

Multiple Affinity States of cGMP-Specific Phosphodiesterase for Sildenafil Inhibition Defined by cGMP-Dependent and cGMP-Independent Mechanisms^[S]

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ABSTRACT

cGMP-specific phosphodiesterase (PDE5) has become a target for drug development for the treatment of a number of physiological dysfunctions, affected by changes in the cGMP/cGMP-dependent protein kinase (PKG) signaling pathway. PDE5 has two highly homologous regulatory domains, GAF-A and GAF-B. We showed previously that PDE5 could be converted from a low-activity (nonactivated) state to a high-activity state upon cGMP binding to the GAF-A domain with higher sensitivities toward sildenafil (*EMBO J* 22:469–478, 2003). Here we investigated whether sildenafil sensitivity of PDE5 could be modified by cGMP-independent mechanisms. Individually expressed recombinant GAF-A and GAF-B proteins were tested for their ability to modulate full-length recombinant PDE5 affinity to sildenafil. The GAF-A domain protein had the most dramatic effect on the affinity of the nonactivated recom-

binant PDE5 for sildenafil, revealing much higher sensitivity to sildenafil inhibition. The apparent affinity for sildenafil increased from the nanomolar range to the picomolar range, providing evidence for the presence of a “super-high” sensitivity state of PDE5 for sildenafil inhibition. In human platelet, higher sensitivity of PDE5 for sildenafil inhibition has been detected after blocking cGMP-binding sites of the GAF-A domain. Thus, our data demonstrate that high sensitivity of PDE5 for sildenafil can be obtained not only through cGMP-induced activation of PDE, but also through cGMP-independent modulation of PDE5 in the nonactivated state, possibly through protein-protein interaction. Furthermore, data suggest that nonactivated PDE5 with “super-high” affinities for sildenafil inhibition may be responsible for therapeutic effects of long-term treatments with low doses of PDE5 inhibitors.

The cGMP/cGMP-dependent protein kinase (PKG) signaling pathway plays important roles in many physiological processes in the cardiovascular, immune, and nervous systems (Hofmann et al., 2009). The components for this pathway are well established and include NO/natriuretic peptides that induce stimulation of cGMP synthesis by guanylyl cyclase, followed by activation of cGMP-dependent protein kinase (PKG) and phosphorylation of a number of target proteins (Schlossmann and Desch, 2009). The intracellular level of cGMP is controlled by cGMP-hydrolyzing PDEs, and PDE5 is a major cGMP PDE expressed in all types of smooth muscle and platelets.

PDE5 became a target for drug development for a variety of physiological disorders, and in 1998, sildenafil (Viagra;

Pfizer Inc., New York, NY) was the first PDE5-specific inhibitor approved for the treatment of erectile dysfunction (Ballard et al., 1998). Several other PDE5-specific inhibitors, such as tadalafil (Cialis; Eli Lilly & Co., Indianapolis, IN) and vardenafil (Levitra; Bayer HealthCare Pharmaceuticals, Wayne, NJ), also have been shown to selectively inhibit PDE5 activity and are available for clinical use (Rotella, 2002). In 2005, sildenafil (Revatio; Pfizer) has been approved for the treatment of pulmonary arterial hypertension (Ghofrani et al., 2006). At present, PDE5 inhibitors are under clinical investigation for the treatments of a number pathological conditions, including lower urinary tract symptoms, cardiac and gastrointestinal disorders, endothelial dysfunction, and Raynaud's disease (Sandner et al., 2007). Many of these pathologies are characterized by vascular deficiencies, and PDE5 inhibitors are likely candidates for the modulation of NO/PKG-controlled smooth muscle tone. Recent reports that sildenafil could improve cardiac functions provided the basis for the initiation of the National Institutes of Health trial of sildenafil for treating heart failure (Takimoto et al., 2005, 2009).

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ABBREVIATIONS: PKG, cGMP-dependent protein kinase; PDE5, cGMP-specific phosphodiesterase; HEK, human embryonic kidney; aa, amino acid; mAb, monoclonal antibody; MOPS, 3-(N-morpholino)propanesulfonic acid; ED, erectile dysfunction.

The physiological importance of PDE5 in clinical applications underscores the importance of understanding the mechanisms of regulation of PDE5 activity. cGMP-specific PDE5 contains two homologous N-terminal GAF domains, GAF-A and GAF-B. These domains belong to a functionally and evolutionary diverse group of proteins found in prokaryotes and eukaryotes. The GAF domains were originally described in cGMP-regulated phosphodiesterases, *Anabaena* adenylyl cyclases, and a bacterial transcription factor FhlA (Aravind and Ponting, 1997). At present, more than 7000 GAF domain-containing proteins have been found, and these proteins may contain a single, tandem, and even multidomain GAF domains (Schultz, 2009).

The binding and regulatory abilities of the tandem GAF domains vary greatly from binding cyclic nucleotides (mammalian PDEs and cyanobacterial adenylyl cyclases) to binding formate for *Escherichia coli* FhlA or haem for the DosS GAF domain of *Mycobacterium tuberculosis*. Although GAF-A and GAF-B domains share a significant sequence homology, they seem to have different binding functions. For example, the GAF-A but not GAF-B domain of PDE5 binds cGMP, whereas in PDE2, the GAF-B domain is the only cGMP-binding domain. However, despite their diversity, all tandem-containing GAF domains in prokaryotes and eukaryotes follow the same domain architecture, such that a GAF-A domain is followed by a GAF-B domain at the N-terminal of the protein. This evolutionary-preserved domain organization suggests that both domains are essential for proper protein function, which may not be limited to just their ligand binding properties.

A new approach to study GAF domain regulation of enzyme activity was developed by creating chimeric constructs between GAF domains of several PDEs and the catalytic domain of cyanobacterial adenylyl cyclases, used as a reporter enzyme. The advantage of these constructs was that they allowed easy monitoring of cGMP/GAF domain-induced signaling through changes in adenylate cyclase activity (Kancher et al., 2002). Human PDE5 GAF-AB domain chimeras showed significant activation by cGMP (Bruder et al., 2006). However, constructs containing either GAF-A or GAF-B PDE5 were unable to respond to cGMP stimulation (Hofbauer et al., 2008), suggesting that both GAF-A and GAF-B domains are necessary for cGMP/GAF domain PDE5 signaling.

We have demonstrated previously that cGMP binding to the GAF-A domain is necessary and sufficient to achieve full activation of PDE5, and PDE5 in nonactivated and cGMP-activated states differs significantly in its catalytic and inhibitory properties (Rybalkin et al., 2003). Here we investigated whether sildenafil affinity of PDE5 could be modified by cGMP/GAF-A-independent mechanisms and showed that PDE5 contains a super-high-affinity site for sildenafil inhibition.

