The GAF Domain of the cGMP-Binding, cGMP-Specific Phosphodiesterase (PDE5) Is a Sensor and a Sink for cGMP†

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ABSTRACT: We describe here a novel sensor for cGMP based on the GAF domain of the cGMP-binding, cGMP-specific phosphodiesterase 5 (PDE5) using bioluminescence resonance energy transfer (BRET). The wild type GAFa domain, capable of binding cGMP with high affinity, and a mutant ($GAFa_{F163A}$) unable to bind cGMP were cloned as fusions between GFP and Rluc for $BRET^2$ assays. $BRET^2$ ratios of the wild type GAFa fusion protein, but not $GAFa_{F163A}$, increased in the presence of cGMP but not cAMP. Higher basal $BRET^2$ ratios were observed in cells expressing the wild type GAFa domain than in cells expressing $GAFa_{F163A}$. This was correlated with elevated basal intracellular levels of cGMP, indicating that the GAF domain could act as a sink for cGMP. The tandem GAF domains in full length PDE5 could also sequester cGMP when the catalytic activity of PDE5 was inhibited. Therefore, these results describe a cGMP sensor utilizing $BRET^2$ technology and experimentally demonstrate the reservoir of cGMP that can be present in cells that express cGMP-binding GAF domain-containing proteins. PDE5 is the target for the anti-impotence drug sildenafil citrate; therefore, this GAF-$BRET^2$ sensor could be used for the identification of novel compounds that inhibit cGMP binding to the GAF domain, thereby regulating PDE5 catalytic activity.

There has been great interest in the development of sensitive reporters to monitor changes in intracellular cyclic nucleotide levels since antibody based assays are time-consuming and cannot readily be extended to high throughput assays (*1, 2*). Fluorescence-based sensors for cAMP and cGMP have been developed, based on cyclic nucleotide binding domains that are found in a number of proteins that include cyclic nucleotide gated channels (*3*), EPAC (*4*), and cGMP- and cAMP-dependent protein kinases (*2, 3, 5*). Recently, a sensor based on bioluminescence energy transfer ($BRET^1$) has been described for cAMP (*6*). In all these proteins, binding of the cyclic nucleotide induces a major conformational change such that the donor and acceptor proteins fused either to the N or the C termini of the cNMP-binding domain, come closer together, or move further apart, thereby altering the resonance energy transfer (RET) that is observed.

GAF domains, so named because of their presence in cGMP-specific PDEs, bacterial adenylyl cyclase, and the bacterial FhLA transcriptional regulator (*7*), are found either singly or in tandem in a number of proteins that are involved in signal transduction (*8, 9*). In mammals, the GAF domains are found almost exclusively upstream to a cyclic nucleotide phosphodiesterase domain (*7*). A number of GAF domains in phosphodiesterases have been shown to bind cyclic nucleotides (*10–12*). In some PDEs, cyclic nucleotide binding to the GAF domains induces a conformational change that allosterically regulates the activity of the C-terminally located phosphodiesterase domain (*13, 14*). It was this observation that prompted the development and characterization of sensors based on the GAF domains, and recently, FRET based assays were described that utilized the GAF domains of PDE5 and PDE2 to detect alterations in the levels of cGMP in the cell (*15–17*).

Bioluminescence energy transfer is an advanced, nondestructive, cell based approach that has been used extensively for characterization of signal transduction pathways (*16, 18, 19*). BRET is based on energy transfer between luminescence donor and fluorescent acceptor proteins, for example, *Renilla* luciferase (Rluc) and green fluorescence protein (GFP). BRET is induced by a little understood step of oxidation of the substrate by luciferase (*20*), followed by resonance energy transfer to the acceptor GFP protein. Excitation of GFP by resonance energy transfer results in emission of green light at 510 nm, and the ratio of light emitted by GFP to that of luciferase (energy transfer efficiency) is called the BRET signal (*19*). Resonance energy transfer (RET) is dependent on the distance between the donor and the acceptor, their spectral overlap, and their orientation with respect to each other, with an optimum distance of 10 to 100 Å. Since the efficiency of RET depends on the sixth power of the distance between donor and acceptor, even a minimal movement of the two moieties toward each other, or away from each other,

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[1] Abbreviations: BRET, bioluminescence resonance energy transfer; GAF, c**G**MP-specific phosphodiesterases, bacterial **a**denylyl cyclase, **F**hLA transcriptional regulator; PDE, phosphodiesterase; RET, resonance energy transfer; Rluc, *Renilla* luciferase; GC-C, receptor guanylyl cyclase C; ST, heat-stable enterotoxin peptide.

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can result in a great change in the RET. BRET assays can be readily carried out in intact cells, and a number of modifications have been developed to optimize energy transfer, by utilizing modified variants of GFP and the luciferase substrate (*16, 21*).

The cyclic GMP-binding, cGMP-specific phosphodiesterase (PDE5) is the target of the drug sildenafil citrate (*13*). A wealth of information is available on the expression and regulation of this enzyme (*22, 23*). Briefly, PDE5 contains a C-terminal catalytic domain which is the site of binding of sildenafil citrate and its derivatives (*24–26*). N-terminal to the catalytic domain are two tandem GAF domains, and cGMP binds only to the GAFa domain (*12, 27*), where the critical F163 residue (numbering based on human PDE5A2 isoform) is required for high affinity interaction with cGMP (*27*). Binding of cGMP to the GAF domain of PDE5 activates the enzyme, and generates a form that can be phosphorylated by cGMP-dependent protein kinase on S60 (numbered according to the human PDE5A2 isoform) N-terminal to the GAF domains (*14, 28, 29*). This stabilizes the enzyme, leading to sustained activation of PDE5 and lowering of intracellular cGMP levels (*30*). The importance of inhibiting PDE5 in the treatment of male impotence is exemplified by the wide use of sildenafil citrate, but side effects such as disturbances in vision are also observed, because of the inhibition of a similar phosphodiesterase, PDE6, that is required for visual signal transduction (*31*).

In this study, we describe the development of a BRET-based sensor for cGMP based on the GAFa domain of PDE5. We utilize a mutant GAFa domain that cannot bind cGMP as a control, in order to validate the specificity of the BRET signals that are observed. Most importantly, this has allowed us to show that GAF domains can act as intracellular sinks for cGMP, an idea that has been suggested earlier, but never directly experimentally demonstrated (*7, 29, 32*). This new cGMP sensor should prove useful in identifying novel molecules that bind to the GAF domain of PDE5 as an alternative strategy for regulating the activity of this enzyme. In addition, this BRET-based approach can be used to identify the ligands for uncharacterized GAF domains from other proteins, since these domains could also show a conformational change in their structure on ligand binding.

