our data are compatible with the observation that cAMP co-purifies with PDE10A GAFb during purification suggesting tight binding [33]. The mechanism underlying the activation of the chimeras is unknown, but assuming that the adenylyl cyclase activation in the chimeras is mediated by a conformational change, an entropy or enthalpy cost due to the cyclic nucleotide induced conformational change specific to the chimeras could explain the high EC_{50} s observed in that assay.

We identified GAFb of PDE10A and GAFa of PDE11A as the domains containing the non-catalytic binding sites. The PDE10A data are in agreement with the recently published crystal structure of PDE10A GAFb in complex with cAMP [33]. The localization of the binding sites seem to follow PDE phylogeny. Thus, the phylogenetically clustered PDEs 5, 6 and 11 all have cyclic nucleotide binding sites in GAFa (Figure 4) [25,29,34], while the phylogenetically more distant PDE2A and PDE10A have cyclic nucleotide binding sites in

GAFb (Figure 4) [24]. PDE2A GAFb and PDE10A GAFb preferentially bind cGMP and cAMP, respectively, but interestingly they exhibit less selectivity between cAMP and cGMP than PDEs 5, 6 and 11 (Figure 3) [30,31].

The crystal structures of the tandem GAF domains of PDE2A in complex with cGMP and PDE10A GAFb in complex with cAMP reveal that the cyclic nucleotides are almost completely buried in the protein and only the C2 groups of the ligands have access to the solvent [24,33]. As would be predicted from this, cyclic nucleotide analogues with substitutions that increase the size relative to the natural substrates at any position except the C2 position generally have dramatically reduced affinity for the GAF domains. Generally, the more groups substituted compared to cAMP or cGMP, the larger the decrease in affinity. A clear exception is 5,6-DM-cBIMP, which despite its large deviance from cGMP exhibits remarkable high binding affinity for PDE2A GAF domains ($K_i = 0.040\mu\text{M}$), but not for PDE10A ($K_i = 51\mu\text{M}$) or PDE11A ($K_i = 218\mu\text{M}$) GAF domains. The crystal structures show that PDE2A, but not PDE10A, has a serine (424) that forms a hydrogen bond to the N7 position of cAMP. A possible explanation for the unexpected high affinity of 5,6-DM-cBIMP for PDE2A is that the nitrogen at position 7 is a better hydrogen bond acceptor in 5,6-DM-cBIMP, which would contribute to affinity for PDE2A but not PDE10A GAF domains. This result is in accordance with a previous result found for another benzimidazole based cyclic nucleotide [23]. PDE11A is even less tolerant of modification of the ligand than PDE2A and PDE10A, suggesting that it has an even more narrow or more rigid binding pocket.

In PDE2A and PDE5A, enzymatic activity is increased by the binding of ligands to the GAF domains [19,23,25]. For PDE10A and -11A, published data are conflicting. Gross-Langenhoff *et al.* [27,28] found that high concentrations of cyclic nucleotides could activate recombinant chimeras of bacterial adenylyl cyclase and the GAF domains from PDE10A and PDE11A. Based on that observation, they propose that PDE10A and PDE11A enzymatic activity may also be regulated by cyclic nucleotide binding to the GAF domains. However, Soderling *et al.* [10] found no increase in PDE10A enzyme activity upon addition of cAMP and Yuasa *et al.* [35] found that cGMP did not stimulate hydrolytic activity of PDE11A, though an effect might be hidden by inhibition of enzyme activity due to the high affinity of the cyclic nucleotides for the catalytic domain. To refine the analysis, we tested GAF domain binding cyclic nucleotide analogues for their interaction with the catalytic domains of the PDEs and found compounds with higher selectivity for the GAF domains than the natural ligands (Table 3). In accordance with earlier findings, we observed a robust activation of PDE2A in response to PDE2A GAF domain ligands, and as expected, cyclic nucleotide analogues with higher selectivity for the PDE2A non-catalytic site exhibited enzyme activation over a larger concentration range than less selective ligands. However, we observed no regulation of the enzymatic activity of PDE10A and PDE11A in response to addition of ligands with similar selectivity for their non-catalytic sites. Thus, our data do not support that PDE10A and PDE11A GAF domains control enzymatic activity of the enzymes.