Materials and Methods

Reagents. Pepstatin, leupeptin, and Nonidet P-40 were from Roche Molecular Biochemicals (Indianapolis, IN). SuperSignal West Pico Chemiluminescent Substrate, AminoLink kit, and Coomassie Plus protein assay were from Thermo Fisher Scientific (Waltham, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgGs and goat anti-mouse IgGs were from Bio-Rad Laboratories (Hercules, CA). Protein G-agarose beads were from Calbiochem (San Diego, CA). Talon Metal Affinity Resin was from Clontech (Mountain View, CA). QIAGEN

plasmid maxi kit was from QIAGEN (Valencia, CA). BenchMark prestained protein standard, Lipofectamine 2000, pcDNA3 vector, Dulbecco's modified Eagle's medium, and SilverXpress kit were from Invitrogen (Carlsbad, CA). [3 H]cGMP was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Mono Q anion exchange column HR 5/5 and Ficol-Paque Plus were from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA). Buffy coats, fractions of blood containing mostly white blood cells and platelets, were obtained from the American Red Cross Blood Bank (Portland, OR). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfection. HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin on 100-mm plates. Mouse PDE5A1 (GenBank accession number AF541937), subcloned into pcDNA3 vector, was used for transfection of HEK 293 cells by the Lipofectamine 2000 method. Cells were harvested after 2 days and lysed in the homogenization buffer, containing 50 mM Tris-HCl, pH 7.5, 2.0 mM EDTA, 1 mM dithiothreitol, 10 mg/ml aprotinin, 5 mg/ml pepstatin, 20 mg/ml leupeptin, 1 mM benzamide, and 0.2 mM sodium vanadate. The cell extract was centrifuged at 230,000g for 20 min, and after appropriate dilutions, the supernatant was used to assay PDE5 catalytic activity.

Expression of PDE5 GAF Domain Proteins. All recombinant proteins of GAF A (125–320 aa) and GAF A domain with the N-terminal end (1–320 aa), GAF B (334–525 aa), and GAF (A + B) (125–539 aa) were expressed in *E. coli* with polyhistidine tags and purified on a Talon Metal Affinity Resin. GAF proteins were analyzed by Western blotting and were silver-stained using SilverXpress kit. Only freshly purified proteins or proteins frozen at -80°C in small aliquots were used to test their ability to modulate the catalytic and inhibitory properties of full-length PDE5.

Antibody Characterization and Production. Mouse monoclonal antibodies (mAbs) were generated using recombinant protein (125–539 aa of bovine PDE5A) as described previously (Rybalkin et al., 2002). The hybridomas were used as a source of monoclonal antibodies and tested for their ability to specifically detect the GAF-A and GAF-B domain protein. Rabbit polyclonal PDE5 antibodies were raised against a glutathione transferase-fusion protein from the C-terminal part of mouse PDE5 (775–865 aa) and were affinity-purified using an AminoLink kit.

Immunoprecipitation and Western Blot Analysis. Protein samples were incubated with different PDE5 antibodies in the immunoprecipitation buffer containing 0.1% Nonidet P-40 and 0.1 mM NaCl in the homogenization buffer. The immunopellets were washed three times in the immunoprecipitation buffer and then boiled for 5 min in SDS sample buffer.

Samples were loaded onto a SDS-polyacrylamide gel (8% acrylamide/0.21% bisacrylamide) and electrophoresed. After transferring proteins onto nitrocellulose, the membranes were immunostained with PDE5-specific antibodies. Immunoreactivity was detected by enhanced chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit IgGs or goat anti-mouse IgGs and SuperSignal West Pico Chemiluminescent Substrate.

Isolation of Human Platelets. Human platelets were isolated from buffy coats by density gradient centrifugation using Ficol-Paque Plus. Platelets were resuspended in incubation buffer containing 137 mM NaCl, 2.7 mM KCl, 0.77 mM NaH_2PO_4 , 5 mM glucose, and 2.0 mM MgCl_2 , pH 7.4. Platelets were lysed by sonication in the homogenization buffer described above. The cell extract was centrifuged at 230,000g for 20 min.

Activation of Human Platelet PDE5 by cGMP in Vitro. PDE5 was immunoprecipitated from platelet extract in the immunoprecipitation buffer. The immunopellets were preincubated without or with 20 μM cGMP on ice for 30 min, followed by two washes (3000g, 3 min) in the homogenization buffer. After the final wash, PDE5 activity was measured at 0.1 μM cGMP.

PDE Assays and Protein Determinations. Phosphodiesterase assays were conducted according established procedures (Rybalkin et al., 2003). Assays were performed for 5 to 10 min at 30°C using different concentrations of cGMP (0.025–1.0 μ M) as substrate. The total reaction volume (125 μ L) contained 40 mM MOPS, pH 7.5, 2.0 mM EGTA, 15 mM magnesium acetate, 0.2 mg/ml bovine serum albumin, and 50,000 cpm [3 H]cGMP. IC_{50} values were calculated using Prism 2.0 C (GraphPad Software Inc., San Diego, CA). Protein concentrations were measured by the Coomassie Plus protein assay.

Results

Analysis of PDE5 kinetic and inhibitory properties has been complicated by the fact that cGMP plays a double role as a substrate for the catalytic domain and as an allosteric activator for the cGMP binding sites of the GAF domain, and the presence of several conformational states for PDE5 has been proposed. For example, it has been suggested that PDE5 could exist in at least two different conformational states: a nonactivated state with relatively low intrinsic catalytic activity and low affinity for sildenafil inhibition; and an activated state formed upon cGMP binding to the GAF-A domain with a higher sensitivity toward sildenafil (Rybalkin et al., 2003). The IC_{50} for sildenafil inhibition changed from 2.1 to 0.63 nM for nonactivated to cGMP-activated PDE5 correspondingly, when PDE5 activity was assayed at 0.1 μ M cGMP.

We also measured sildenafil inhibition for PDE5 in these two states at lower substrate concentration, 0.025 μ M (Fig. 1A). This cGMP concentration is supposed to be closer to the physiological condition present in unstimulated corpus cavernosum, estimated to be 18 nM (Gopal et al., 2001). We found that at 0.025 μ M cGMP, the sildenafil inhibition

curves were better fit not by a one-site model but by a two-site model, demonstrating the presence of two affinity states for sildenafil inhibition for cGMP-activated PDE5.

We noted that diluted samples of recombinant PDE5, if not assayed immediately after dilution, could quickly lose their high-affinity sites for sildenafil inhibition, even diluted in the presence of 1 mg/ml albumin, whereas concentrated samples (lysates) of recombinant PDE5 could be used for at least 1 day and still show differential responses to sildenafil inhibition for nonactivated and cGMP-activated PDE5. Still, these samples would also lose their high-affinity sites (Fig. 1B), and then their inhibitory curves could be described again by the typical sigmoidal dose-response curve, which could stay unchanged for a much longer period of time. These data suggest that concentrated samples of PDE5 could better preserve PDE5 in the conformation state favorable for high-affinity sites for sildenafil inhibition.

