Distinct Allostery Induced in the Cyclic GMP-binding, Cyclic GMP-specific Phosphodiesterase (PDE5) by Cyclic GMP, Sildenafil, and Metal Ions

Received for publication, October 10, 2010, and in revised form, November 26, 2010. Published, JBC Papers in Press, December 29, 2010, DOI 10.1074/jbc.M110.193185

Kabir H. Biswas and Sandhya S. Visweswariah

From the Department of Molecular Reproduction, Development, and Genetics, Indian Institute of Science, Bangalore 560012, India

The activity of many proteins orchestrating different biological processes is regulated by allostery, where ligand binding at one site alters the function of another site. Allosteric changes can be brought about by either a change in the dynamics of a protein, or alteration in its mean structure. We have investigated the mechanisms of allostery induced by chemically distinct ligands in the cGMP-binding, cGMP-specific phosphodiesterase, PDE5. PDE5 is the target for catalytic site inhibitors, such as sildenafil, that are used for the treatment of erectile dysfunction and pulmonary hypertension. PDE5 is a multidomain protein and contains two N-terminal cGMP-specific phosphodiesterase, bacterial adenylyl cyclase, FhLA transcriptional regulator (GAF) domains, and a C-terminal catalytic domain. Cyclic GMP binding to the GAFa domain and sildenafil binding to the catalytic domain result in distinct conformations of the protein. Metal ions bound to the catalytic site also allosterically modulated cGMP- and sildenafil-induced conformational changes. The sildenafil-induced conformational change was temperature-sensitive, whereas cGMP-induced conformational change was independent of temperature. This indicates that different allosteric ligands can regulate the conformation of a multidomain protein by distinct mechanisms. Importantly, this novel PDE5 sensor has general physiological and clinical relevance because it allows the identification of regulators that can modulate PDE5 conformation in vivo.

Allostery is a thermodynamic phenomenon utilized by nature to regulate the function of a large number of proteins. Thus, ligand binding at one site of a protein can alter the function of another distinct site in the protein (1–3). Due to inherent dynamics, proteins sample multiple conformations in solution (4). A “shift” in the ensemble of conformations, involving conformational selection and induced–fit mechanisms (5), is generally thought to result in allostery regulation (6). Thermodynamically, however, allostery could manifest itself as a change in the mean conformation of the ensemble (enthalpy-driven), a change in the dynamics of the ensemble without any observable change in the backbone structure of the ensemble (entropy-driven), or a combination of the two (7, 8). In addition, the presence of multiple domains in a single polypeptide chain could allow allosteric integration of different input signals into similar outputs or allosteric dissemination of similar inputs into different output signals (9, 10).

The mammalian cyclic nucleotide phosphodiesterases (PDEs) serve as examples of multidomain proteins where different N-terminal regulatory domains sense a variety of ligands, and then allosterically modulate the hydrolysis of cAMP or cGMP (11). The cGMP-binding, cGMP-specific phosphodiesterase (PDE5) specifically hydrolyzes cGMP (12, 13) and is expressed in a variety of tissues (14). PDE5 has been targeted by the specific inhibitors sildenafil, vardenafil, and tadalafl (15, 16) for the treatment of erectile dysfunction and pulmonary hypertension. PDE5 is a multidomain protein containing two N-terminal tandem GAF domains (GAFa and GAFb) followed by the C-terminal catalytic domain. Cyclic GMP binding to the GAFa domain allosterically activates the catalytic domain by increasing both the Vmax and affinity for cGMP, resulting in increased cGMP hydrolysis (17–19). In addition, sildenafil binding to the catalytic domain enhances cGMP binding to the regulatory GAFa domain (20). Further, cGMP binding to the GAFa domain (21, 22) or physical interaction of the isolated GAFa domain with PDE5 (23) increases sildenafil affinity to the catalytic domain. PDE5 shows a reduced migration in native polyacrylamide gels in the presence of cGMP (24, 25), providing a qualitative indication of conformational changes in the protein. Until now, allosteric changes in PDE5 have been studied using purified preparations of the protein and therefore may not reflect the conformational states or the biochemical properties of the enzyme in the cell (26).

* This work was supported by the Department of Biotechnology, Government of India, and the Indian Institute of Science.

** The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5, Methods, and additional references.

1 Recipient of a senior research fellowship from the Council of Scientific and Industrial Research, Government of India.

2 To whom correspondence should be addressed: Dept. of Molecular Reproduction, Development and Genetics, Sir C. V. Ramakrishna Aven, Indian Institute of Science, Bangalore 560012, India. Tel.: 91-80-22932542; Fax: 91-80-23600999; E-mail: sandhya@mrdg.iisc.ernet.in.

3 The abbreviations used are: PDE, phosphodiesterase; BRET, bioluminescence resonance energy transfer; DETA/NO, diethylenetriamine-NO; EGFP, enhanced green fluorescent protein; GAF, cGMP-specific phosphodiesterases, bacterial adenylyl cyclase, FhLA transcriptional regulator; IBMX, isobutylmethylxanthine; PDE5, cGMP-binding, cGMP-specific PDE; Rluc, Renilla luciferase.
**Allostery in PDE5**

We asked whether we could develop a method in which conformational changes in full-length PDE5 could be measured quantitatively, not only *in vitro*, but also in the intact cell. Here, we report the generation of a functional reporter of PDE5 conformation that exploits intramolecular bioluminescence resonance energy transfer (BRET). The sensor reveals structural changes and associated allostery induced by cGMP and sildenafil binding to the full-length PDE5. Importantly, we show that metal binding to the catalytic site of PDE5 negatively modulates cGMP-induced and positively modulates sildenafil-induced conformational changes. The conformational changes induced by cGMP and sildenafil are structurally and thermodynamically distinct. This novel sensor not only monitors dynamic changes in PDE5 conformation in intact cells, but also provides new insights into allostery in a multidomain protein.