MATERIALS AND METHODS

Generation of PDE5A Constructs. Full length human PDE5A2 cDNA (accession AY264918) cloned in the pCR-II vector (*22, 27*) was used as a template for PCR to obtain the tandem GAF domains with forward primer hPDE5A2:731 (5′ AGA AGG AAT TCA TGC CTC TAA C 3′) and reverse primer hPDE5A2:2106 (5′ GAA GTT CTG CTC GAG GTT GAG GTC 3′). This would amplify a region encompassing residues M92 to N541, numbered according to the PDE5A2 isoform. The amplified fragment was digested with *Eco*RI and *Xho*I and cloned into pBlueScript II KS(+) vector (Stratagene), sequenced and then subcloned into the pEGFP-N3 mammalian expression vector (Invitrogen) using *Eco*RI and *Kpn*I. This generated a plasmid (pEGFP-GAFab) which would express the tandem GAF domains fused to the N terminus of EGFP (GAFab-EGFP). A mutation of F163 to an alanine residue was introduced in the above plasmid construct as described earlier (*27*).

To clone the GAFa and mutant $GAFa_{F163A}$ domains encompassing residues S_{118} to H_{273} of PDE5A2, PCR was performed with forward primer GAFa_f339_2 (5′ AGT GAA TTC TAT GTC TAG TCA TTT GG 3′) and reverse primer GAFa_r831_2 (5′ GAG CTG AAG CTT ATG AAG AAC AAT AC 3′) using pCR-II-PDE5A2 and pEGFP-N3-GAF_{F163A}, respectively, as templates. Amplified fragments were then cloned into pBlue-Script II KS(+) following digestion with *Eco*RI and *Hind*III, sequenced, and then subcloned into pGEX-6p-3 vector (GE Healthcare) following digestion with the same enzymes to generate plasmids pGEX-GAFa and pGEX-$GAFa_{F163A}$. These plasmids were used for bacterial expression of the GAFa domains fused to the C-terminus of glutathione S-transferase.

To generate the constructs to be used for $BRET^2$ analysis, a region encompassing residues I_{117} to N_{274} was amplified with the forward primer GAFa_PDE5f339 (5′ AGT GAA GCT TAA TTT CTA GTC ATT TGG 3′) and reverse primer GAFa_PDE5r806 (5′ GAG AGA TCT ATT ATG AAG AAC AAT AC 3′) using pCR-II-PDE5A2 as template. The fragment was cloned into pBlue-Script II KS(+) following digestion with *Hind*III and *Bgl*II, sequenced and subsequently cloned using the same restriction enzymes into $pGFP^2$-MCS-Rluc vector (Perkin-Elmer Life Sciences) to generate $pGFP^2$-GAFa-Rluc plasmid. A BRET construct expressing the mutant $GAFa_{F163A}$ domain of PDE5A2 was obtained by using the same primers and the pEGFP-$GAFab_{F163A}$ plasmid as template. The PCR product obtained was digested with *Hind*III and *Bgl*II and directly cloned into similarly digested $pGFP^2$-MCS-Rluc ($BRET^2$-MCS) vector. The cloned fragment was sequenced using the forward primer GAFa_PDE5f339. The amino acid sequence of the linker in between GFP^2 and the GAF domain is SGSSL, and that between GAF and Rluc is RSDIGPSRAT.

To generate a clone that would express the full length PDE5A2-EGFP protein, pCRII-PDE5A2 was digested with *Sac*I and *Pst*I and the fragment of ~ 2.4 kb was cloned into similarly digested pEGFP-N2 vector, generating the plasmid pEGFP-$PDE5A2_{M1\text{-}C804}$. PCR was carried out using pCRII-PDE5A2 as template and hPDE5A2:2231 forward (5′ AAG CAG GCA AAA TTC AGA AC 3′) and hPDE5A2:2976 reverse (5′ GGC CAC TCG GTA CCG CTT GGC 3′) primers, and the product obtained was digested with *Pst*I and *Kpn*I to release an 80 bp fragment containing the mutated stop codon in PDE5A2. This 80 bp fragment was then cloned into pEGFP-$PDE5A2_{M1\text{-}C804}$ plasmid that was digested with *Pst*I and *Kpn*I, to generate the plasmid pEGFP-PDE5A2, which would express the full length PDE5A2 fused to GFP at its N-terminus. Mutagenesis was performed using the *Dpn1* digestion method (*33*), using two mutagenic primers described earlier (*27*) and the pEGFP-PDE5A2 plasmid as the template. The mutant insert was sequenced to confirm the presence of the mutation and absence of additional mutations. All proteins that were expressed in this study are diagrammatically represented in Figure 1.

Cell Culture and Transfection. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's media (DMEM) with 10% fetal calf serum, 120 mg/L penicillin and 270 mg/L streptomycin at 37 °C in a 5% CO_2 humidified incubator. Transfection was performed with polyethyleneimine lipid according to manufacturers' protocols. Expression of proteins was monitored by Western Blot

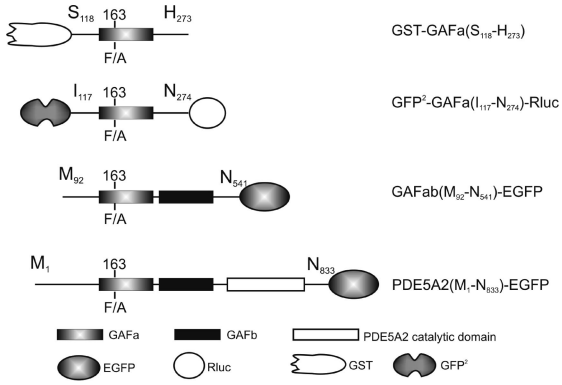

FIGURE 1: Diagrammatic representation of the various proteins utilized in this study. The proteins used in this study are shown with residues numbered for demarcation of the GAF domain in the PDE5A2 sequence that was used for generation of the sensor. GST, GFP^2, Rluc, and other domains in PDE5 are represented as cartoons.

analysis using either an antibody raised in rabbits to GFP, generated in the laboratory, or a previously described antibody to PDE5 (*22*).