We also wondered whether PDE5 could have a different affinity for sildenafil in the diluted and concentrated samples, perhaps because of protein interactions between PDE5 molecules. In the present study, we used purified GAF domains as a tool to analyze the effect of sildenafil inhibition on fresh concentrated samples of PDE5. To achieve linearity, PDE assays typically require serial dilution of samples to achieve hydrolysis no higher than 10 to 20% of a substrate. Taking advantage of the fact that isolated GAF domains have no catalytic activity, we measured sildenafil inhibitory potency of full-length PDE5 in the presence of GAF domain proteins, creating conditions likely to resemble high concentrations of PDE5 observed *in vivo* but still be acceptable for PDE assay analysis. Therefore, we hypothesized that PDE5 might exist in the cell in a state that had different kinetic properties than those seen in the very high dilution states normally required for PDE activity analysis.

First, we used individually expressed GAF domain proteins to investigate whether any protein-protein interactions between GAF domains were possible by performing coimmunoprecipitation analysis. For this purpose, we developed a number of GAF domain-specific antibodies for immunoprecipitation and Western blotting analysis (Supplemental Fig. 1). Several protein constructs have been used for coimmunoprecipitation experiments, including the GAF-A domain with and without the N-terminal end (1–320 and 125–320 aa), the GAF-B (334–525 aa), and the tandem GAF-AB (125–539 aa).

Protein/Protein Interaction of GAF Domain Proteins *in Vitro*. We found that incubation of purified GAF-A (2 μ M) domain protein and GAF-B domain (2 μ M) protein for 1 h on ice could result in forming a protein complex, detected by coimmunoprecipitation, when either mAb/P4A9 or mAb/P3B2 was used for immunoprecipitation (Fig. 2A). GAF-B-specific mAb was used to identify GAF-B in the immunopellet by Western blotting.

Decreasing the concentration of the GAF-B protein (0.2 μ M) (the molar ratio 1:0.1 for GAF-A/GAF-B) in the incubation mixture significantly reduced the amount of GAF-B coimmunoprecipitated with the GAF-A domain and increased the concentration of GAF-B (20 μ M) (molar ratio 1:10) produced the same results as with 2 μ M GAF-B (Fig. 2A), suggesting that saturation of binding was achieved when equal concentrations of GAF-A and GAF-B (molar ratio 1:1) were used.

GAF-A and GAF-B protein-protein interactions were not cGMP-dependent. The addition of cGMP (10–100 μ M) to the

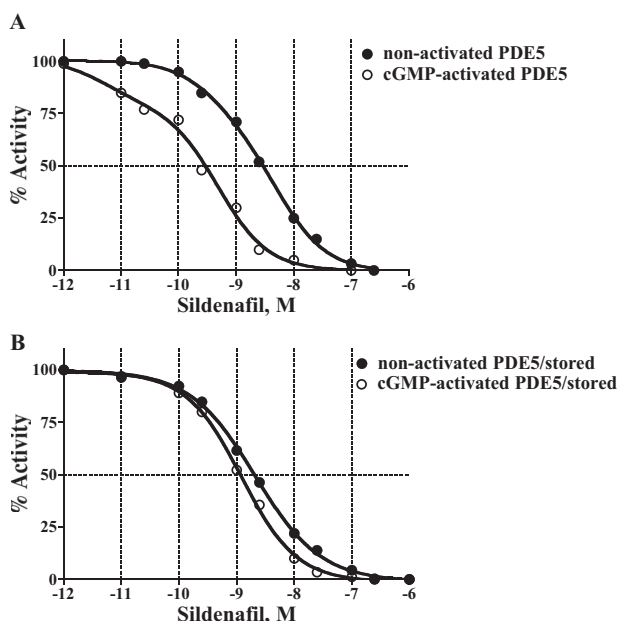


Fig. 1. The difference in sildenafil sensitivity between nonactivated and cGMP-activated PDE5 is observed only in freshly prepared samples. A, recombinant full-length PDE5 was analyzed immediately after preparing a cell lysate from the transfected cells at 0.025 μ M cGMP. B, the potency of sildenafil to inhibit PDE5 diminishes upon storage of concentrated samples at 4°C, and after 1 day, no differences between nonactivated and cGMP-activated PDE5 was detected. Inhibitory curves were fitted to either a one- or a two-site competition equation using GraphPad Prism 2.0C.

incubation mixture did not affect the binding pattern for coimmunoprecipitation by mAb/P4A9 (Fig. 2B). Moreover, mAb/P3B2, which was also used for these coimmunoprecipitation experiments, was found to completely block cGMP binding to the GAF-A domain, even at high concentrations of cGMP (100 μ M), thus preserving the GAF-A domain into a nonbinding state. Thus, GAF domain interactions were not affected whether cGMP-binding sites at the GAF-A domain were occupied by cGMP or not.

We also tested whether other GAF-A containing constructs, such as the tandem GAF-AB (125–539 aa) and the GAF-B domain protein, could form a protein complex. As shown in Fig. 2C, GAF-AB and GAF-B were also coimmunoprecipitated, confirming physical interaction between the GAF-A-containing domain and the GAF-B domains *in vitro*.

For these experiments, GAF domain proteins, which were purified on a Talon Affinity Resin, were used immediately after purification or frozen in aliquots at -80°C and used only once after thawing. Purified GAF domain proteins (especially the GAF-B protein) stored at 4°C could quickly lose their ability to interact with other GAF domains.

GAF-A and GAF-B Domain Protein-Protein Functions and Their Effects on the Regulation of PDE5.