**MATERIALS AND METHODS**

*Generation of PDE5 Sensor Constructs*—The GFP gene was released from pGFP-C1 plasmid (PerkinElmer Life Sciences) with SnaBI-Xhol and cloned into similarly digested pEGFP-PDE5A2 (27) resulting in pGFP-PDE5A2-EGFP. The EGFP gene was then replaced with the Rluc gene from pRluc-N1 vector (PerkinElmer Life Sciences) with KpnI-XbaI digestion to generate pGFP-PDE5A2-Rluc. This plasmid expresses the GFP-PDE5A2-Rluc fusion protein (PDE5 sensor). The F163A mutant sensor was similarly generated from the pEGFP-PDE5A2-Rluc plasmid (27).

For mutagenesis in the catalytic domain, a HindIII-Kpn fragment was released from the pGFP-PDE5A2-Rluc plasmid and cloned into similarly digested pBlueScript II KS(+) vector (Stratagene). The single mutagenic oligonucleotide-based protocol was utilized for generation of mutations (28). Primers PDE5A2F778A (5'-CCCCAGTTGCAAGTTGGGCCATCGATGCACTGTGTCG-3'), PDE5A2D612A (5'-GCTGCACATCCACCGCTATAGCTACGGTGTGTG-3'), and PDE5A2G617P (5'-GCCCAGCATCTAGATCACTACGCCGTTC-TGTGAATAACTCTTAC-3') were used for generating F778A, D612A, and G617P mutations, respectively, and mutations were confirmed by sequencing (Macrogen).

Mutant fragments in pBlueScript II KS(+) vector were then suitably subcloned to generate either single or double mutants of the PDE5 sensor.

*Cell Culture and Transfection*—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (FCS), 120 mg/liter penicillin, and 270 mg/liter streptomycin at 37 °C in a 5% CO₂ humidified incubator. Transfections were performed with polyethyleneimine lipid.

*Cyclic GMP PDE Assays*—Cytosol prepared from cells transfected with either the wild-type or mutant PDE5 sensors were prepared in PDE lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM sodium pyrophosphate, 80 μM β-glycerophosphate, 1 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, 100 μM sodium orthovanadate, and 10% glycerol) and were used for cGMP PDE assays (29). All PDE assays were performed with ~100 nM [³H]cGMP (GE Healthcare) essentially as reported previously (30). Expression of proteins was monitored by Western blot analysis using either an antibody raised in rabbits to GFP, generated in the laboratory, or a previously described antibody to PDE5 (29).

*Gel Filtration Analysis*—HEK293T cells that were either untransfected or expressing pGFP-PDE5A2-Rluc were lysed in PDE lysis buffer. Cytosol from lysed cells was subjected to gel filtration (Superose 12, 10 × 30; GE Healthcare) in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% glycerol. Fractions (500 μl) were collected and analyzed for GFP fluorescence using a Victor3 microplate reader (excitation, 485 nm; emission, 510 nm; PerkinElmer Life Sciences).

*Intracellular cGMP Estimation*—Intracellular levels of cGMP were measured in cells transfected with either wild-type or mutant PDE5 sensors 72 h after transfection. Cells were lysed in 0.1 N HCl, and cGMP was measured by radioimmunoassay as described earlier (31). In some cases, lysates were acetylated prior to radioimmunoassay to increase the sensitivity of the radioimmunoassay.

*In Vitro BRET Assays*—Cells transfected with sensor plasmids were lysed in PDE lysis buffer. Following brief sonication, the lysates were centrifuged at 13,000 × g, and the cytosol was removed. Aliquots of the cytosol were incubated with the indicated amounts of ligand in a total volume of 40 μl at 37 °C for 10 min.

To determine the effect of sildenafil binding on cGMP-induced conformational change, lysates were preincubated with 100 μM sildenafil at 37 °C for 5 min followed by incubation with varying concentrations of cGMP at 37 °C for another 5 min. Similarly, to determine the effect of cGMP binding on sildenafil-induced conformational change, lysates were preincubated with 100 μM cGMP at 37 °C for 5 min followed by incubation with varying concentrations of sildenafil at 37 °C for another 5 min, and BRET was measured.

In experiments to determine the effect of EDTA and metals on the PDE5 sensor, cells were lysed in 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10% glycerol. Lysates were incubated in the absence or presence of 2 mM EDTA and 10 μM metals in 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, and 10% glycerol in a total volume of 40 μl at 37 °C for 10 min. To determine the concentrations of Mg²⁺ or Ca²⁺ required to inhibit the cGMP-induced decrease in BRET, lysates were preincubated with 0.1 mM EDTA and 100 μM cGMP for 5 min at room temperature followed by incubation with varying concentrations of MgCl₂ or CaCl₂ at 37 °C for 10 min. The free metal ion concentrations were calculated with the program WinMAXC32 2.51 (C. Patton, Stanford University, Palo Alto, CA). The dependence of cGMP- or sildenafil-induced conformational changes on temperature was determined by incubating lysates with either 100 μM cGMP or sildenafil at the specified temperatures for 10 min, followed by BRET measurements.