Cyclic Nucleotide Binding Assays. Cyclic nucleotide binding assays were carried out essentially as described earlier (*27*) using 1 μg of purified GST fusion proteins bound to glutathione beads in a 50 μL reaction volume in buffer containing 25 mM HEPES, 100 mM NaCl, 10% glycerol and 200 μM PMSF in the presence of varying concentration of [^{3}H]-cGMP along with unlabeled cGMP. Reactions were incubated at 37 °C for 1 h and then filtered through GF/C filters (Whatman) which were then washed with 6 mL of ice-cold washing buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 10% glycerol). The filters were then dried, and radioactivity was measured by scintillation counting in scintillation cocktail (1:1, 2-methoxy ethanol: toluene, 5 g/l PPO). The binding data were analyzed using GraphPad Prism (San Diego, CA, USA), and the data shown represent the mean ± SEM.

In Vitro BRET Assays. HEK 293T cells transfected with pGFP^2-GAFa-Rluc or pGFP^2-GAFa$_{F163A}$-Rluc plasmids were lysed in a buffer of 50 mM HEPES (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10 mM sodium pyrophosphate, 80 μM β-glycerophosphate, 1 mM benzamidine, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL soyabean trypsin inhibitor, 100 μM sodium orthovanadate and 10% glycerol. Following brief sonication, the lysates was centrifuged at 13,000*g* and the cytosol removed. Aliquots of the cytosol (with luciferase activity of ~10, 000 cps) were incubated with the indicated amounts of cNMP in buffer of 50 mM HEPES, pH 7.5, containing 100 mM NaCl in a total volume of 60 μL, at 37 °C for 10 min. DeepBlueC (coelenterazine 4a; Molecular Imaging Products) was added to a final concentration of 5 μM and readings for 0.5 s were collected in a Victor^3 microplate reader (Perkin-Elmer). Emission filters used for Rluc and GFP^2 emission were 410 nm (bandpass 80 nm) and 515 nm (bandpass 30 nm), respectively. BRET^2 measurements were averaged from 3 readings, and BRET^2 ratios, calculated as GFP^2 emission divided by Rluc emission, was recorded.

Cellular BRET^2 Assays. HEK 293T cells were transfected with pcDNA3-hGCC plasmid (*34*) along with either pGFP^2-GAFa-Rluc or pGFP^2-GAFa$_{F163A}$-Rluc plasmid in 10 cm tissue culture dishes. Forty-eight hours post-transfection, medium was removed and monolayers treated with Dulbecco's phosphate buffered saline containing 5 mM EDTA for 5 min at 37 °C in the incubator following which the EDTA solution was removed, and cells resuspended in phenol-red free DMEM, containing 10% fetal calf serum. Cells (~10^5) were then treated with varying concentrations of ST for 5 min following which DeepBlueC was added (5 μM) and BRET measurements made. A parallel set of cells were similarly treated, and then lysed directly in 0.1 N HCl for cGMP/cAMP measurements.

To follow time dependent changes of the BRET ratio upon sodium nitroprusside (SNP) or forskolin treatment, HEK 293T cells were transfected with pGFP^2-GAFa-Rluc or pGFP^2-GAFa$_{F163A}$-Rluc plasmids. Forty-eight hour post-transfection, cells were detached from dishes as described above. Cells (~10^5) were incubated with either SNP or forskolin (100 μM) for various times, and BRET ratio was measured by addition of 5 μM DeepBlueC to the cell suspension. A parallel set of cells were lysed in 0.1 N HCl to monitor total cGMP (SNP-treated cells) or cAMP (forskolin-treated cells) production. Luciferase activities were similar in cells expressing either the wild type or mutant GAF proteins, indicating equivalent expression of the proteins.

In experiments to monitor the sink-like property of the GAF sensor, HEK 293T cells were transfected with either the wild type or mutant GAF domain plasmid, in 24-well white Optiplates (Perkin-Elmer). Post 72 h transfection, media was removed and Hanks' Balanced Salt Solution was added to the confluent monolayer of cells. DeepBlueC at a final concentration of 5 μM was added just prior to BRET measurement. Basal BRET^2 ratios in cell monolayer assays varied from 0.06 to 0.08 for the wild type GAFa domain construct, and from 0.03 to 0.04 for the mutant GAFa domain constructs, which could be correlated with the basal intracellular level of cGMP (see later).

Intracellular cGMP and cAMP Estimation. Intracellular levels of cyclic nucleotide monophosphates were measured in HEK 293T cells transfected with either GAF domain plasmids or PDE5-EGFP plasmids, 72 h post-transfection. Cells were lysed in 0.1 N HCl, and cNMP was measured by radioimmunoassay as described earlier (*34*). In some cases, lysates were acetylated prior to radioimmunoassay in order to increase the sensitivity of the radioimmunoassay.

cGMP Phosphodiesterase Assays. Lysates were prepared from cells transfected with the wild type or mutant PDE5A2-EGFP constructs and cytosol prepared as described previously (*22*). Cyclic GMP phosphodiesterase assays were performed in the presence of varying concentrations of unlabeled cGMP (100 nM to 40 μM) and 100 nM ^{3}H-cGMP (GE Health) essentially as reported previously (*30*). Data was analyzed using GraphPad Prism (San Diego, USA) by nonlinear regression. Sildenafil citrate (Orchid Chemicals, Chennai, India) at concentrations ranging from 10 pM to 1 μM was added to assays performed in the presence of ~30 nM cGMP to determine the IC_{50} of sildenafil citrate for the PDE5-EGFP fusion proteins.

RESULTS

The GAFa Domain Binds cGMP and Not cAMP. We have modeled earlier the GAFa domain of PDE5 and identified