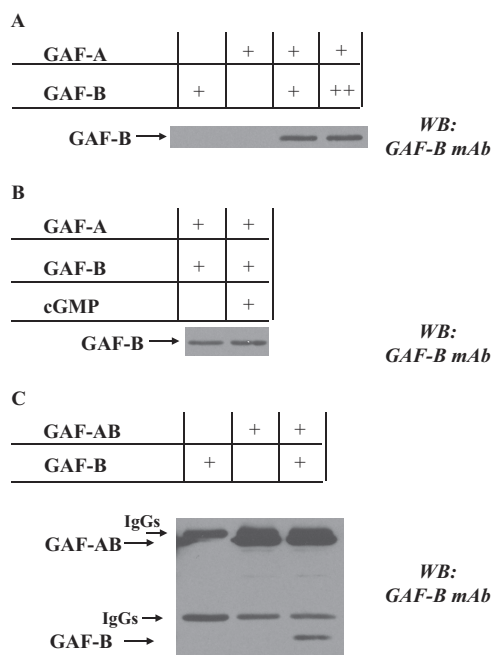


Fig. 2. Direct interaction of the GAF domain proteins analyzed by coimmunoprecipitation. **A**, coimmunoprecipitation of purified GAF-B with GAF-A domain protein. GAF-A (2 μ M) was preincubated with 2 μ M GAF-B (+) at molar ratio 1:1 or with 20 μ M GAF-B (++) at molar ratio 10:1 on ice for 1 h in the homogenization buffer. GAF-A-specific mAbs (mAb/P3B2 or mAb/P4A9) were used for coimmunoprecipitation with similar results. Immunoprecipitation buffer contained 0.1 M NaCl, 1 mg/ml bovine serum albumin, and 0.1% Nonidet P-40. After performing SDS-PAGE, GAF-B domain protein was detected by Western blotting with the GAF-B domain-specific mAb. **B**, coimmunoprecipitation of purified GAF-B with GAF-A domain proteins was performed as described above in the presence of 100 μ M cGMP, showing cGMP binding to the GAF-A domain did not change the coimmunoprecipitation pattern. **C**, coimmunoprecipitation of purified GAF-B with GAF-AB domain protein. GAF-AB (2 μ M) (125–539 aa) domain protein was preincubated with 2 μ M GAF-B on ice for 1 h and analyzed using the same conditions as above. Light- and heavy-chain IgGs are visible on the blot because mouse monoclonal antibodies were used for immunoprecipitation and Western blotting.

Because we found that *in vitro* GAF-A and GAF-B domains demonstrated interdomain binding capabilities, we investigated whether GAF domains could affect any inhibitory properties of the full-length recombinant PDE5. We hypothesized that interactions between GAF domain proteins and full-length PDE5 would be dependent on the conformation status of PDE5, providing or preventing access to the binding interfaces. Moreover, these experimental settings for probing protein-protein interactions between PDE5 and GAF domain proteins could imitate conditions that could occur between PDE5 molecules at high concentrations of PDE5.

Here we analyzed the effects of individually expressed GAF-A, GAF-B, and GAF-AB domain proteins on the inhibitory properties of full-length PDE5 in the nonactivated and cGMP-activated states, assayed at 0.025 μ M cGMP. We found that the most dramatic changes in the sildenafil sensitivity occurred for nonactivated PDE5 (Fig. 3A). The whole pattern for sildenafil inhibition moved sharply to the left for this form of enzyme when it was preincubated with either the GAF-A or GAF-AB protein. Because the GAF-A and the GAF-AB proteins showed very similar responses, it is likely that in these experiments, the GAF-A domain was largely responsible for this shift in the sildenafil inhibitory profile. Moreover, the inhibitory curve could no longer be described by the typical sigmoidal dose-response curve for one-site model and was better fit for a two-site model, revealing the presence of two affinity states for sildenafil inhibition, one of which contains a super-high-affinity site—in the low picomolar range of concentrations—for sildenafil inhibition (IC_{50} values of 2.9 nM and 1.7 pM).

We also performed coimmunoprecipitation experiments to test whether under our experimental conditions any interactions between individual GAF-A domain protein and the full-length PDE5 could be found. PDE5 in the nonactivated state was preincubated with the GAF-A domain protein and then immunoprecipitated using a C-terminal polyclonal PDE5 antibody. We found the presence of the GAF-A domain protein in the immunopellet by using GAF-A-specific mAb (Fig. 3B). Using a combination of GAF-AB and the full-length PDE5 under the same experimental conditions also resulted in their coimmunoprecipitation, suggesting that changes in the inhibitory properties of PDE5 in the nonactivated state after the addition of the GAF-A domain (or GAF-AB) protein could be the result of protein-protein interactions.

The addition of the GAF-B domain together with the GAF-A domain reversed the effect of GAF-A on sildenafil sensitivity of full-length PDE5 (Fig. 3C). This suggests that binding competition between the GAF-A and GAF-B domain proteins prevented the development of any GAF-A domain-induced effects on sildenafil potency for PDE5.

The sildenafil inhibitory profiles of PDE5 in the cGMP-activated state did not undergo any significant changes when either GAF-A, GAF-B, or GAF-AB domain proteins were used (Fig. 4A). Under all treatments, nearly similar inhibitory curves were obtained. A combined figure (Fig. 4B) shows differences for nonactivated and cGMP-activated PDE5 in their responses to the addition of GAF domain proteins. Thus, our data suggest that super-high-affinity sites for sildenafil inhibition for nonactivated PDE5 could be revealed in a cGMP-independent manner, most likely through GAF domain interactions.

Multiple Sildenafil Inhibitory Sites in Human Platelet PDE5. PDE5 is expressed in human platelets at a very high concentration. The reported IC_{50} value for sildenafil inhibition of platelet PDE5 seems higher than IC_{50} values for PDE5 from any smooth muscle cells. We determined that the IC_{50} of human aortic smooth muscle cells was 3 nM, whereas the value for PDE5 from human platelets was 10 nM (Fig. 5A). These values were similar to the IC_{50} data we reported for recombinant mouse PDE5 in the nonactivated state when assayed at 1.0 μ M cGMP (Rybalkin et al., 2003). It has also been shown that platelet PDE5 is able to regulate the NO/

cGMP signaling response through its phosphorylation by PKG and direct activation by cGMP (Mullershausen et al., 2003). A 2-fold increase in cGMP-induced activation of PDE5 has been detected in PKG-depleted platelet lysates. The importance of compartmentalization of PDE5 in human platelets has been shown, and it was suggested that distinct pools of cGMP could be selectively regulated by cGMP/PKG-activated PDE5 (Wilson et al., 2008).

Here we tested whether PDE5 from human platelets could be a source of nonactivated PDE5 and investigated whether its sildenafil inhibitory sites could be modulated by cGMP/GAF-A-dependent and independent mechanisms. To show that PDE5 in human platelets is activated by cGMP, we immunoprecipitated PDE5 from platelets lysates using mAb/P4A9 and preincubated PDE5 immunopellets without and with 20 μ M cGMP followed by two washes to remove excess cGMP. Under these experimental conditions, we could detect up to 3-fold activation of platelet PDE5 activity by cGMP (Fig. 5B). Phosphospecific PDE5 antibody did not show any phosphorylation of PDE5 in the immunopellets after preincubation with cGMP, indicating that activation of PDE5 was not due to phosphorylation (data not shown). Although