BRET was measured by addition of 5 μM coelenterazine 400a (Molecular Imaging Products) to a final concentration of 5 μM. Readings for GFP and Rluc emission (0.8 s) were collected in the Victor3 Microplate Reader. Emission filters used for Rluc and GFP emission were 410 nm (bandpass 80 nm).
and 515 nm (bandpass 30 nm), respectively. BRET was calculated as the ratio of GFP emission per second to Rluc emission per second, and the average of three such measurements is reported.

**Cellular BRET Assays**—BRET assays with intact cells were performed by incubating cell monolayers expressing various sensors with Dulbecco’s PBS containing 5 mM EDTA, resuspending cells in phenol red-free DMEM with 10% FCS, followed by treatment with diethylenetriamine-NO (DETA/NO) (27, 35) to alanine abrogated the BRET decrease (Fig. 2A). The EC_{50} (0.75 ± 0.25 μM) of the cGMP-induced conformational change was similar to the binding affinity of cGMP to the GAFα domain in full-length PDE5 (36), and no apparent co-operativity in binding to the two GAFα domains in the PDE5 dimer (n_{H} = 1.0 ± 0.1; Fig. 2B) was seen. Therefore, cGMP binding to the GAFα domain resulted in a shift in the conformational ensemble of PDE5 from a “closed” high BRET form to an “open” low BRET form (24, 25).

The isolated GAFα and the tandem GAF domains of PDE5 show high specificity, with cAMP binding affinity ~ 1,000-fold lower than that of cGMP (27, 37). Interestingly, cAMP reduced BRET of the wild-type sensor with an EC_{50} ~ 40-fold higher than that of cGMP (EC_{50} 28 ± 11 μM; Fig. 2C).

8-Bromo-cGMP was also able to decrease BRET with an EC_{50} of 20 ± 10 μM (Fig. 2C), similar to its binding affinity to the isolated GAFα domain (27).

**Allostery in PDE5**—Crystallographic studies have revealed that binding of sildenafil to the catalytic domain of PDE5 induces a major change in the positioning and secondary structure of the H-loop in the catalytic domain (38). However, no such structural changes are observed on binding of isobutylmethylxanthine (IBMX; supplemental Fig. S1). We investigated whether the conformational change induced by sildenafil could alter BRET of the PDE5 sensor. Indeed, a reduction in BRET was seen when lysates were incubated with sildenafil (~40%) but not IBMX (Fig. 3A), even though both compounds were effective in inhibiting PDE5 catalytic activity (Fig. 3B).

To determine whether the conformational change induced by sildenafil was solely a consequence of inhibitor binding to the catalytic site, we generated a mutant sensor where Phe-163 residue (which has a stacking interaction with cGMP in the GAFα domain (27, 35)) to alanine abrogated the BRET decrease (Fig. 3A). The EC_{50} (0.75 ± 0.25 μM) of the cGMP-induced conformational change was similar to the binding affinity of cGMP to the GAFα domain in full-length PDE5 (36), and no apparent co-operativity in binding to the two GAFα domains in the PDE5 dimer (n_{H} = 1.0 ± 0.1; Fig. 2B) was seen. Therefore, cGMP binding to the GAFα domain resulted in a shift in the conformational ensemble of PDE5 from a “closed” high BRET form to an “open” low BRET form (24, 25).

The isolated GAFα and the tandem GAF domains of PDE5 show high specificity, with cAMP binding affinity ~ 1,000-fold lower than that of cGMP (27, 37). Interestingly, cAMP reduced BRET of the wild-type sensor with an EC_{50} ~ 40-fold higher than that of cGMP (EC_{50} 28 ± 11 μM; Fig. 2C).

8-Bromo-cGMP was also able to decrease BRET with an EC_{50} of 20 ± 10 μM (Fig. 2C), similar to its binding affinity to the isolated GAFα domain (27).

**Allosteric Effects Induced by Sildenafil Require H-loop Flexibility**—Crystallographic studies have revealed that binding of sildenafil to the catalytic domain of PDE5 induces a major change in the positioning and secondary structure of the H-loop in the catalytic domain (38). However, no such structural changes are observed on binding of isobutylmethylxanthine (IBMX; supplemental Fig. S1). We investigated whether the conformational change induced by sildenafil could alter BRET of the PDE5 sensor. Indeed, a reduction in BRET was seen when lysates were incubated with sildenafil (~40%) but not IBMX (Fig. 3A), even though both compounds were effective in inhibiting PDE5 catalytic activity (Fig. 3B).

To determine whether the conformational change induced by sildenafil was solely a consequence of inhibitor binding to the catalytic site, we generated a mutant sensor where Phe-778 (which shows an important stacking interaction with sildenafil (13, 39, 40)) was mutated to alanine. The F778A mutant sensor showed reduced basal BRET (Fig. 3C), indicating an alteration in PDE5 conformation due to this point mutation. Nevertheless, cGMP was able to reduce BRET by ~20%, and the EC_{50} (0.81 ± 0.57 μM) of the F778A mutant...
sensor was similar to that of the wild-type sensor (Figs. 2B and 3D). This indicated that there was no change in the ability of the GAFa domain in the Phe-778 mutant to bind cGMP and induce a conformational change in PDE5. As expected, the F163A/F778A double mutant PDE5 showed no response to cGMP (Fig. 3D).