Unlike all other GAF domains from human phosphodiesterase genes, PDE10A GAFa and PDE11A GAFb do not contain the consensus sequence NK/RX_nFX₃DE (the so called NKFDE motif) [36]. Furthermore, the PDE10A GAFb crystal structure suggests that dimerization of PDE10A is asymmetric rather than symmetric as for PDE2A [24,33]. These differences of the enzymes' tertiary and quaternary structure may explain why PDE10A and PDE11A differ in their regulation from other PDEs.

From an evolutionary perspective, it seems unlikely that the conserved cyclic nucleotide binding sites in PDE10A and PDE11A GAF domains have no function. Our *in vitro* experiments cannot rule out that PDE10A and PDE11A enzymatic activity is regulated through the GAF domains *in vivo*; the regulation might - unlike that of PDE2A - be dependent on phosphorylation, membrane attachment or interaction with other proteins that are absent in our system. The enzymatic activation of the chimeras observed by Gross-

Langenhoff *et al.* [27,28] might suggest that PDE10A and -11A GAF domains are able to alter conformation in response to cyclic nucleotide binding also *in vitro*, although our data indicate that it does not affect PDE activity of the phosphodiesterases. Changes in conformation might affect PDE activity indirectly for example by altering protein stability or the subcellular localization through altered protein-protein interactions. However, we show here that in contrast to other mammalian GAF domain-containing PDEs, cyclic nucleotide binding does not directly regulate the enzymatic activity of PDE10A and PDE11A.

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FIGURE LEGENDS

Figure 1 PDE full-length, GAF and catalytic domain constructs

(A) Schematic diagram showing the various PDE constructs generated in this study. Residue boundaries of the individual constructs are indicated for PDE2A3, PDE10A2 and PDE11A4, respectively. Boundaries of the individual GAF domains were chosen to include the entire globular domains based on alignments with PDE2A, where a crystal structure have been determined for both GAF domains [17,24]. (B) Western blot of the expressed PDE GAF constructs generated in this study. The molecular masses in kilo Daltons of protein standards are indicated on the left. *Lane 1*, Biotinylated Protein Ladder (Cell Signaling Technology); *lane 2*, PDE2A GAFab; *lane 3*, PDE10A GAFab; *lane 4*, PDE10A GAFa; *lane 5*, PDE10A GAFb; *lane 6*, PDE11A GAFab; *lane 7*, PDE11A GAFa; *lane 8*, PDE11A GAFb.

Figure 2 Binding of cAMP and cGMP

Binding curves for binding of [5', 8-³H]-cAMP (A) and [8-³H]-cGMP (B) to the tandem GAF domains of PDE2A (■), PDE10A (▲), and PDE11A (▼). The figure illustrates the window of the GAF domain binding assay. The same amount of the respective proteins was added for the different ligands. For Control (●), *E. coli* lysate was added to the assay. Dissociation constants (K_D) found was 66 ± 18 nM for binding of cGMP to PDE2A GAFab, 48 ± 15 nM for binding of cAMP to PDE10A GAFab, and 110 ± 27 nM for binding of cGMP to PDE11A GAFab. The curves shown are

representative whereas K_D values represent the mean \pm SEM for $n = 2$ or 3 for fitted curves with the control subtracted.

Figure 3 Displacement of radio ligands by cAMP or cGMP

Competition binding assays for cAMP and cGMP with the tandem GAF domains of PDE2A3, PDE10A2 and PDE11A4, respectively. Various concentrations of either unlabeled cAMP (■) or cGMP (▲) were used as competitive ligands to inhibit the binding of 14nM [3 H]-cAMP (PDE10A GAFab) or 60nM [3 H]-cGMP (PDE2A GAFab and PDE11A GAFab) to the non-catalytic binding site of the tandem GAF domains. Inhibition constants (K_i) are stated in Table 2. Hill coefficients were for all curves between -0.9 and -1.1. Each data point represent the mean \pm SEM for $n = 3$.

Figure 4 Localization of non-catalytic binding site

Binding curves for the individually expressed GAFa (■) and GAFb (▲) domains of PDE10A2 and PDE11A4. Binding of [3 H]-cAMP or [3 H]-cGMP was measured to determine the localization of the non-catalytic binding site to either of the GAF domains. The figure illustrates that the non-catalytic binding site is located in PDE10A GAFb and PDE11A GAFa, respectively. The results are subtracted a baseline control with *E. coli* lysate. Dissociation constants (K_D) found were 61 ± 11 nM (PDE10 GAFb) and 77 ± 18 nM (PDE11 GAFa). The curves shown are representative whereas K_D values represent the mean \pm SEM for $n = 3$.