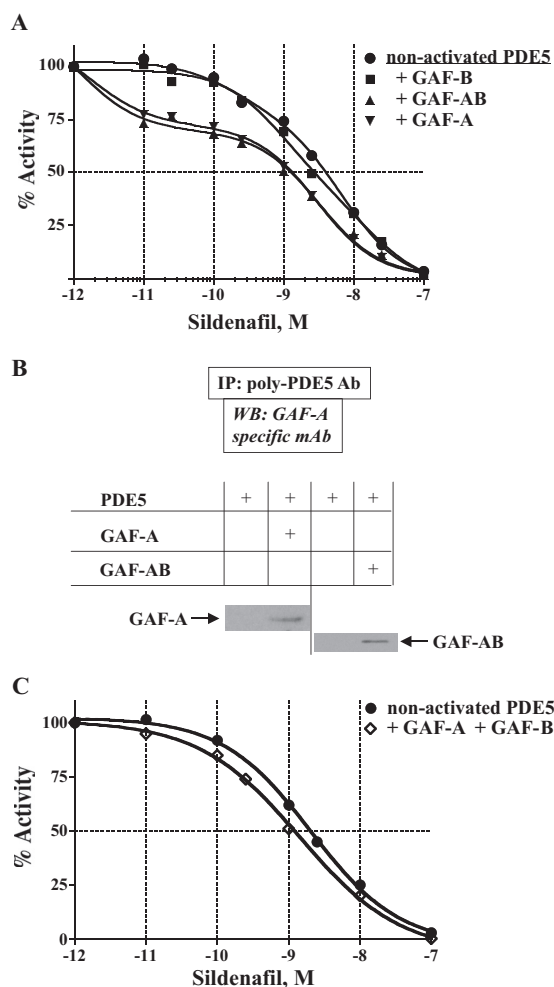


Fig. 3. Interactions between the GAF-A domain containing proteins and full-length PDE5 in the nonactivated state lead to significant increases in affinity for sildenafil inhibition. A, recombinant full-length PDE5 was preincubated with 1 μ M purified GAF-A (125–320 aa), GAF-B (334–525 aa), or GAF-AB (125–539 aa) domain proteins for 1 h on ice. PDE5 activity was measured immediately after preincubation in the presence of different concentrations of sildenafil. Inhibitory curves of sildenafil inhibition analysis of PDE5 in the nonactivated state were fitted to either a one- or two-site competition equation using GraphPad Prism 2.0C. Similar results were obtained when the concentrations of the GAF domain proteins used in the preincubation step were 10 μ M. B, coimmunoprecipitation of PDE5 and GAF-A domain protein after their preincubation under the same condition as in A reveals the presence of the GAF-A domain protein in the complex with the full-length PDE5. Polyclonal C-terminal PDE5 Abs were used for immunoprecipitation and GAF-A-specific mAb for Western blotting. C, preincubation of full-length PDE5 with both GAF (1 μ M) and GAF-B (5 μ M) domain proteins prevented changes in sildenafil sensitivity of PDE5, which could be induced by GAF-A domain protein alone. PDE5 activity was measured and analyzed as described in A.

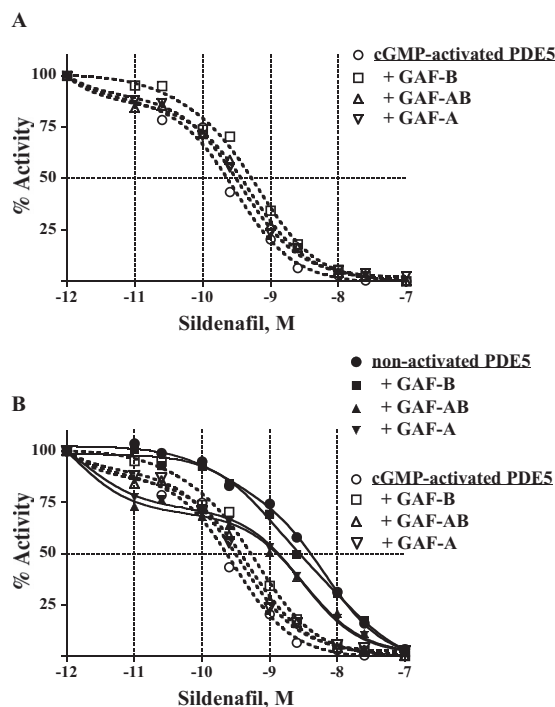


Fig. 4. GAF domain proteins do not change affinity for sildenafil inhibition for PDE5 in the cGMP-activated state. A, recombinant PDE5 was preincubated with 1 μ M purified GAF-A (125–320 aa), GAF-B (334–525 aa), or GAF-AB (125–539 aa) domain proteins for 1 h on ice. To convert nonactivated PDE5 to the cGMP-activated state, 50 μ M cGMP was added. After 30-min additional preincubation on ice, the samples were diluted, and PDE5 activity was measured in the presence of different concentrations of sildenafil. Using 10 μ M concentration of the same GAF domain proteins produced similar results. PDE5 activities of the control samples for cGMP-activated PDE5 were 0.14 pmol/min/ μ g protein and 0.013 pmol/min/mg protein for nonactivated PDE5, measured at 0.025 μ M cGMP. B, data from A, above, and from Fig. 3A are shown in the same graph to illustrate different responses in sildenafil sensitivity for PDE5 in the nonactivated and cGMP-activated states. All inhibitor curves were obtained in the same experiment and are presented as a representative experiment. At least three more experiments produced similar results. Inhibitory curves were fitted to either a one- or two-site competition equation using GraphPad Prism 2.0C.

cGMP-induced PDE5 activation in human platelets is less than the 10-fold activation by cGMP observed for recombinant PDE5, it was possible for the first time for a native PDE5 to show differences in sildenafil sensitivities between nonactivated ($IC_{50} = 5.0$ nM) and cGMP-activated PDE5 ($IC_{50} = 1.6$ nM), when assayed at $0.1 \mu\text{M}$ cGMP (Fig. 5C).

Because we found that treatments of mouse PDE5 with mAb/P3B2 could preserve the enzyme in the nonactivated state by blocking the cGMP binding sites, we treated samples of human platelet PDE5 with mAb/P3B2, thus preventing any interactions with cGMP binding sites of the GAF-A domain (Fig. 6A). We found that low-affinity sites, which could be attributed to nonactivated PDE5, did not change; however, the appearance of high-affinity inhibitory sites for sil-

denafil was detected. Similar biphasic inhibitory curves and similar conversion of nonactivated PDE5 into a state with high affinities for sildenafil inhibition were also obtained when recombinant PDE5 in the nonactivated state was treated with mAb/P3B2 under the same conditions (Fig. 6B), also suggesting that cGMP binding sites in the nonbound state are involved in the formation of nonactivated conformation of PDE5 with low affinity for sildenafil. mAb/P3B2 may block that region from any interaction, thus preserving PDE5 in the nonactivated state but with high affinity for sildenafil. These effects seem specific, because another PDE5 mAb (mAb/P4A9), which does not bind to the cGMP-binding sites of the GAF-A domain, was not effective in changing sildenafil inhibitory profiles (data not shown).

These data suggest that nonactivated platelet PDE5 and recombinant PDE5 could also be present in at least two different states, characterized by low and high affinities for sildenafil, and these states can be modulated independently. In these experiments, the high-affinity sites for sildenafil were found when cGMP binding sites of the GAF-A domain were blocked, demonstrating a cGMP/GAF-A-independent mechanism of regulation.