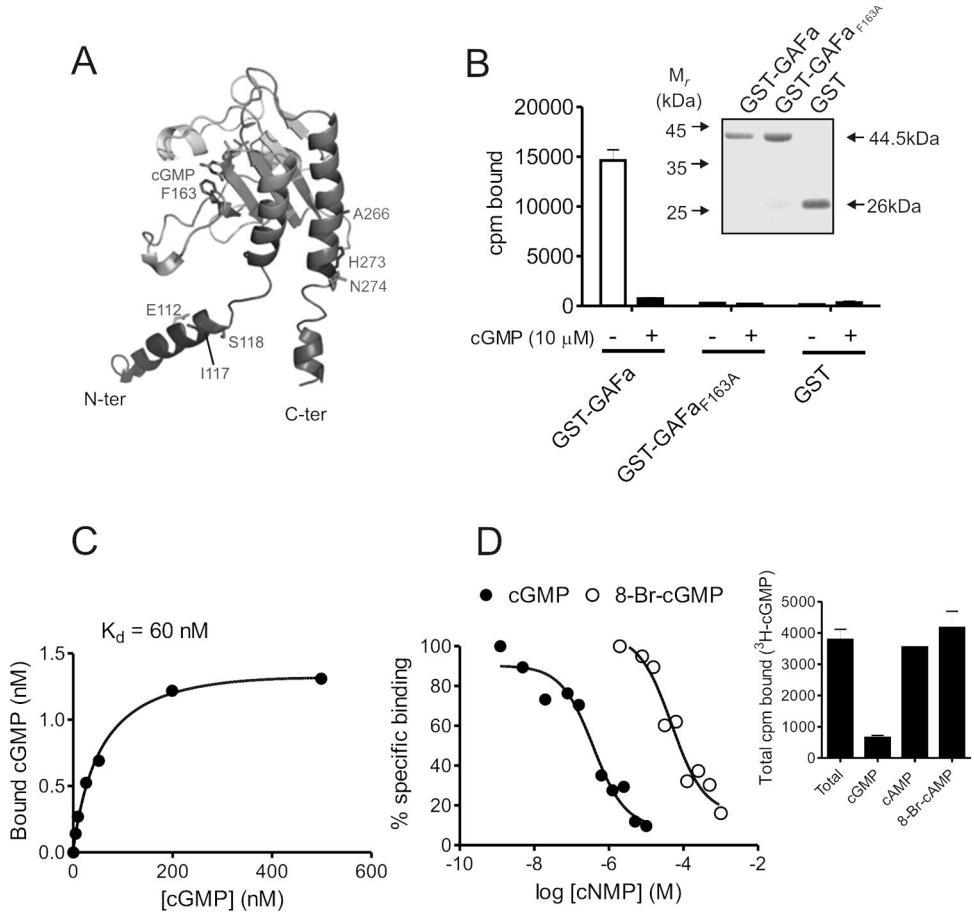

FIGURE 2: GAFa domain of PDE5 binds cGMP specifically. (A) A model of the GAFa domain is shown, and the BRET sensor encompasses residues I117 to N274. An earlier sensor (*17*) starts at residue E112 and ends at A266. (B) Proteins (~1 μg) bound to glutathione beads were incubated with ^{3}H-cGMP (~1 nM) in the absence or presence of 10 μM unlabeled cGMP. Data shown is a representative of assays performed thrice in duplicate, and the values are the mean ± SEM. The inset shows a Coomassie stained gel picture of the purified proteins taken for assay. (C) Purified GST-GAFa protein bound to glutathione beads was incubated with increasing concentrations of ^{3}H-cGMP. Data fitted best to a one-site binding hyperbola, and data shown is the mean of duplicate determinations and is representative of three independent assays. (D) GST-GAFa protein bound to glutathione beads was incubated with ^{3}H-cGMP (~1 nM) either alone or along with the indicated concentrations of unlabeled cGMP or 8-Br-cGMP. Data shown is the mean of duplicate determinations and is representative of three independent assays. The inset shows the binding seen with the GST-GAFa protein in the absence or presence of cAMP (1 mM), 8-Br-cAMP (1 mM), and cGMP (10 μM).

the Phe163 residue in PDE5A2 that was critical for cGMP binding (*27*). The model that we obtained is shown in Figure 2A, and we chose to clone a fragment from residue I117 to N274 to develop the BRET sensor. We believed that this construct would allow the GAFa domain to fold completely and independently, even when GFP and luciferase proteins were fused to its termini. Also shown in Figure 2A are the locations of the amino acids of the GAFa domain construct utilized in earlier FRET studies (*17*), which, as can be seen, do not complete the first N-terminal helix in our model.

We cloned and expressed the wild type GAFa and mutant GAFa$_{F163A}$ domains as GST fusion proteins in bacteria. We monitored cGMP binding to the purified wild type and mutant GAF (GAFa$_{F163A}$) domain proteins. In agreement with earlier reports (*27, 35*), cGMP bound with high affinity to the GAFa domain, and mutation of the F163 residue abolished cGMP binding (Figure 2B and C). GAFa was able to interact with 8-Br-cGMP (*27, 35*), with an IC_{50} of 40 μM (Figure 2D). However, this markedly lower affinity could also be caused by the presence of a low amount of contaminating cGMP in the 8-Br-cGMP as supplied. The GAFa domain showed no detectable binding to cAMP or 8-Br-cAMP, indicating the specificity of the interaction (Figure 2D, inset).

The affinity of the isolated GAFa domain for cGMP was similar to that obtained for the tandem GAFa-GAFb domain protein studied earlier (*12, 27, 35*) and is also in agreement with the affinities seen for the GAF domains in full length PDE5 reported in earlier studies (*36*). However, while the tandem GAF domain used in earlier constructs showed some binding to cAMP with low affinity (*31, 35*), the GAFa domain in isolation did not bind cAMP (Figure 2D, inset). A number of residues distal to the actual binding site have been shown to modulate the binding of cGMP to the GAF domain of the cGMP-stimulated phosphodiesterases, PDE2 (*11*), and it is therefore possible that the presence of the GAFb domain in the tandem GAF construct of PDE5 allowed entry of cAMP into the binding pocket.

The GAFa Domain As a cGMP BRET Sensor. The fact that the GAFa domain did not interact with cAMP suggested that it would be a specific sensor for cGMP in the cell. Wild type and mutant GAFa domains were cloned into the pGFP^2-MCS-Rluc vector and proteins expressed in HEK 293T cells. Lysates were prepared from transfected cells and protein