In contrast, the addition of sildenafil did not result in any change in BRET of the F778A mutant sensor, whereas a change was seen with the F163A mutant sensor (Fig. 3B). The EC50 for sildenafil-induced conformational change was similar in the wild-type and F163A mutant sensors (EC50 0.68 ± 0.18 μM and 0.30 ± 0.6 μM, respectively; Fig. 3E), and binding of sildenafil showed negative co-operativity (nH = 0.7 ± 0.1).

This therefore confirmed that sildenafil binding at the catalytic site and the consequent conformational change in H-loop positioning were reflected in reduced BRET, thereby further validating the use of the PDE5 sensor to monitor conformational changes in the full-length enzyme.

The movement of the H-loop is believed to be required for cGMP-mediated activation of PDE5 (25) and PDE2 (41). We mutated the conserved Gly-617 present in the beginning of the H-loop to proline, thereby restricting the flexibility of the loop without substantially affecting sildenafil binding (25). Basal BRET of the G617P mutant sensor was lower than the wild-type sensor (0.06 versus 0.09, respectively), but both the G617P mutant sensor and the F163A/G617P sensor failed...
to show any reduction in BRET in the presence of sildenafil (Fig. 4A). However, the EC\textsubscript{50} of the cGMP-induced conformational change on binding to the GAF\textalpha{} domain remained unaltered in the G617P mutant sensor (EC\textsubscript{50} 1.45 ± 0.7 \mu{}M; Fig. 4B), and cGMP could not induce a decrease in BRET in the F163A/G617P mutant sensor. Thus, flexibility of the H-loop is required for the sildenafil-induced conformational change in PDE5, but not for that induced by cGMP, indicating distinct structural transitions induced in PDE5 by these ligands.

In the simultaneous presence of both cGMP and sildenafil, BRET was significantly lower than that seen in the presence of the individual ligands (Fig. 4C). This indicated that the conformational changes induced in PDE5 by cGMP and sildenafil were distinct. Indeed, preincubation of lysates with sildenafil reduced the EC\textsubscript{50} of cGMP-induced conformational change (Fig. 4D), but preincubation with cGMP did not alter the EC\textsubscript{50} of sildenafil-induced BRET reduction (Fig. 4E). However, it is possible that cGMP competes with sildenafil for binding to the catalytic site, and cGMP binding to the catalytic site may not induce any change in BRET. If this was the case, no change in the EC\textsubscript{50} for sildenafil would be seen.

Metal Ion Binding to the Active Site of PDE5 Regulates the cGMP-induced Conformational Change—We then monitored ligand-induced conformational changes in PDE5 in intact cells. HEK293T cells were transfected with the PDE5 sensor and treated with DETA/NO. Intracellular cGMP levels increased to micromolar concentrations (assuming a cell volume of 1 pl), but no decrease in BRET was observed (Fig. 5A). Sildenafil, however, showed a decrease in BRET (supplemental Fig. S2A). The PDE5 sensor was found in the cytosol of cells (supplemental Fig. S1B), ruling out mislocalization as a cause for seeing no reduction in BRET in the presence of cGMP.

We checked that the GAF\alpha{} domain of the PDE5 sensor could bind cGMP in intact cells by treating cells with sildenafil for 24 h. As shown in Fig. 5B, we observed an increase in intracellular levels of cGMP in cells expressing the wild-type but not the F163A mutant sensor (Fig. 5B). This is reflective of cGMP binding to the GAF\alpha{} domain in this protein which acts as a sink for cGMP (27, 42). Therefore, despite the functionality of the PDE5 sensor in terms of binding cGMP in intact cells, it appeared that the conformational change (as monitored by a change in BRET) did not occur.

We hypothesized that factors present in intact cells constrained (or modified) the conformational changes that were seen in lysates on addition of cGMP. We inspected components of the lysis buffer that could have modulated the conformational transitions in the PDE5 sensor. Sodium ions have been shown to regulate cNMP binding to the GAF domains of CyaB2 and PDE2 (43). Analysis of the available structures of the GAF\alpha{} and the catalytic domain of PDE5 (35, 38) showed potential disulfide pairing of cysteine residues, suggesting the possibility of redox regulation of PDE5 conformation in cells. Additionally, PDE5 requires two divalent cations (Mg\textsuperscript{2+} and Zn\textsuperscript{2+}) bound to the catalytic domain for cGMP hydrolysis, and these metal ions could control the conformation of PDE5.