Discussion

This study demonstrates that a super-high-affinity site for sildenafil inhibition can be present on PDE5. Our data suggest that nonactivated PDE5 is able to adopt two different states, characterized by nanomolar and picomolar affinities for sildenafil inhibition (Fig. 7). In our experiments, the equilibrium between these two states was shifted by the addition

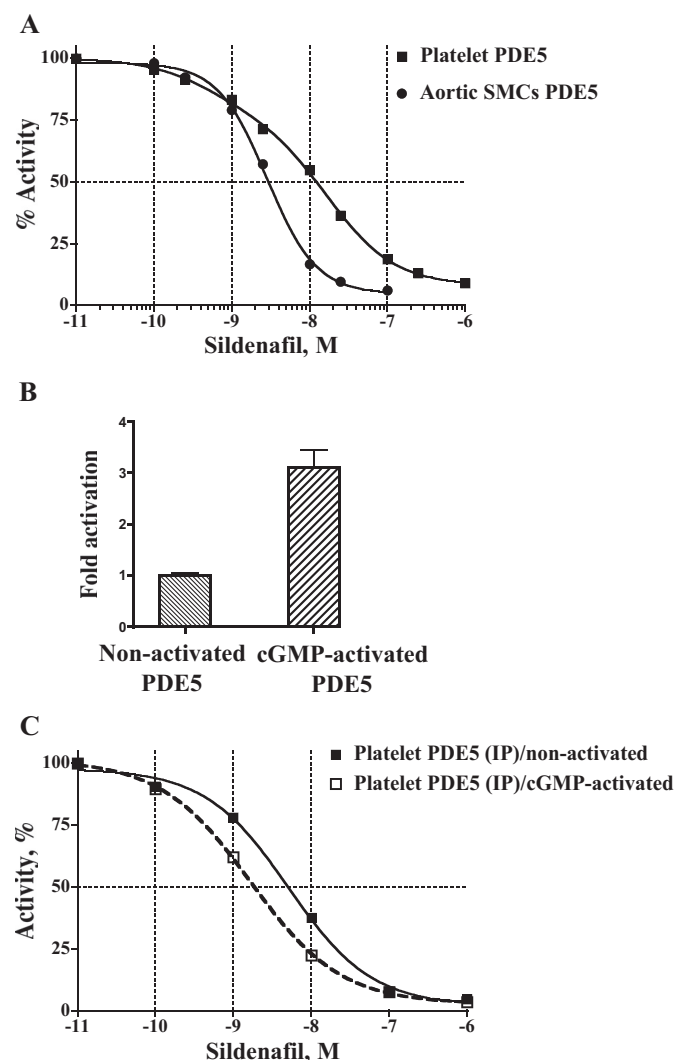


Fig. 5. Human platelet PDE5 is directly activated by cGMP and shows differential affinities for sildenafil inhibition. A, PDE5 from human platelet and PDE5 from cultured human aortic smooth muscle cells were immunoprecipitated, and their activities were analyzed in the presence of different concentrations of sildenafil. PDE5 activity was assayed at $1 \mu\text{M}$ cGMP. B, human platelet PDE5 is directly activated by cGMP. PDE5 was immunoprecipitated with mAb/P4A9. The immunopellet was preincubated in the homogenization buffer without and with $20 \mu\text{M}$ cGMP for 30 min on ice, followed by two washes with the homogenization buffer of 1 ml each using quick, low-speed centrifugation. PDE5 activity was assayed at $0.1 \mu\text{M}$ cGMP. C, nonactivated and cGMP-activated PDE5 were obtained as described above and analyzed in the presence of different concentrations of sildenafil at $0.1 \mu\text{M}$ cGMP.

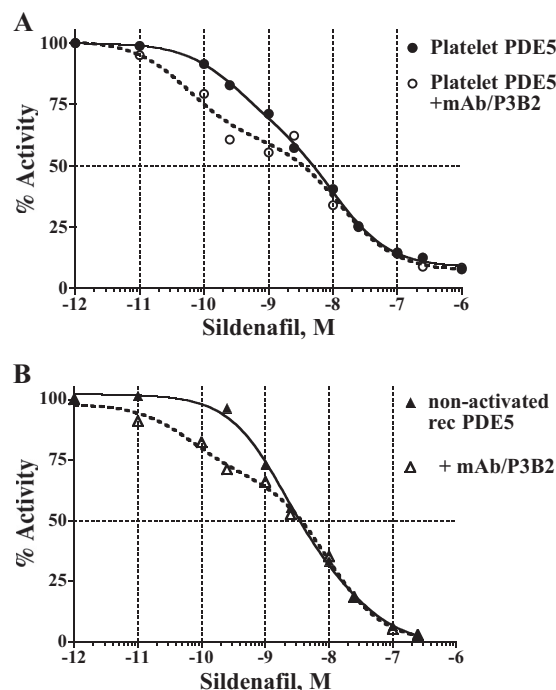


Fig. 6. Blocking cGMP binding site of PDE5 in the nonactivated state with mAb/P3B2 reveals high-affinity sites for sildenafil inhibition. A and B, human platelet PDE5 (A) and recombinant PDE5 (B) were preincubated with mAb/P3B2 on ice for 30 min. PDE5 activity was measured at $0.1 \mu\text{M}$ cGMP in the presence of different concentrations of sildenafil. Inhibitory curves were fitted to either a one- or two-site competition equation using GraphPad Prism 2.0C. Data shown are representative of at least three experiments.

of the GAF-A domain protein or by blocking the cGMP binding sites with mAb/P3B2, revealing super- high-affinity sites for sildenafil inhibition. In both cases, PDE5 remains in the less catalytically active state (i.e., with low cGMP hydrolytic activity).

Thus it is possible to suggest that the protein-protein interactions observed in our experiments at high GAF-A protein concentrations between the nonactivated PDE5 and GAF domain proteins may emulate the normal physiological situations in tissues as lung or corpus cavernosum, in which high concentrations of PDE5 exist, probably creating protein clusters or oligomeric formations between different PDE5 molecules.

The presence of two different conformation states of PDE5 has been suggested for the full-length PDE5 and its catalytic domain based on the analysis of exchange-dissociation kinetics of [^3H]sildenafil and unlabeled sildenafil (Corbin et al., 2003; Blount et al., 2004). These studies showed two kinetic components (slow and fast), and for sildenafil, two calculated K_d values have been determined: 14.7 and 0.7 nM. This method was also applied for two other PDE5 inhibitors (vardenafil and tadalafil). The ratio between fast and slow components varied between PDE5 inhibitors and could be changed by long preincubation with PDE5 inhibitors (Blount et al., 2007). For example, 12-h preincubation with 30 nM tadalafil resulted in detection of only the slow (high-affinity) component for full-length PDE5 and for a construct containing GAF-B and the catalytic domain but not for the catalytic domain alone.