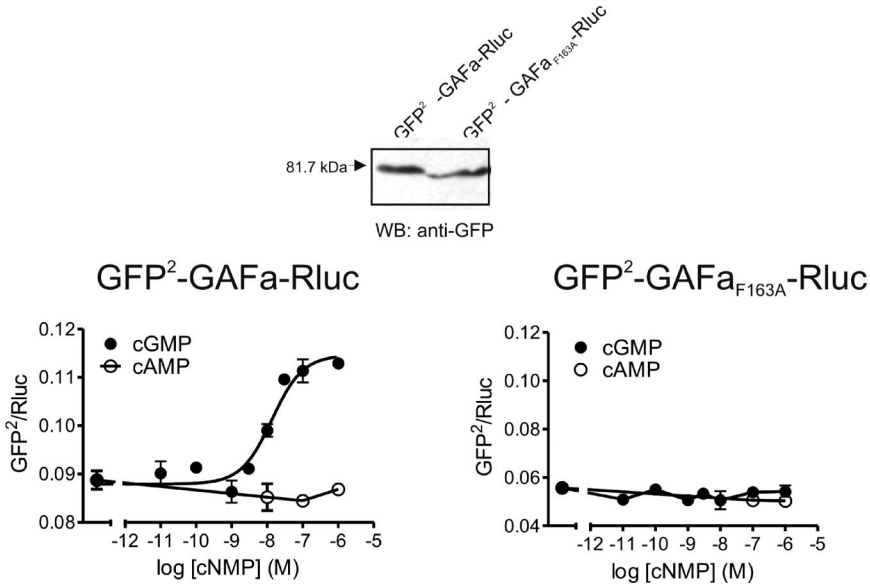

FIGURE 3: GAFa domain is a sensor for cGMP. (A) Lysates were prepared from HEK 293T cells expressing GFP^2-GAFa-Rluc or GFP^2-GAFa$_{F163A}$-Rluc proteins. Suitable aliquots of the lysates were incubated with the indicated concentrations of cGMP or cAMP for 10 min at 37 °C, following which the BRET^2 ratio was measured. The data shown is representative of three independent experiments, with each determination performed in duplicate. The inset shows a Western blot performed with 200 μg of lysate protein and anti-GFP antibody, indicating similar expression of the wild type and mutant GAFa domain.

expression monitored by Western blot analysis (Figure 3, inset). The majority of the GFP^2-GAF-Rluc fusion protein was localized to the cytosol (data not shown). Addition of varying concentrations of cGMP to the cytosolic fraction prepared from cells expressing the wild type GAF-BRET construct altered the BRET ratio in a dose dependent manner, with an apparent EC_{50} of ~30 nM (Figure 3). No change in BRET ratio was observed with cAMP, indicating the specificity of the BRET ratio change. The BRET ratio did not change on the addition of equivalent concentrations of either cGMP or cAMP in lysates prepared from cells expressing the mutant GAFa construct (Figure 3), indicating that the altered BRET ratio was a consequence of cGMP binding to the GAFa domain.

An increase in the BRET ratio is an indication of the conformational change that occurred in the GAF domain on cGMP binding. The conformational alteration could indicate that the helices from residue A125 to L137 and E255 to N274 have moved closer to each other such that RET occurred more efficiently between Rluc and GFP. Alternatively, or in addition, cGMP binding may rotate the helices along their axis such that the orientation of GFP to Rluc becomes more favorable for RET.

The BRET ratio seen with lysates expressing the wild type GAFa domain construct in the presence of cGMP was increased by ~30% over the basal ratio, and this change was robust and reproducible (Figure 3). This prompted us to use this sensor in intact cells to determine if it could detect changes in intracellular levels of cGMP.

Intracellular levels of cGMP can be altered by treatment of cells with sodium nitroprusside (SNP), which releases NO in the cell, resulting in activation of soluble guanylyl cyclases and consequent cGMP production (*37*). In addition, ligand-mediated activation of receptor guanylyl cyclases also results in increased cGMP production (*38*). We transfected HEK 293T cells with pcDNA-hGCC plasmid along with the GAF domain BRET constructs and elevated intracellular cGMP levels by treatment with various doses of the heat-stable enterotoxin, ST, which is an agonist of the receptor GC-C. An increase in the BRET ratio was seen on ST treatment in cells expressing the wild type GAF domain but not in cells expressing the mutant GAF protein (Figure 4). The alteration in BRET ratio of the wild type GAF sensor was correlated with increases in cGMP levels seen on addition of varying concentrations of ST (Figure 4). Interestingly, the level of cGMP reached in the cells transfected with the wild type GAF domain construct was higher than in cells transfected with the mutant construct. This could be because the cGMP produced in cells (consequent to ST addition) remained bound to the GAF domain, thereby preventing its hydrolysis by cellular PDEs, and/or its secretion from the cell. The saturation of the BRET response when cGMP levels reached ~1.5 pmol/10^5 cells is indicative of the fact that all the GAFa protein in the cells was bound to cGMP (Figure 4).

To test the temporal response of the cGMP sensor to SNP treatment, we transfected HEK 293T cells with the sensor constructs, and then treated cells with SNP. SNP addition led to a rapid increase in BRET ratio concomitant with an increase in the levels of cGMP in the cell (Figure 5A). No change was observed in cells with the mutant sensor construct. In these experiments, total cGMP levels achieved were also higher in cells transfected with the wild type GAF domain construct when compared to the mutant sensor construct.

Treatment of cells with forskolin activates adenylyl cyclases, leading to a dramatic increase in intracellular cAMP levels. Cells expressing the wild type GAFa sensor showed an insignificant change in BRET ratio (Figure 5B) even when intracellular cAMP levels reached 100-fold higher than cGMP levels. Interestingly, the BRET ratio in cells expressing the mutant GAF domain construct showed a small but significant increase across different experiments. It is possible that the mutant GAFa construct becomes responsive to very high concentrations of cAMP since a mutation of the

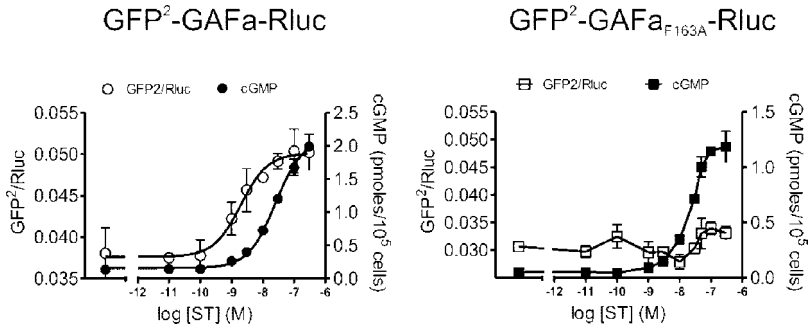

FIGURE 4: GAFa sensor responds to increases in intracellular cGMP. (A) HEK 293T cells were transfected with pcDNA3-GCC plasmid along with the pGFP^2-GAFa-Rluc plasmid or pGFP^2-GAFa$_{F163A}$-Rluc plasmid. Forty-eight hours following transfection, cells were treated with the indicated concentrations of ST peptide. BRET^2 ratios were measured 5 min following the addition of ST. Cyclic GMP levels were measured in cells treated in parallel. Data shown is representative of three experiments, with triplicate determinations at each point, and is representative of the mean ± SEM.