**FIGURE 4. Role of the H-loop in sildenafil-induced conformational change.** A, scheme representation of the PDE5 dimer shows the relative positioning of the H-loop in the apo- and sildenafil-bound crystal structure of the catalytic domain of PDE5 (38). The positions of Gly-617 and bound sildenafil in the catalytic domain, and Phe-163 and bound cGMP in the GAF\alpha{} domain are shown. Lysates prepared from cells expressing G617P or F163A/G617P mutant PDE5 sensors were incubated with 100 \mu{}M cGMP or sildenafil at 37 °C for 10 min and BRET determined. ***, \( p = 0.0002\), Student’s two-tailed \( t \) test. B, lysates prepared from cells expressing either the G617P and F163A/G617P mutant PDE5 sensors were incubated with varying concentrations of cGMP at 37 °C for 10 min in PDE lysis buffer followed by BRET determination. Percentage decrease in BRET in the presence of different concentrations of cGMP is plotted, and the EC\textsubscript{50} value of the cGMP-induced conformational change was determined. Values represent mean ± S.E. (error bars) of a representative experiment with each experiment performed three times with duplicate determinations. C, lysates prepared from cells expressing the PDE5 sensor were incubated with either 100 \mu{}M cGMP or 100 \mu{}M sildenafil or both at 37 °C for 10 min, and BRET was determined. ** and *** denote \( p = 0.046 \) and 0.008, respectively, Student’s two-tailed \( t \) test. D, lysates were preincubated with 100 \mu{}M cGMP at 37 °C for 5 min followed by incubation with varying concentrations of cGMP at 37 °C for 5 min. The further percentage decrease in BRET was determined. E, lysates were preincubated with 100 \mu{}M cGMP at 37 °C for 5 min followed by incubation with varying concentrations of sildenafil at 37 °C for 5 min, and the percentage decrease in the BRET was determined. Lysates not preincubated with cGMP (D) or sildenafil (E) serve as controls. Values represent mean ± S.E. from a representative set of experiments performed three times with at least duplicate determinations.
in intact cells. We either removed or altered the concentrations of NaCl, DTT, or EDTA in the buffer used for cell lysis and in assays and monitored cGMP-induced BRET changes in the lysates. Changes in NaCl concentration and absence of reducing agent in the lysis buffer still permitted the cGMP-induced reduction in BRET (supplemental Fig. S3A). However, lysates prepared in a buffer without EDTA showed no cGMP-induced reduction in BRET (supplemental Fig. S3A and Fig. 6A). Addition of EDTA to the same lysates now restored the cGMP-induced conformational change, detected by a reduction in BRET (Fig. 6A). The maximum cGMP-induced BRET reduction was seen in the presence of 0.1 mM EDTA (supplemental Fig. S3B). Further, addition of MgCl₂ along with EDTA abolished the cGMP-induced reduction in the BRET, and this effect was also seen in the presence of MnCl₂, and CaCl₂ (Fig. 6A). In the absence of cGMP, neither EDTA nor MgCl₂ considerably altered BRET (Fig. 6B).

Mg²⁺ and Ca²⁺ inhibited the cGMP-induced conformational change with an EC₅₀ of 1.2 ± 0.6 μM (Fig. 6C) and 19.2 ± 6 μM (Fig. 6D), respectively. Although both Mg²⁺ and Mn²⁺ hydrolyzed cGMP to similar extents (Fig. 6E), inclusion of Ca²⁺ in the lysate did not allow cGMP hydrolysis (44, 45). Therefore, the alleviation of the cGMP-induced BRET decrease did not result from hydrolysis of cGMP at the catalytic site, but was presumably due to a specific structural alteration brought about by the metal ions. Therefore, we suggest that intracellular Mg²⁺ or Ca²⁺ bound to PDE5 restricts the conformational change induced by cGMP binding to the GAFα domain in cells, and therefore the reduction in BRET. Because the GAFα sensor was still able to respond to increases in intracellular cGMP, and in buffer without EDTA (supplemental Fig. S3, C–E), the “regulatory” metal ions are bound to PDE5 at a site distinct from the GAFα domain, perhaps in the catalytic domain.

Indeed, mutation of the metal anchoring Asp-612 residue in the catalytic site (38) to alanine (D612A) permitted the cGMP-induced reduction in BRET, independent of the presence of EDTA and metal ions (Fig. 7A). Importantly, the D612A mutant PDE5 sensor was now responsive to cGMP elevation in cells (Fig. 7B), whereas responsiveness was lost in the F163A/D612A double mutant sensor. Therefore, metal ion binding at the catalytic site modified the conformational change induced on cGMP binding to the GAFα domain in PDE5.

To demonstrate unequivocally that the presence of metal ions in cells modulates the conformation of PDE5, we reduced intracellular metal ion concentrations by incubating cells in media lacking either MgCl₂, CaCl₂, or both. Treatment of cells with DETA/NO caused a detectable and significant reduction in BRET in the wild-type PDE5 sensor only in cells incubated in buffer lacking both Mg²⁺ and Ca²⁺, whereas cells expressing the D612A mutant sensor showed BRET changes independent of the medium composition (Fig. 7C). The GAFα sensor was able to respond under similar conditions (supplemental Fig. S4). Therefore, a reduction in intracellular metal ion levels resulted in the generation of a fraction of metal ion-free PDE5, which was now sensitive to increased intracellular cGMP levels.

Distinct Allosteric Effects of cGMP, Sildenafil, and Catalytic Metal Ions on PDE5—Given the allosteric effect of metal ions on the cGMP-induced structural change, we proceeded to investigate the effect of metal ions on sildenafil-induced structural change. We incubated lysates with varying concentrations of sildenafil in the presence of 10 mM MgCl₂ and observed a striking decrease in the EC₅₀ value (EC₅₀ 29.1 ± 14 pM; Fig. 8A). Incubation of the D612A mutant sensor with MgCl₂ did not alter the EC₅₀ of the sildenafil-induced conformational change (Fig. 8B), confirming that metal ion binding to the catalytic site allosterically altered ligand binding to PDE5.

We then extended our studies to suggest the distinct thermodynamic bases for cGMP- and sildenafil-induced conformational changes. Although the cGMP-induced conformational change occurred independently of temperature, the sildenafil-induced conformational change was temperature-dependent in the range of 20 – 40 °C (Fig. 8C). No considerable BRET change was observed in the absence of ligand at different temperatures, and the gross structural integrity of the PDE5 sensor in this temperature range was ascertained by similar luciferase activities at all the temperatures tested (supplemental Fig. S5).