The determination of conformation states of PDE5 would be more straightforward if crystal structures of PDE5 in the liganded and nonliganded states were available. However, at present, there are no crystals of full-length PDE5 but only a number of crystal structures of the PDE5 catalytic domain, including complexes without and with several PDE inhibitors (3-isobutyl-1-methylxanthine, sildenafil) (Huai et al.,

2004; Zhang et al., 2004; Wang et al., 2006). Although there are certain sequence homologies between the catalytic domains of different PDEs, crystal data analysis showed that contrary to other PDEs, the catalytic site of PDE5 could have four different conformations depending on different conditions, such as the presence and absence of inhibitors and also their type. For example, sildenafil produced significant changes in secondary and tertiary structures through H-loop conversions distinct from other inhibitors. It is likely that additional conformations might be discovered, because cGMP binding to the GAF domain can induce changes in the catalytic domain, resulting in higher catalytic activity and higher sensitivity for sildenafil.

So far, no crystals of the GAF domain of PDE5 have been produced, and only several crystal structures of the tandem GAF domains and separate GAF domains from PDE2A, PDE6C, and PDE10 and the cyanobacterial tandem GAF domains from the cyanobacterial adenylyl cyclase-2 have been reported (Martinez et al., 2002, 2005, 2008; Handa et al., 2008). In all, GAF domain crystal structures ligands, such as cGMP for PDE2 and PDE6 or cAMP for cyanobacterial adenylyl cyclase-2, were buried deeply inside their binding pockets, indicating that these structures presented only one type of conformation when GAF domain was in a ligand-bound state. However, no crystal structures of the GAF domain of PDE5 in the unbound state have been obtained. The reasons for that may include GAF domain instability without cGMP bound. NMR data showed that GAF-A domain of PDE5 was quite flexible in the unbound state, and cGMP binding was needed to produce large stable conformational change (Heikaus et al., 2008).

To solve these problems, in some cases, proteins were crystallized with appropriate antibodies to provide a stabilization effect for the crystallization. Because we found that mAb/P3B2 could bind to the GAF-A domain, block cGMP binding, and preserve PDE5 in a nonactivated state, we believe that mAb/P3B2 will be helpful in producing stable complexes with the GAF-A domain without cGMP present for the crystallization studies.

Super High Affinity for Sildenafil Inhibition of Non-activated PDE5 and Its Physiological Significance. Because approval of sildenafil as an oral drug for the treatment of erectile dysfunction in 1998, many pharmacokinetic and efficacy data for this compound have been accumulated (Nichols et al., 2002; Padma-Nathan, 2006). For example, the maximum concentration of sildenafil in plasma is achieved by 1 h, and the elimination half-life is 3.7 h. Therefore, the optimal time of therapeutic use has been recommended to be between 30 min and no longer than 4 h. This drug is removed from the plasma mostly through liver metabolism, and by 24 h, its plasma level is decreased to below nanomolar concentrations.

However, there are a number of reports showing that the effects of PDE5 inhibitors could be observed at times longer than the recommended time for effective ED therapy. Sildenafil has been found effective in 60 and 33% of men with ED 8 and 12 h, respectively, after an oral dose of sildenafil (Gingell et al., 2004). A high rate of response (74%) has been reported to sildenafil treatment at 12 h after dosing in the open-label uncontrolled study of sildenafil responders (Moncada et al., 2004). The effects of sildenafil lasting up to 24 h have also been shown in men beginning sildenafil therapy for erectile dysfunction

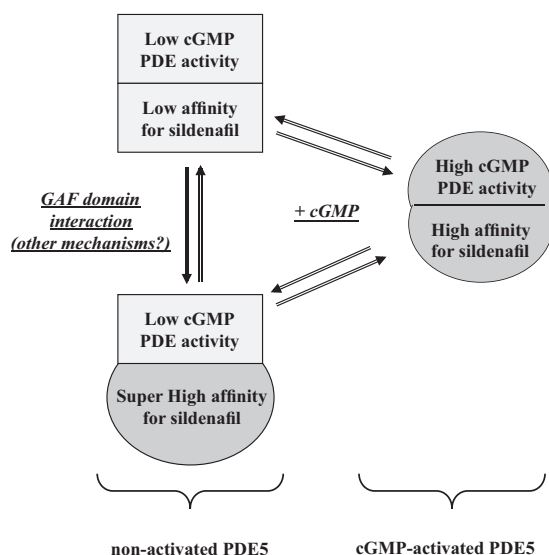


Fig. 7. Multiple states of nonactivated and cGMP-activated PDE5. Non-activated PDE5 may exist in two different conformation states, characterized by low and high affinities for sildenafil inhibition. GAF domain interactions may shift equilibrium between low and high affinity for sildenafil of nonactivated PDE5. cGMP binding would transform both states into the cGMP-activated state with high catalytic activity and high affinity for sildenafil.

(Steidle et al., 2007). Similar prolonged effects of tadalafil and vardenafil have also been reported.

To explain possible mechanisms of such effects, it has been proposed that sildenafil could be retained and accumulated in penile vascular smooth muscle cells beyond the time of clearance from plasma because of its binding to high-affinity sites of PDE5 (Francis et al., 2008). Higher intracellular concentrations of sildenafil would result in higher activity for PKG, thus extending the duration of sildenafil action when the concentration of sildenafil falls below therapeutic levels outside of the cell. However, it is not clear why PDE5 retains sildenafil for 12 to 24 h after taking one dose of sildenafil. Studies of sildenafil dissociation patterns showed that only long preincubation with high concentrations of PDE5 inhibitors could substantially slow down dissociation (Blount et al., 2007). In addition, sildenafil as a moderately lipophilic compound is expected to achieve equilibrium between its intracellular and extracellular/plasma concentrations relatively fast.

Our data provide another possible explanation for sildenafil effectiveness at the time of its decreasing plasma concentrations into the subnanomolar levels. We show that non-activated PDE5 could be effectively inhibited by sildenafil in the picomolar range of its concentrations. Therefore, in vivo saturation of PDE5 with sildenafil in the areas of high concentrations of PDE5 would provide long-term favorable conditions for inhibition of cGMP-activated PDE5 when a surge of NO/cGMP occurs even a long time after taking of one dose of sildenafil.

Although physiological mechanisms for the modulation of inhibitor sensitivities of nonactivated PDE5 are not known, understanding these mechanisms seems to be important for designing therapeutic strategies for patients with ED and pulmonary hypertension treated with smaller doses of sildenafil (Revatio, 20 mg sildenafil, three times daily) and for any other pathologies, which are now under investigation using long-term low doses of PDE5 inhibitors.