Figure 5 Structures of cAMP and cGMP and their derivatives used in this study

The names corresponding to each numbered compound are listed in Table 2. Arrows indicate where the substitution has been made for that particular analogue. *Group I*, analogues substituted in the pyrimidine region; *Group II*, analogues substituted in the imidazole moiety; *Group III*, cAMP and cGMP derivatives substituted at the ribose 2' position; *Group IV*, cAMP and cGMP derivatives with substitutions in the cyclic phosphate moiety; *Group V*, non-cyclic AMP and GMP. *RcP*, ribose cyclic phosphate moiety; *Pyr*, pyrimidine base.

Figure 6 Stimulation of PDE catalytic activity by GAF domains

Effect of binding of cyclic nucleotide analogues to GAF domains on the phosphodiesterase activity of recombinant full-length PDEs. Various concentrations of unlabeled ligands, which were selected based on high affinity for the non-catalytic binding site but low for the catalytic site, were added and catalytic activity measured in PDE activity assays as described in detail under "Experimental procedures". Percent of maximal activity is defined as the enzyme activity at a specific concentration of unlabeled ligand relative to the activity when only the [3 H]-labelled substrate is present. 14nM [3 H]-cAMP was used as substrate for PDE2A3 and PDE11A4 and 60nM [3 H]-cGMP was used as substrate for PDE10A2. Selected ligands used were (numbers refer to Table 2): cGMP (4, ▲), 5,6-DM-cBIMP (10, ▼), 2'-dcGMP (16, ●), Rp-cGMPS (20, ◇), cAMP (1, ■), 1-NO-cAMP (3, ○), cPuMP (8, ◆), 7-CH-cAMP (12, △), cIMP (5, □). Each data point represents the mean \pm SEM for $n = 2$ or 3 .

Table 1 cAMP and cGMP inhibition constants for various GAF domain constructs

Unlabeled cAMP or cGMP was used as competitive ligand to inhibit the binding of either [³H]-cAMP (PDE10A) or [³H]-cGMP (PDE11A). The inhibition constant (K_i) was determined from fitted curves similar to those shown in Figure 3, using the equation $K_i = IC_{50}/(1+([^3H]\text{-cNMP}/K_D)$. [³H]-cNMP] was 14nM for assays with [³H]-cAMP and 60nM for assays with [³H]-cGMP. K_D was as indicated in Figure 2 and Figure 4. Values represent the mean \pm SEM for n = 3 or 4.

Construct ^a	K_i	
	cAMP	cGMP
	μM	μM
PDE10 GAFab	0.030 \pm 0.004	1.6 \pm 0.12
PDE10 GAFa	ND ^b	ND
PDE10 GAFb	0.039 \pm 0.004	4.2 \pm 0.34
PDE11 GAFab	41 \pm 15.8	0.035 \pm 0.006
PDE11 GAFa	148 \pm 28	0.21 \pm 0.039
PDE11 GAFb	ND	ND

^a Boundaries of constructs used in the competition binding assay are as shown in Figure 1A.

^b ND, not detectable (indicates the construct did not bind detectable levels of cyclic nucleotide).

Table 2 Summary of inhibition constants for various cAMP- and cGMP-analogues

Various concentrations of unlabeled ligand were used to inhibit the binding of [³H]-cAMP (PDE10A2 GAFab) or [³H]-cGMP (PDE2A3 GAFab and PDE11A4 GAFab) to the tandem GAF domains in competition binding assays. The inhibition constant (K_i) was determined as described for Table 1. The values represent the mean \pm SEM for $n = 2$ to 4.