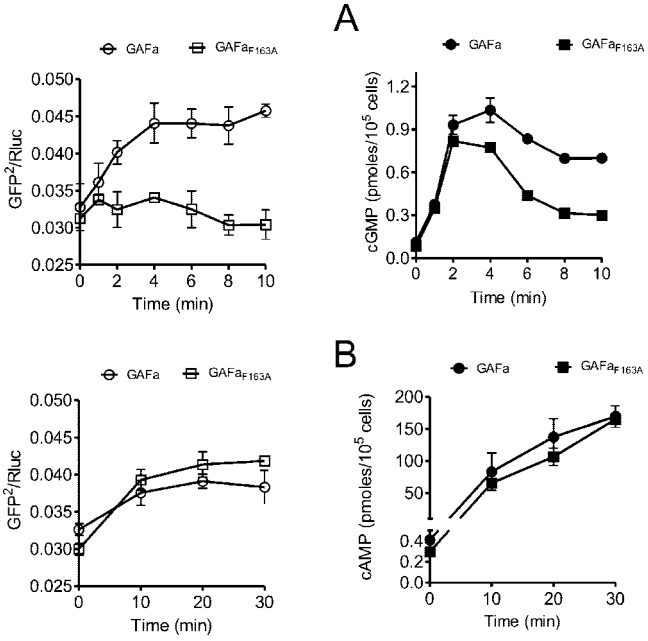

FIGURE 5: GAF sensor responds temporally to changes in intracellular cGMP but not cAMP. HEK 293T cells were transfected with pGFP^2-GAFa-Rluc or pGFP^2-GAFa$_{F163A}$-Rluc plasmid, and 48 h following transfection, cells were treated with 100 μM SNP (A) or forskolin (B) for the indicated times and BRET^2 ratio monitored. Similarly treated cells were taken for radioimmunoassay to monitor total cNMP accumulation. Data shown is the mean ± SEM of triplicate determinations in a representative experiment. Each experiment was repeated thrice.

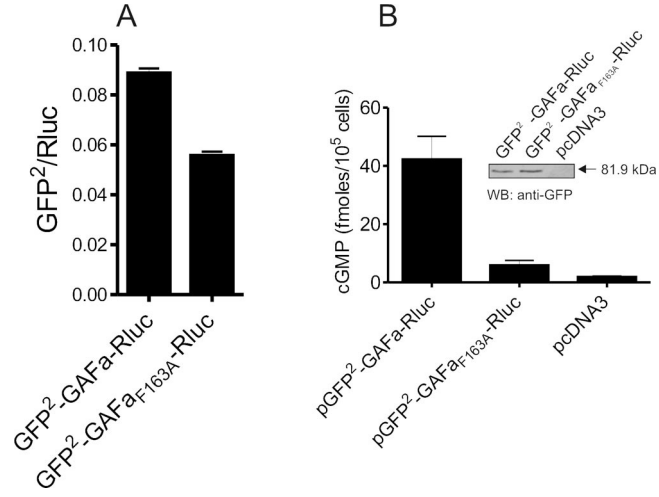

FIGURE 6: Basal BRET^2 ratios and intracellular cGMP levels in GAF domain transfected cells. (A) The basal BRET^2 ratio was measured in HEK 293T cells expressing GFP^2-GAFa-Rluc or GFP^2-GAFa$_{F163A}$-Rluc proteins. Measurements were made 72 h post transfection and represent the mean ± SEM of triplicate determinations, with the experiment performed thrice. (B) Intracellular cGMP was measured in HEK 293T cells transfected with various plasmid DNAs as indicated. Data shown is representative of three independent experiments, and the values are the mean ± SEM. The inset shows a Western blot with anti-GFP antibody showing similar expression of wild type and mutant proteins in cells taken for cGMP estimation.

equivalent Phe residue in PDE2 (F438A) has been shown to impart cAMP binding to the related PDE2 GAFb domain (*11*). Therefore, these results indicate that the wild type GAFa-BRET sensor could be utilized in cells to monitor changes in intracellular cGMP levels, with very little interference from physiological concentrations of intracellular cAMP.

The ~30% increase of the basal BRET ratio on elevation of intracellular cGMP was consistent and in agreement with a similar change seen in the first FRET-based sensors that were described earlier (*17*). In contrast, a recent report has stated that the PDE5 GAFa construct did not show any change in FRET, whereas the tandem GAFa-GAFb domains of PDE5 showed a slow, but significant change in FRET (*15*). Our results appear to agree with the earlier FRET studies.

GAF Domains Can Act As Sinks for cGMP. An intriguing observation that we made was that basal BRET ratios were always higher in cells expressing the wild type GAF protein than in cells expressing the mutant GAF protein, at equal level of expression of the two proteins (Figure 6A). Since the BRET ratio reflects a conformational change that occurs on cGMP binding to the GAF domain, we hypothesized that the increased BRET ratio was due to the wild type GAF domain acting as a sink to sequester basal levels of cGMP in the cell. We therefore measured intracellular levels of cGMP in cells that were expressing the wild type and mutant GAF domains, and indeed, higher cGMP levels were observed only in cells that contained the wild type GAF domain (Figure 6B). Equivalent expression of the two proteins was seen on Western blot analysis (Figure 6B, inset), thus providing direct evidence that the PDE5 GAFa domain could act as a sink for cGMP.

In order to see if this sink-like property of the GAFa domain was seen in the tandem GAFa-GAFb domain protein

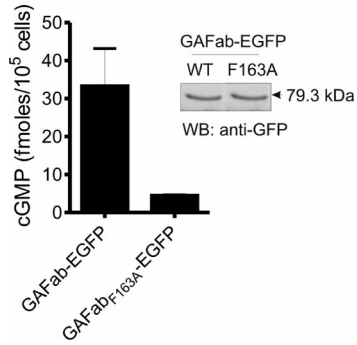

FIGURE 7: Tandem GAF domain of PDE5 acts as a sink for cGMP. HEK 293T cells were transfected with plasmids that enabled the expression of EGFP-GAFab and EGFP-GAFab$_{F163A}$ proteins. Cells were lysed 48 h post-transfection for the measurement of cGMP by radioimmunoassay. Data shown is representative of three independent experiments, and the values are the mean ± SEM. Expression of the wild type and the mutant proteins was confirmed by Western blot analysis (inset).

that we had shown earlier to bind cGMP (*27*), as well as in the full length protein, we generated constructs of GAFa-GAFb with a C-terminal fusion to EGFP. The EGFP tag was used to be able to detect the proteins by Western Blot using antibodies to GFP, in order to normalize expression levels. As seen in Figure 7, the wild type tandem GAF domain expressing cells also accumulated and sequestered intracellular cGMP. Importantly, the mutant F163A tandem GAF domain construct showed no increase in basal cGMP levels, indicating that the higher level of cGMP in the cell was due to binding of cGMP to the GAFa domain.