Arrhenius plots were generated using the relative change in BRET versus 1/T, and provided an estimation of the apparent activation energies. The Eyring equation provided an estimate...
of apparent enthalpy and entropy changes in the activation process (supplemental Methods and Fig. 8D). The apparent activation energy for sildenafil was higher than that of cGMP (Fig. 8E), whereas the apparent free energy changes were similar. Interestingly, although sildenafil-induced conformational change involved both enthalpic and entropic changes, the cGMP-induced change was largely entropically driven (Fig. 8F). The repositioning and coil-to-310-helix transition of the H-loop, probably involving local unfolding in the catalytic domain (46), could account for the enthalpic barrier, involving an “induced-fit” mechanism in the sildenafil-induced conformational change. However, the high entropic contribution seen in the case of cGMP could arise from the “selection of conformations” with stabilized helix α4 and β2–β3 loop (in-
Allostery in PDE5

Metal ion binding to the catalytic domain of PDE5 did not cause a major change in BRET (Fig. 6B), but brought about significant effects on cGMP- and sildenafil-induced conformational changes (Figs. 6, A, C, and D, and 8A). We suggest that the metal-ion-induced conformational change in PDE5 could represent an entropically driven mechanism, where the conformational entropy of the protein is altered, with no marked change in the mean structure of PDE5 (7, 8). Indeed, divalent metal ion binding to an endonuclease was found to be largely entropy-driven without any gross structural change (55). This entropic effect of metal ion binding at the catalytic site of PDE5 may inhibit the conformational selection of low BRET conformers by cGMP binding at the distant GAFa domain (long range allosteric effect). However, metal ion binding facilitates the induction of conformational change by local sildenafil binding (short range allosteric effect).

Sildenafil orthosterically inhibits cGMP hydrolysis at the catalytic site, but allosterically increased the affinity of the GAFa domain for cGMP (Fig. 4B), giving rise to a “new function” for the GAFa domain to act as sink for cGMP (27, 42). Although the paradigm is to identify allosteric sites distinct from the catalytic site and target those sites for regulation of the function of the protein, in a multidomain protein such as PDE5, it appears that one can develop allosteric modulators that can bind to the catalytic site and alter the properties of a second domain in the protein.

FIGURE 8. Distinct allosteric effect induced by different ligands in PDE5. A and B, lysates prepared from cells expressing wild-type (A) or D612A (B) PDE5 sensor in buffer lacking EDTA were preincubated with 10 mM MgCl2 for 5 min at room temperature, followed by incubation with varying concentrations of sildenafil at 37 °C for 10 min. The percentage decrease in BRET is plotted. Lysates not preincubated with MgCl2 serve as controls. C, lysates prepared from cells expressing the PDE5 sensor in buffer lacking EDTA were incubated in the absence or presence of either 100 μM cGMP or 100 μM sildenafil along with 2 mM EDTA for 10 min at the indicated temperatures, and BRET was determined. D, lysates prepared from cells expressing the PDE5 sensor in buffer lacking EDTA were incubated in the absence or presence of either cGMP or sildenafil (100 μM each) for 10 min at the indicated temperatures, and BRET was determined. Plot of the ln of the ratio of change in BRET in the presence of cGMP or sildenafil to basal BRET versus 1/T (K−1) is shown. E, apparent activation energies for the conformational change in PDE5 induced by cGMP and sildenafil estimated from the slope of the Arrhenius plot shown in D. Data represent mean ± S.E. (error bars) from a representative experiment performed at least twice with duplicate determinations. F, apparent free energy, enthalpy, and entropy (−TΔS) changes in the activation for the conformational change induced by cGMP and sildenafil at 37 °C. Data represent mean ± S.E. of a representative experiment, with experiments performed at least twice, with duplicate determinations.
PDE5 could, however, alter activities (56). Cyclic GMP binding to the GAFa domain of the GAF domains of PDE10 and PDE11 does not alter their proteins (58) and/or its localization in the cell (59, 60). Alter the ability of PDE5 to form complexes with other phosphorylation by cGMP-dependent protein kinase (57), and therefore no reduction in BRET is observed. Sequential binding of sildenafil and cGMP, and vice versa, further changes the mean structure of the protein, which for simplicity, is not depicted in the figure. Metal ion binding to the catalytic site reverses the cGMP-bound low BRET conformers to ~basal BRET conformers. In contrast, metal ion binding to the catalytic site enhances sildenafil-induced reduction in BRET.

Point mutations (F778A and G617P) altered basal BRET of the PDE5 sensor (Figs. 3 and 4). This indicates that specific mutations can shift the conformational ensemble of PDE5. Interestingly, the ability of the F163A mutant to respond to sildenafil and the ability of F778A and G617P mutants to respond to cGMP suggest that cognate ligand binding and the subsequent conformational changes brought about in otherwise allosterically coupled domains of a protein are independent of mutations in the “other” domain.

The prevention of cGMP-induced conformational change in PDE5 in the presence of metal ions (Fig. 6) and similar kinetic properties of the wild-type and F163A mutant PDE5 (Fig. 1D) raise questions as to the role of cGMP in directly regulating PDE5 catalytic activity. Indeed, cGMP binding to the GAF domains of PDE10 and PDE11 does not alter their activities (56). Cyclic GMP binding to the GAFa domain of PDE5 could, however, alter in vivo stability, allow enhanced phosphorylation by cGMP-dependent protein kinase (57), and alter the ability of PDE5 to form complexes with other proteins (58) and/or its localization in the cell (59, 60).