Group ^a	No.	Nucleotide	K_i		
			PDE2 GAFab ^b	PDE10 GAFab	PDE11 GAFab
			μM	μM	μM
I	1.	cAMP	4.3 \pm 0.55	0.030 \pm 0.004	41 \pm 5.8
	2.	2-Cl-cAMP	2.5 \pm 0.11	0.047 \pm 0.004	27 \pm 2.0
	3.	1-NO-cAMP	64 \pm 13	0.36 \pm 0.065	>300
	4.	cGMP	0.031 \pm 0.004	1.6 \pm 0.12	0.035 \pm 0.006
	5.	cIMP	0.53 \pm 0.11	4.6 \pm 0.85	0.43 \pm 0.050
	6.	1-NH ₂ -cGMP	3.3 \pm 0.65	176 \pm 1.9	52 \pm 8.8
	7.	cXMP	51 \pm 11	140 \pm 15	28 \pm 5.2
	8.	cPuMP	3.1 \pm 0.026	0.56 \pm 0.11	66 \pm 7.8
	9.	2-NH ₂ -cPuMP	0.52 \pm 0.16	1.1 \pm 0.023	8.2 \pm 1.5
	10.	5,6-DM-cBIMP	0.040 \pm 0.002	51 \pm 3.6	218 \pm 34
	11.	6-Cl-cPuMP	5.2 \pm 0.18	0.69 \pm 0.15	17 \pm 2.8
II	12.	7-CH-cAMP	5.6 \pm 0.49	0.009 \pm 0.003	>300
	13.	8-Br-cAMP	130 \pm 7.1	4.2 \pm 0.035	>300
	14.	8-Br-cGMP	192 \pm 13	>300	2.9 \pm 0.75
III	15.	2'-dcAMP	48 \pm 3.7	0.083 \pm 0.017	>300
	16.	2'-dcGMP	0.46 \pm 0.20	20 \pm 1.4	1.7 \pm 0.25
	17.	2'-O-Me-cAMP	>300	>300	>300
	18.	2'-O-Me-cGMP	8.5 \pm 1.0	>300	16 \pm 2.8
IV	19.	Rp-cAMPS	129 \pm 8.1	2.4 \pm 0.054	>300
	20.	Rp-cGMPS	1.1 \pm 0.12	292 \pm 37	1.5 \pm 0.10
	21.	Sp-cAMPS	>300	9.5 \pm 2.7	>300
	22.	Sp-cGMPS	20 \pm 4.8	>300 \pm 107	36 \pm 7.3
	23.	Sp-8-OH-cAMPS	>300	234 \pm 23	>300
V	24.	5'-AMP	>300	>300	>300
	25.	5'-GMP	>300	>300	>300

^a Group and number refer to the structures in Figure 5.

^b Boundaries of constructs used in the competition binding assay are as shown in Figure 1A.

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Table 3 Inhibition constants for catalytic domain vs. GAF domains

Comparison of the inhibition constant for catalytic site vs. non-catalytic site for selected ligands. The catalytic domain was expressed separately and dose/response curves obtained for selected ligands in PDE activity assays. The inhibition constant (K_i) was determined using the equation $K_i = IC_{50}/(1+[(^3H)\text{-cNMP}]/K_M)$. $[(^3H)\text{-cNMP}]$ was 14nM for assays with $[(^3H)\text{-cAMP}]$ (PDE2A and PDE11A) and 60nM for assays with $[(^3H)\text{-cGMP}]$ (PDE10A). K_M values used were 30 μ M (PDE2A) [19], 7.2 μ M (PDE10A) [11] or 0.45 μ M (PDE11A) [37]. Values represent the mean \pm SEM for $n = 2$.

The inhibition constants for the tandem GAF domains are obtained from Table 2. The ratio of the inhibition constants for the separate parts of the enzyme indicates the size of the window where the ligand will bind to the non-catalytic binding site of the GAF domains and potentially stimulate the catalytic activity without inhibiting by occupying the catalytic site.