To extend these observations to the full length enzyme, we expressed a wild type full length PDE5-EGFP fusion protein and a mutant PDE5-EGFP fusion protein containing the F163A mutation, in HEK293T cells. Equivalent expression of the proteins was again monitored by Western blot analysis (Figure 8A). The endogenous levels of PDE5 in the cell were at least 10-fold lower than the overexpressed protein, as estimated by the Western Blot. Enzymatic PDE assays performed with lysates prepared from transfected cells showed that the overexpressed wild type and the F163A mutant enzymes showed a similar K_m for cGMP (~4 μM; data not shown). The IC_{50} for inhibition by sildenafil citrate was also similar (~10 nM), thereby confirming that the presence of the EGFP tag at the C-terminus of PDE5 did not substantially alter its catalytic activity or its inhibition by sildenafil citrate (Figure 8B).

Since overexpression of full length PDE5 would degrade any endogenous levels of cGMP, we treated cells with sildenafil citrate to inhibit PDE5. The concentration of sildenafil citrate used was 50 nM, which is the concentration found in circulation of individuals being treated with the drug (*39, 40*). In the absence of sildenafil citrate, cells overexpressing either the full length PDE5 or the mutant enzyme showed similar and low levels of cGMP. However, when enzyme activity was inhibited by the addition of sildenafil citrate for 24 h prior to measuring cGMP levels, cGMP levels were found to be higher in cells expressing the wild type protein, but not in cells expressing the mutant protein (Figure 8D). Therefore, this increase in intracellular cGMP must be due to the binding of cGMP to the GAF domains in full length PDE5, which are able to sequester cGMP. Thus, we show that the GAF domains of PDE5 and perhaps other GAF-domain-containing phosphodiesterases could act as sinks for cyclic nucleotides in addition to their role in regulating the catalytic activity of the phosphodiesterase domain.

DISCUSSION

The results described in this study validate the use of the GAFa domain of PDE5 as a sensor for cGMP in a BRET format. Other groups have described sensors of cGMP based on FRET (*17, 41, 42*), and a more recent report has developed FRET sensors based on the tandem GAF domains of PDE5 and the cGMP-binding domain of cGMP-dependent kinase (*15*). These sensors including ours are all specific for cGMP and do not demonstrate any response with cAMP. However, FRET-based assays do not readily allow a high throughput format (*18*) since a high level of background autofluorescence from intact cells may result from the use of filters, rather than lasers, for excitation of the fluorophore. Therefore, the BRET assay that we have described may be more suitable for rapid analysis of changing cGMP levels in cells.

An important observation that we have made in this study is that the GAFa domain could act as a sink for cGMP in the cell. We were able to notice the sink-like nature of the GAF domain only because we utilized the mutant GAF in our studies which we knew did not bind cGMP. Since the mutant GAF construct showed no change in BRET ratio on cGMP elevations in the cell, the higher basal BRET ratio that was seen in cells expressing the wild type GAF reflected cGMP binding to the wild type GAF domain, and we were able to confirm this by measuring cGMP levels in cells (Figures 6 and 8). Intracellular levels of cGMP were higher in resting cells expressing the wild type GAFa domain and the tandem GAF domains, indicating that the GAFa domain was able to sequester cGMP from the action of phosphodiesterases in the cell. An insightful suggestion along these lines was indeed proposed by Corbin and Francis some years ago, based purely on knowledge of the relative concentrations of PDE5, protein kinase G and cGMP in the cell (*29, 32*). To our knowledge, our experiments are the first to experimentally demonstrate the sink-like property of the GAFa domain as well as the tandem GAFa-GAFb domain.

Overexpression of full length PDE5 in cells did not result in higher intracellular cGMP levels, presumably because of the activity of the downstream catalytic domain. The addition of sildenafil citrate verified this since a significant increase in basal levels was then detected, indicating that the GAF domain in full length PDE5 was also capable of acting as a sink for cGMP (Figure 8). The fact that there was no increase in intracellular cGMP in cells expressing PDE5 containing a mutation in the GAF domain indicates that the GAF domain in the full length enzyme is able to capture a significant amount of the cGMP that is produced in the cells during the 24 h of sildenafil citrate treatment. This implies that the wild type GAF domain could sequester the cGMP produced transiently on inhibition of PDE5 and prevent its degradation by other PDEs in the cell. Moreover, the cGMP must have *remained* bound to the GAF domain to prevent it being hydrolyzed, indicating that the off-rate for cGMP from the GAF domain is low. It is therefore conceivable that the

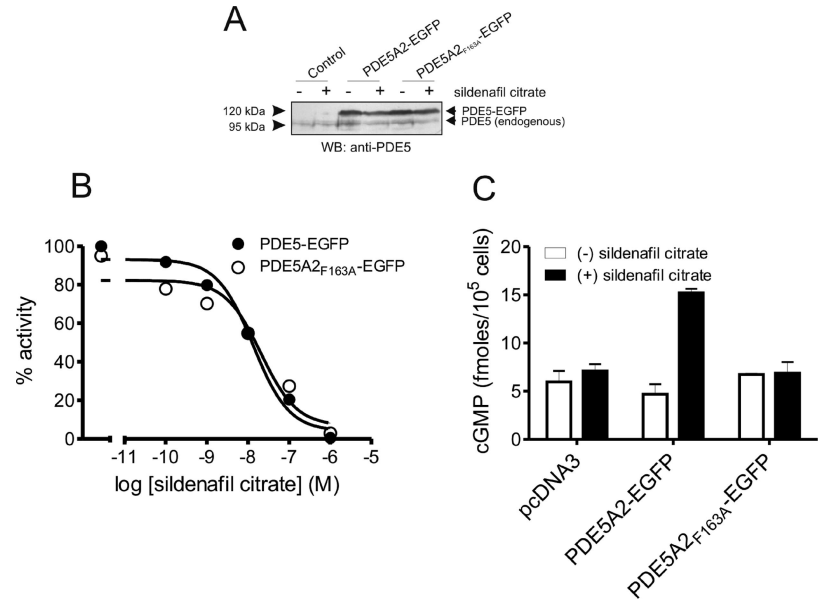

FIGURE 8: GAF domains in full length PDE5A2 can sequester cGMP on the inhibition of cGMP hydrolytic activity. HEK 293T cells were transfected with plasmids that allowed the expression of full length PDE5A2-EGFP or the PDE5A2$_{F163A}$-EGFP mutant proteins. (A) Western blot performed with a PDE5 polyclonal antibody that reacts with the recombinant proteins as well as the endogenous PDE5 present in cells. (B) Lysates prepared from cells expressing PDE5A2 recombinant proteins were taken for a cGMP phosphodiesterase activity, in the presence or absence of the indicated concentrations of sildenafil citrate. Data shown is the mean of duplicate determinations and is representative of assays performed at least thrice. (C) Cells expressing recombinant PDE5A2 proteins were lysed 72 h post transfection and aliquots taken for the estimation of cGMP. Cells were treated with medium or 50 nM sildenafil citrate for 24 h prior to the estimation of cGMP. The values represent the mean ± SEM of a representative experiment, with the experiment performed thrice, with each treatment performed in triplicate.