In conclusion, we propose that cGMP, cAMP, Mg2+, Ca2+, and sildenafil can regulate PDE5 conformation and hence its activity in cells. The BRET sensor that we have developed allows us to suggest the following scheme of conformational changes that may occur in PDE5. An ensemble of conformational changes that may occur in PDE5 can exist, with a spectrum of BRET, and basal BRET being the average (Fig. 9). Cyclic GMP may selectively bind the low BRET conformers shifting the equilibrium toward reduced average BRET. Sildenafil induces conformational changes in the protein that require high activation energy, reflected in a reduction in BRET. A change in the mean structure of the protein occurs in both the cases. Metal ions binding to the catalytic site may alter the dynamics of PDE5 without causing any considerable change in the mean structure, and therefore no reduction in BRET is observed. Sequential binding of sildenafil and cGMP, and vice versa, further changes the mean structure of the protein, reflected in a reduced BRET. Metal ion binding to the catalytic site can revert the cGMP-bound low BRET conformers to ~basal BRET conformers. In contrast, metal ion binding to the catalytic site enhances sildenafil-induced reduction in BRET. We envisage the use of this sensor to gain further insight into the regulation of PDE5 conformation in cells that endogenously express PDE5 and for identifying allosteric modulators of PDE5 that regulate its activity, without giving rise to the sink-like property of the GAFa domain.

**REFERENCES**

1. Changeux, J. P., and Edelstein, S. J. (2005) Science 308, 1424–1428
2. Fenton, A. W. (2008) Trends Biochem. Sci. 33, 420–425
3. Cui, Q., and Edelstein, S. J. (2005) Science 308, 1424–1428
4. Hinzler-Wildman, K., and Kern, D. (2007) Nature 450, 964–972
5. Hammes, G. G., Chang, Y. C., and Oas, T. G. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 13737–13741
6. Hilsen, V. I. (2010) Science 327, 653–654
7. Cooper, A., and Dryden, D. T. (1984) Eur. Biophys. J. 11, 103–109
8. Tsai, C. J., del Sol, A., and Nussinov, R. (2008) J. Mol. Biol. 376, 1–11
9. Del Sol, A., Araujo-Bravo, M. J., Amaros, D., and Nussinov, R. (2007) Genome Biol. 8, R92
10. Kuriyan, J., and Eisenberg, D. (2007) Nature 450, 983–990
11. Conti, M., and Beavo, J. (2007) Annu. Rev. Biochem. 76, 481–511
12. Turko, I. V., Francis, S. H., and Corbin, J. D. (1998) Biochemistry 37, 4200–4205
13. Zhang, K. Y., Card, G. L., Suzuki, Y., Artis, D. R., Fong, D., Gillette, S., Hsieh, D., Neiman, J., West, B. L., Zhang, C., Milburn, M. V., Kim, S. H., Schlessinger, J., and Bollag, G. (2004) Mol. Cell 15, 279–286
14. Lin, C. S. (2004) Int. J. Impot. Res. 16, S8–S10
15. Rotella, D. P. (2002) Nat. Rev. Drug Discov. 1, 674–682
16. Galiè, N., Ghofrani, H. A., Torbicki, A., Barst, R. J., Rubin, L. J., Badesch, D., Fleming, T., Parpia, T., Burgess, G., Branzi, A., Grimmer, F., Kurzyna, M., and Simonneau, G. (2005) N. Engl. J. Med. 353, 2148–2157
17. Mullershausen, F., Friebe, A., Feil, R., Thompson, W. J., Hofmann, F., and Koesling, D. (2003) J. Cell Biol. 160, 719–727
18. Rybalkin, S. D., Rybalkina, I. G., Shimizu-Albergine, M., Tang, X. B., and Beavo, J. (2003) EMBO J. 22, 469–478
19. Okada, D., and Asakawa, S. (2002) Biochemistry 41, 9672–9679
20. Turko, I. V., Ballard, S. A., Francis, S. H., and Corbin, J. D. (1999) Mol. Pharmacol. 56, 124–130
21. Blount, M. A., Zoraghi, R., Bessay, E. P., Beasley, A., Francis, S. H., and Corbin, J. D. (2007) J. Pharmacol. Exp. Ther. 323, 730–737
22. Corbin, J. D., Corbin, J. D., Blount, M. A., Weeks, J. L., Beasley, A., Kuhn, K. P., Ho, Y. S., Saidi, L. F., Hurley, J. H., Koteria, J., and Francis, S. H. (2003) Mol. Pharmacol. 63, 1364–1372
23. Rybalkina, I. G., Tang, X. B., and Rybalkin, S. D. (2010) Mol. Pharmacol. 77, 670–677
24. Francis, S. H., Bessay, E. P., Koteria, J., Grimes, K. A., Liu, L., Thompson, W. J., and Corbin, J. D. (2002) J. Biol. Chem. 277, 47581–47587
25. Corbin, J. D., Zoraghi, R., and Francis, S. H. (2009) Cell. Signal. 21, 1768–1774
26. Giersch, L. M., and Gershenson, A. (2009) Nat. Chem. Biol. 5, 774–777
27. Biswas, K. H., Sopory, S., and Viswanawarhia, S. S. (2008) Biochemistry
**Allostery in PDE5**