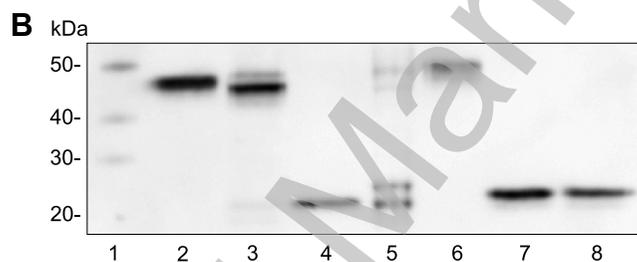
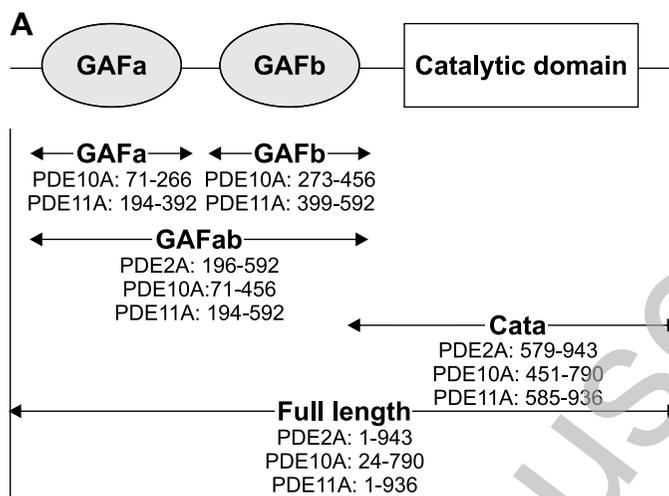
	No. ^a	Nucleotide	K_i		$K_i(\text{Cata})/K_i(\text{GAFab})$
			Cata ^b μ M	GAFab μ M	
PDE2	4.	cGMP	63 \pm 0.45	0.031 \pm 0.004	2032
	10.	5,6-DM-cBIMP	314 \pm 10	0.040 \pm 0.002	7850
	16.	2'-dcGMP	112 \pm 1.5	0.46 \pm 0.20	243
	20.	Rp-cGMPS	>300	1.1 \pm 0.12	>273
PDE10	1.	cAMP	0.26 \pm 0.043	0.030 \pm 0.004	8.7
	3.	1-NO-cAMP	89 \pm 37	0.36 \pm 0.065	247
	8.	cPuMP	5.4 \pm 1.2	0.56 \pm 0.16	9.6
	12.	7-CH-cAMP	0.28 \pm 0.026	0.009 \pm 0.003	30
PDE11	4.	cGMP	7.3 \pm 3.3	0.035 \pm 0.006	209
	5.	cIMP	3.1 \pm 1.4	0.43 \pm 0.050	7.2
	16.	2'-dcGMP	11 \pm 0.53	1.7 \pm 0.25	6.5
	20.	Rp-cGMPS	>300	1.5 \pm 0.10	>200

^aNumber refers to the structures in Figure 5.

^bCata, catalytic domain. Boundaries of constructs used are as indicated in Figure 1A.

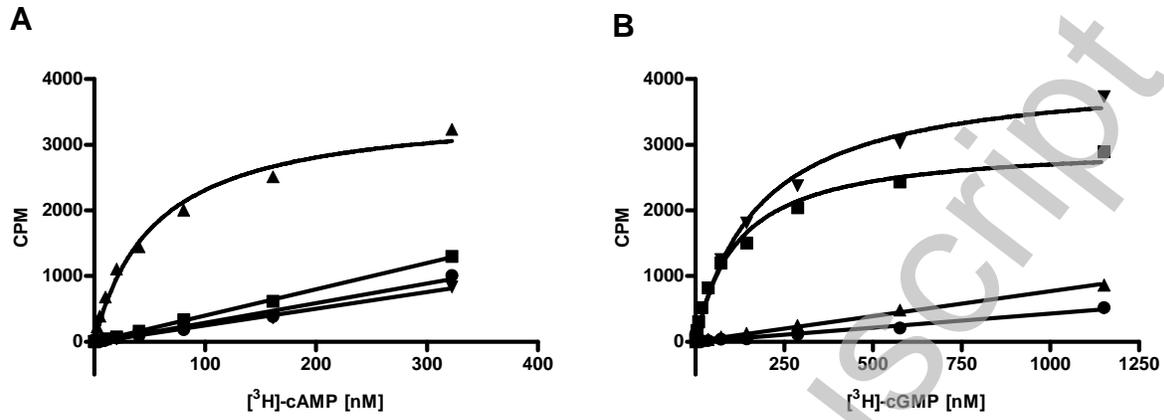
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Figure 1