affinity of the GAF domain for cGMP was increased by the presence of the inhibitor in the catalytic site of PDE5, perhaps brought about by a conformational change that resulted in more efficient sequestration of cGMP by the GAF domain. Indirect evidence for alterations in the conformation of PDE5 have been reported recently. First, preincubation of the full length enzyme with cGMP resulted in an increased affinity for its inhibitors at its catalytic site (*43*). In addition, phosphorylation of PDE5 at the N-terminal serine residue was promoted by a conformational change induced in the presence of sildenafil citrate, again suggesting a complex interplay among the various domains in PDE5 (*44*). Therefore, the presence of sildenafil citrate at the catalytic site of the full length enzyme may enhance cGMP binding to the GAF domain, now resulting in increased phosphorylation of the enzyme and therefore an increased catalytic activity of PDE5 once the inhibitor is removed. These speculations remain to be tested and could have important consequences in tissues of individuals being administered the drug. For example, if the cGMP bound to the GAF domain is released slowly following inhibitor removal, other cGMP-dependent signaling events could be activated in tissues leading to side-effects that could vary on an individual basis. This would of course also depend on the rate of hydrolysis of cGMP by noninhibited PDE5 in the cell.

It is interesting to note that to date, cyclic nucleotide binding GAF domains are found predominantly associated with phosphodiesterase domains in higher eukaryotes, whereas in bacteria, they are found associated with adenylyl cyclases (*45, 46*). In the latter case, the downstream domain *generates* the nucleotide to which the upstream GAF domain binds, instead of the C-terminal domain *degrading* the cyclic nucleotide, as is seen in eukaryotic GAF domain containing proteins. We do not believe that this is a chance event and in fact could have arisen out of an evolutionary necessity. Cyclic AMP and GMP-dependent signaling pathways are more diverse and interconnected in mammalian cells than in bacteria, and therefore, levels of cyclic nucleotides need to be kept in check in the eukaryotic cell. If GAF domains have the potential to act as sinks for cGMP, then a number of pathways may be activated if the cyclic nucleotide dissociates from the GAF domain. Therefore, a phosphodiesterase domain is associated with the GAF domain in eukaryotic enzymes, with the phosphodiesterase domain in turn being allosterically regulated by cGMP bound to the GAF domain, thereby imposing a strict control of intracellular cGMP levels in the cell.

The concentration of PDE5 in different cells can vary, which can also affect the basal intracellular levels of cGMP. Based on Western blot analysis of cells transfected with PDE5-EGFP using a PDE5 specific antibody (Figure 8A), the protein level of transfected PDE-EGFP is at least 10-fold higher than endogenous levels of PDE5. Therefore, this may have allowed us to detect the increased level of cGMP that is associated with the GAF domain sink. It is difficult at this time to estimate how much cGMP in a cell is bound to the GAF domains of endogenous PDE5. This will depend not only on the levels of PDE5 in the cell but also on the concentrations of cGMP-synthesizing enzymes and additional putative sinks (such as the cGMP-binding domains found in cGMP-dependent kinases) that could also be present in the cells under study. This complex interplay of proteins involved in cGMP generation, binding and degradation has been alluded to in earlier studies (*47*). HEK 293 cells clearly do express an NO-activable guanylyl cyclase and PDE5, and a recent proteomic analysis has shown that cGMP-dependent protein kinase-α is also present in these cells (*48*). Therefore, HEK cells contain some components of a cGMP-signaling

system, though clearly not at the concentrations that would be seen in tissues such as the corpus cavernosum, platelets or the lung.

It is also important to realize at this time that other sensors that utilize cAMP or cGMP-binding domains may also act as sinks for their respective cyclic nucleotide. For example, the earlier cGMP sensor based on the PDE5 GAFa domain should also have led to an increase in intracellular cGMP levels, on the basis of the affinity of cGMP for the GAFa domain. Intracellular levels of cGMP are also not reported in a more recent study (*15*). Investigators need to be aware of the potential of cyclic nucleotide binding domains to act as sinks and therefore interpret their results with some caution.

We envisage a number of uses for the GAF-BRET sensor that we have described here. It can be utilized in identifying modulators of cGMP signaling in cells and in the first case can be used as a read out to measure alterations in cGMP levels on activation of a receptor or inhibition of a phosphodiesterase. A cAMP-sensor has already been described that can monitor the activation of the PDE2 (a dual-specificity PDE) in the cell on atrial natriuretic peptide stimulation (*49*). Since the atrial natriuretic peptide signals through a receptor guanylyl cyclase, GC-A (*38, 50*), it would be possible to monitor the activation of GC-A using this cGMP BRET sensor. As shown in this study, the GAF-BRET sensor could be used to detect the activation of GC-C since BRET ratio changes mirrored changes in cGMP as monitored by radioimmunoassay (Figure 4).

Sildenafil citrate, widely used to treat male impotence, targets PDE5 by binding to it catalytic domain (*31*). It is known that cGMP binding to the GAF domain increases the catalytic activity of PDE5, and therefore, one could envisage the utilization of the GAF-BRET sensor for identifying compounds that inhibit the binding of cGMP to the GAF domain of PDE5 but do not result in the conformational change that activates the catalytic domain. Identification of inhibitors to the GAF domain would be a novel way of targeting PDE5, and it is likely that the BRET-based assay would prove useful in initial high-throughput screens for compounds. An inhibitor of cGMP binding to the GAF domain could inhibit catalytic *activation* of the enzyme, as opposed to sildenafil citrate, which would inhibit the basal activity of the enzyme as well.

In conclusion, this study not only describes the generation of a sensitive monitor of intracellular cGMP levels in the cell but also demonstrates that GAF domains can act as sinks for cGMP in the cell. The utilization of this approach to monitor conformational changes in the GAF domain of PDE5 can be extended to detect changes that occur in other proteins that contain the GAF domain. Given the importance of PDEs as drug targets, it is likely that BRET-based approaches can be utilized to identify novel inhibitors that allosterically modulate the activity of the PDEs that contain GAF domains.

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