47. Shenoy, A. R., and Visweswariah, S. S. (2003) *Anal. Biochem.* **319**, 335–336

28. Sopory, S., Kaur, T., and Visweswariah, S. S. (2004) *Cell. Signal.* **16**, 681–692

30. Bakre, M. M., Sopory, S., and Visweswariah, S. S. (2000) *J. Cell. Biochem.* **77**, 159–167

31. Bakre, M. M., Ghanekar, Y., and Visweswariah, S. S. (2000) *Eur. J. Biochem.* **267**, 179–187

29. Sopory, S., Kaur, T., and Visweswariah, S. S. (2004) *Cell. Signal.* **16**, 681–692

32. Lin, C. S., Lau, A., Tu, R., and Lue, T. F. (2000) *Biochem. Biophys. Res. Commun.* **268**, 628–635

33. Lin, C. S., Chow, S., Lau, A., Tu, R., and Lue, T. F. (2002) *Int. J. Impot. Res.* **14**, 15–24

34. Dacres, H., Dumancic, M. M., Horne, I., and Trowell, S. C. (2009) *Anal. Biochem.* **385**, 194–202

35. Heikaus, C. C., Stout, J. R., Sekharan, M. R., Eakin, C. M., Rajagopal, P., Brzovic, P. S., Beavo, J. A., and Klevit, R. E. (2008) *J. Biol. Chem.* **283**, 22749–22759

36. Zoraghi, R., Bessay, E. P., Corbin, J. D., and Francis, S. H. (2005) *J. Biol. Chem.* **280**, 12051–12063

37. Sopory, S., Balaji, S., Srinivasan, N., and Visweswariah, S. S. (2003) *FEBS Lett.* **539**, 161–166

38. Wang, H., Liu, Y., Huai, Q., Cai, J., Zoraghi, R., Francis, S. H., Corbin, J. D., Robinson, H., Xin, Z., Lin, G., and Ke, H. (2006) *J. Biol. Chem.* **281**, 21469–21479

39. Wang, H., Ye, M., Robinson, H., Francis, S. H., and Ke, H. (2008) *Mol. Pharmacol.* **73**, 104–110

40. Sung, B. J., Hwang, K. Y., Jeon, Y. H., Lee, J. I., Heo, Y. S., Kim, J. H., Moon, J., Yoon, J. M., Hyun, Y. L., Kim, E., Eum, S. J., Park, S. Y., Lee, J. O., Lee, T. G., Ro, S., and Cho, J. M. (2003) *Nature* **425**, 98–102

41. Pandit, J., Forman, M. D., Fennell, K. F., Dillman, K. S., and Menniti, F. S. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 18225–18230

42. Corbin, J. D., Kotera, J., Gopal, V. K., Cote, R. H., and Francis, S. H. (2003) in *Handbook of Cell Signaling* (Dennis, E. A., ed) pp. 465–470, Academic Press, Burlington, VT

43. Cann, M. (2007) *Mol. Microbiol.* **64**, 461–472

44. Francis, S. H., Colbran, J. L., McAllister-Lucas, L. M., and Corbin, J. D. (1994) *J. Biol. Chem.* **269**, 22477–22480

45. Francis, S. H., Turko, I. V., Grimes, K. A., and Corbin, J. D. (2000) *Biotechnology* **39**, 9591–9596

46. Hilser, V. J., and Thompson, E. B. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8311–8315

47. Martinez, S. E., Wu, A. Y., Glavas, N. A., Tang, X. B., Turley, S., Hol, W. G., and Beavo, J. A. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 13260–13265

48. Boehr, D. D., Mussinov, R., and Wright, P. E. (2009) *Nat. Chem. Biol.* **5**, 789–796

49. Sullivan, S. M., and Holyoak, T. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13829–13834

50. Wolf, F. I., and Trapani, V. (2008) *Clin. Sci.* **114**, 27–35

51. Yago, M. D., Manas, M., and Singh, J. (2000) *Front. Biosci.* **5**, D602–D618

52. Touyz, R. M. (2003) *Mol. Aspects Med.* **24**, 107–136

53. Francis, S. H., Morris, G. Z., and Corbin, J. D. (2008) *Int. J. Impot. Res.* **20**, 333–342

54. Rybalkin, S. D., Yan, C., Bornfeldt, K. E., and Beavo, J. A. (2003) *Circ. Res.* **93**, 280–291

55. Feng, M., Patel, D., Dervan, J. J., Ceska, T., Suck, D., Haq, I., and Sayers, J. R. (2004) *Nat. Struct. Mol. Biol.* **11**, 450–456

56. Matthiesen, K., and Nielsen, J. (2009) *Biochem. J.* **423**, 401–409

57. Corbin, J. D., Turko, I. V., Beasley, A., and Francis, S. H. (2000) *Eur. J. Biochem.* **267**, 2760–2767

58. Wilson, L. S., Elbatarny, H. S., Crawley, S. W., Bennett, B. M., and Maurice, D. H. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13650–13655

59. Dolci, S., Belmonte, A., Santone, R., Giorgi, M., Pellegrini, M., Carosa, E., Piccione, E., Lenzì, A., and Jannini, E. A. (2006) *Biochem. Biophys. Res. Commun.* **341**, 837–846

60. Francis, S. H., Zoraghi, R., Kotera, J., Ke, H., Bessay, E., Blount, M., and Corbin, J. D. (2006) in *Cyclic Nucleotide Phosphodiesterases in Health and Disease* (Houslay, M., ed) pp. 135–164, CRC Press, Boca Raton, FL