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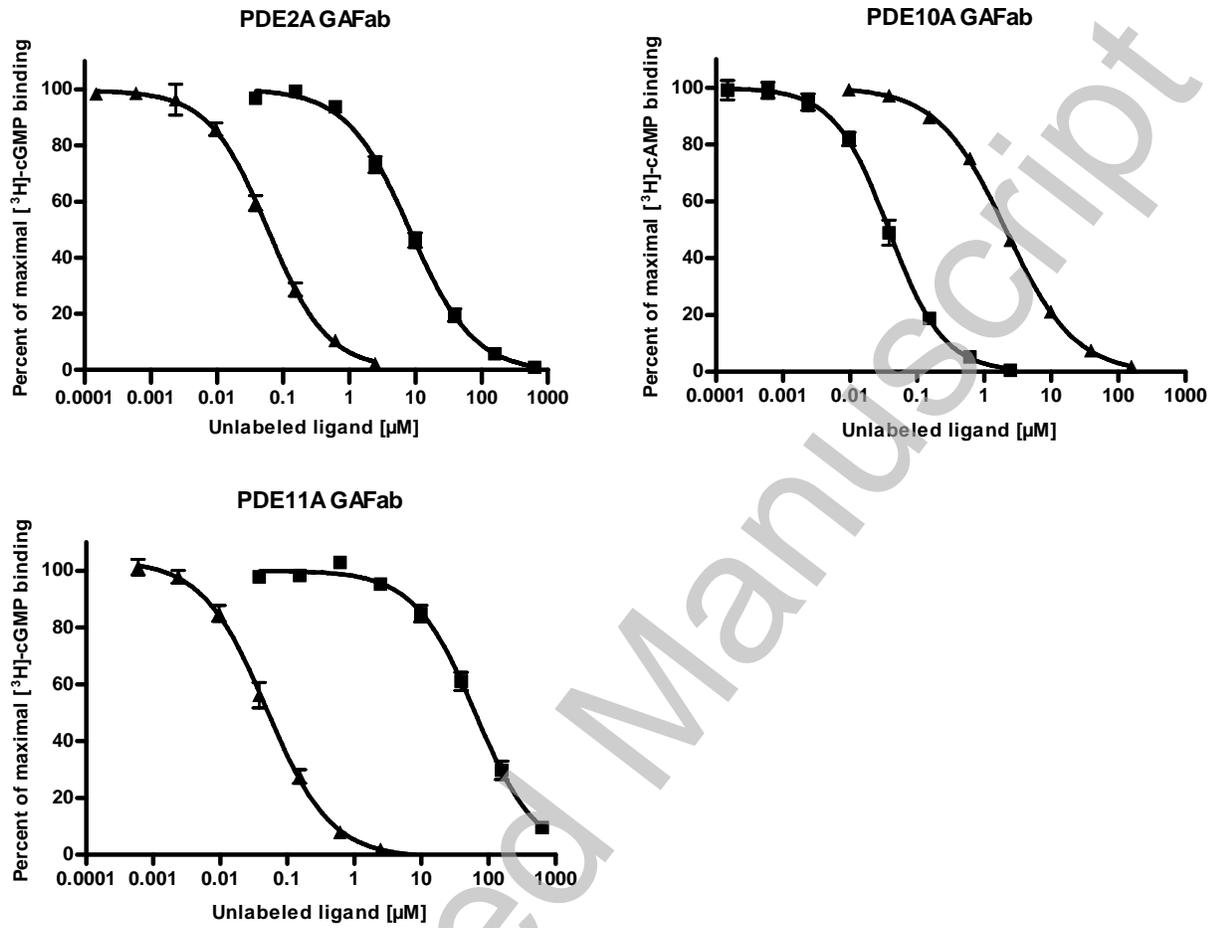
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Figure 2

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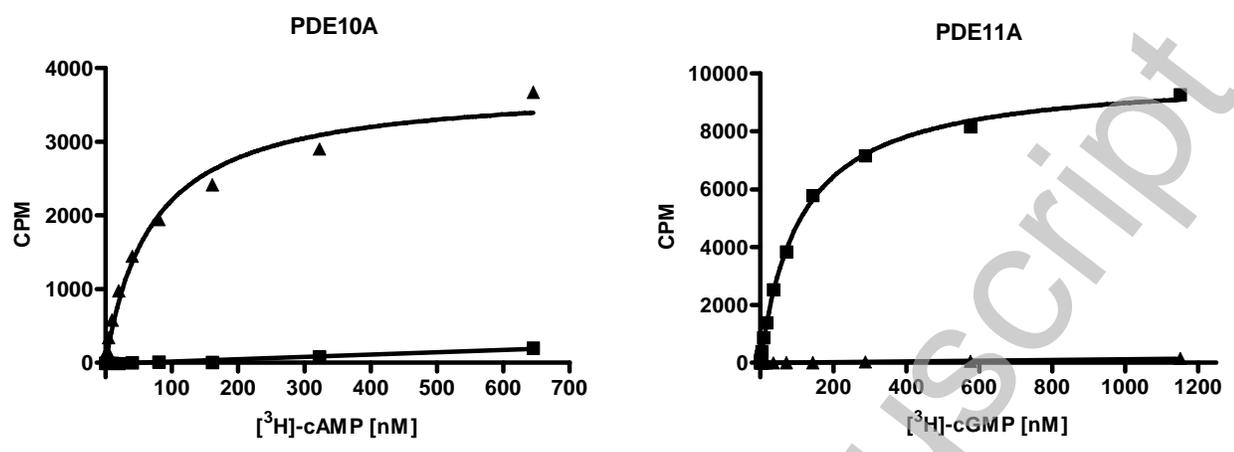
Figure 3



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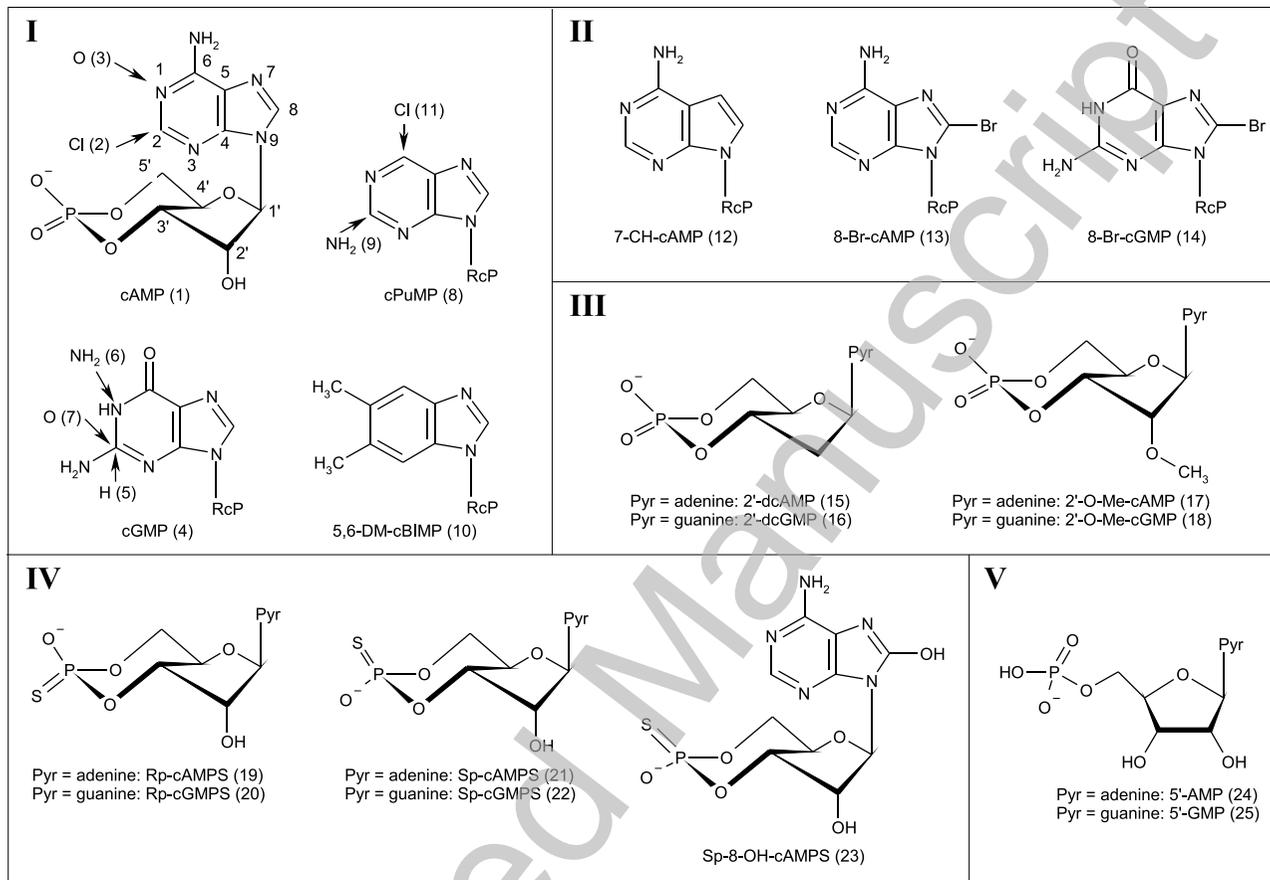
Figure 4



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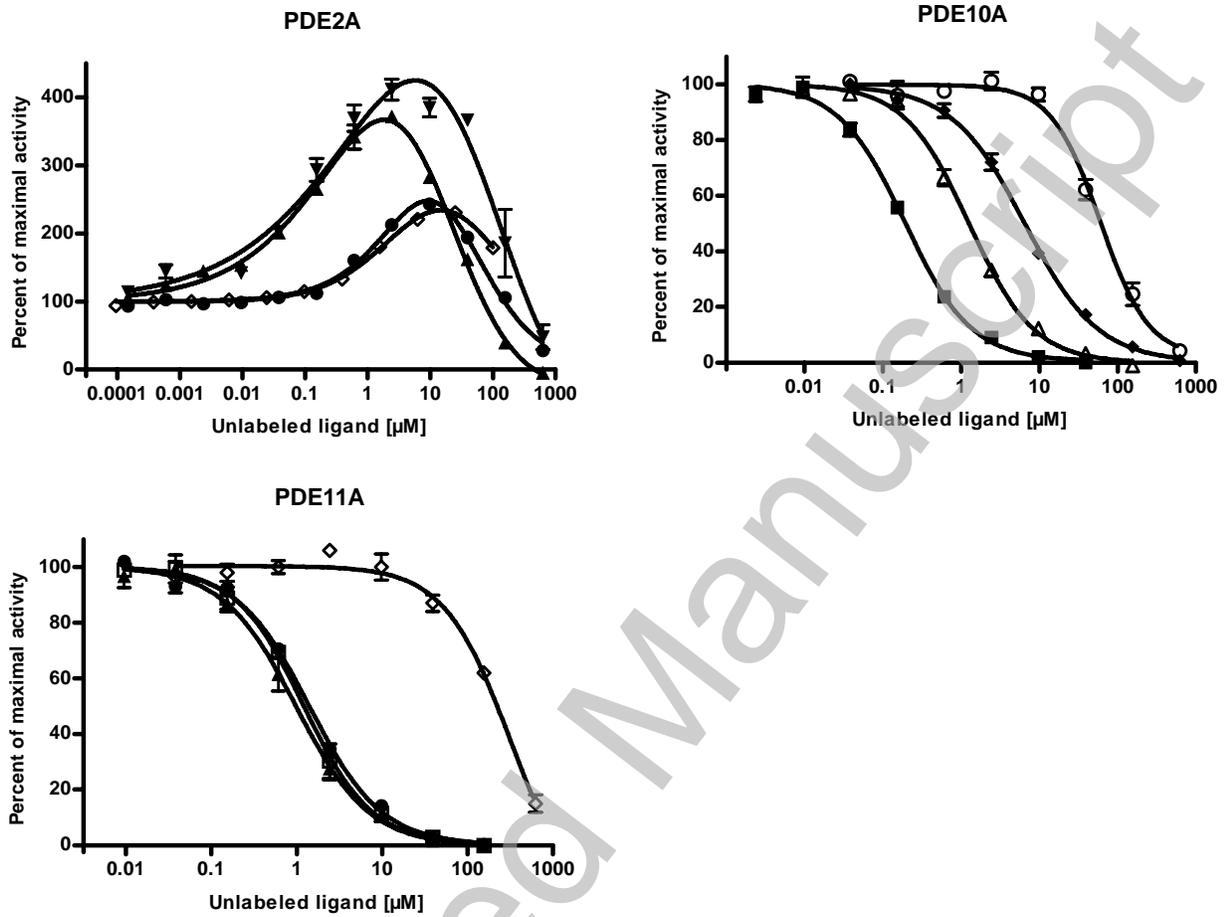
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Figure 5



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