References

- Aravind L and Ponting CP (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci* **22**:458–459.
- Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, and Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* **159**:2164–2171.
- Blount MA, Beasley A, Zoraghi R, Sekhar KR, Bessay EP, Francis SH, and Corbin JD (2004) Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* **66**:144–152.
- Blount MA, Zoraghi R, Bessay EP, Beasley A, Francis SH, and Corbin JD (2007) Conversion of phosphodiesterase-5 (PDE5) catalytic site to higher affinity by PDE5 inhibitors. *J Pharmacol Exp Ther* **323**:730–737.
- Bruder S, Schultz A, and Schultz JE (2006) Characterization of the tandem GAF domain of human phosphodiesterase 5 using a cyanobacterial adenylyl cyclase as a reporter enzyme. *J Biol Chem* **281**:19969–19976.
- Corbin JD, Blount MA, Weeks JL 2nd, Beasley A, Kuhn KP, Ho YS, Saidi LF, Hurley JH, Kotera J, and Francis SH (2003) [3H]sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous, and stimulated by cGMP. *Mol Pharmacol* **63**:1364–1372.
- Francis SH, Morris GZ, and Corbin JD (2008) Molecular mechanisms that could contribute to prolonged effectiveness of PDE5 inhibitors to improve erectile function. *Int J Impot Res* **20**:333–342.
- Ghofrani HA, Osterloh IH, and Grimminger F (2006) Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nat Rev Drug Discov* **5**:689–702.
- Gingell C, Sultana SR, Wulff MB, and Gepi-Attee S (2004) Duration of action of sildenafil citrate in men with erectile dysfunction. *J Sex Med* **1**:179–184.
- Gopal VK, Francis SH, and Corbin JD (2001) Allosteric sites of phosphodiesterase-5

- (PDE5). A potential role in negative feedback regulation of cGMP signaling in corpus cavernosum. *Eur J Biochem* **268**:3304–3312.
- Handa N, Mizohata E, Kishishita S, Toyama M, Morita S, Uchikubo-Kamo T, Akasaka R, Omori K, Kotera J, Terada T, et al. (2008) Crystal structure of the GAF-B domain from human phosphodiesterase 10A complexed with its ligand, cAMP. *J Biol Chem* **283**:19657–19664.
- Heikaus CC, Stout JR, Sekharan MR, Eakin CM, Rajagopal P, Brzovic PS, Beavo JA, and Kleivit RE (2008) Solution structure of the cGMP binding GAF domain from phosphodiesterase 5: insights into nucleotide specificity, dimerization, and cGMP-dependent conformational change. *J Biol Chem* **283**:22749–22759.
- Hofbauer K, Schultz A, and Schultz JE (2008) Functional chimeras of the phosphodiesterase 5 and 10 tandem GAF domains. *J Biol Chem* **283**:25164–25170.
- Hofmann F, Bernhard D, Lukowski R, and Weinmeister P (2009) cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol* **191**:137–162.
- Huai Q, Liu Y, Francis SH, Corbin JD, and Ke H (2004) Crystal structures of phosphodiesterases 4 and 5 in complex with inhibitor 3-isobutyl-1-methylxanthine suggest a conformation determinant of inhibitor selectivity. *J Biol Chem* **279**:13095–13101.
- Kanacher T, Schultz A, Linder JU, and Schultz JE (2002) A GAF-domain-regulated adenylyl cyclase from *Anabaena* is a self-activating cAMP switch. *EMBO J* **21**:3672–3680.
- Martinez SE, Bruder S, Schultz A, Zheng N, Schultz JE, Beavo JA, and Linder JU (2005) Crystal structure of the tandem GAF domains from a cyanobacterial adenylyl cyclase: modes of ligand binding and dimerization. *Proc Natl Acad Sci USA* **102**:3082–3087.
- Martinez SE, Heikaus CC, Kleivit RE, and Beavo JA (2008) The structure of the GAF A domain from phosphodiesterase 6C reveals determinants of cGMP binding, a conserved binding surface, and a large cGMP-dependent conformational change. *J Biol Chem* **283**:25913–25919.
- Martinez SE, Wu AY, Glavas NA, Tang XB, Turley S, Hol WG, and Beavo JA (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *Proc Natl Acad Sci USA* **99**:13260–13265.
- Moncada I, Jara J, Subirá D, Castaño I, and Hernández C (2004) Efficacy of sildenafil citrate at 12 hours after dosing: re-exploring the therapeutic window. *Eur Urol* **46**:357–360; discussion 360–361.
- Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, and Koesling D (2003) Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J Cell Biol* **160**:719–727.
- Nichols DJ, Muirhead GJ, and Harness JA (2002) Pharmacokinetics of sildenafil after single oral doses in healthy male subjects: absolute bioavailability, food effects and dose proportionality. *Br J Clin Pharmacol* **53** (Suppl 1):S5–12S.
- Padma-Nathan H (2006) Sildenafil citrate (Viagra) treatment for erectile dysfunction: An updated profile of response and effectiveness. *Int J Impot Res* **18**:423–431.
- Rotella DP (2002) Phosphodiesterase 5 inhibitors: current status and potential applications. *Nat Rev Drug Discov* **1**:674–682.
- Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, and Beavo JA (2002) Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem* **277**:3310–3317.
- Rybalkin SD, Rybalkina IG, Shimizu-Albergine M, Tang XB, and Beavo JA (2003) PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. *EMBO J* **22**:469–478.
- Sandner P, Hütter J, Tinel H, Ziegelbauer K, and Bischoff E (2007) PDE5 inhibitors beyond erectile dysfunction. *Int J Impot Res* **19**:533–543.
- Schlossmann J and Desch M (2009) cGK substrates. *Handb Exp Pharmacol* **191**:163–193.
- Schultz JE (2009) Structural and biochemical aspects of tandem GAF domains. *Handb Exp Pharmacol* **191**:93–109.
- Steidle CP, McCullough AR, Kaminetsky JC, Crowley AR, Siegel RL, Deriesthal H, and Tseng LJ (2007) Early sildenafil dose optimization and personalized instruction improves the frequency, flexibility, and success of sexual intercourse in men with erectile dysfunction. *Int J Impot Res* **19**:154–160.
- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, and Kass DA (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* **11**:214–222.
- Takimoto E, Koitabashi N, Hsu S, Ketner EA, Zhang M, Nagayama T, Bedja D, Gabrielson KL, Blanton R, Siderovski DP, et al. (2009) Regulator of G protein signaling 2 mediates cardiac compensation to pressure overload and antihypertrophic effects of PDE5 inhibition in mice. *J Clin Invest* **119**:408–420.
- Wang H, Liu Y, Huai Q, Cai J, Zoraghi R, Francis SH, Corbin JD, Robinson H, Xin Z, Lin G, et al. (2006) Multiple conformations of phosphodiesterase-5: implications for enzyme function and drug development. *J Biol Chem* **281**:21469–21479.
- Wilson LS, Elbatarny HS, Crawley SW, Bennett BM, and Maurice DH (2008) Compartmentation and compartment-specific regulation of PDE5 by protein kinase G allows selective cGMP-mediated regulation of platelet functions. *Proc Natl Acad Sci USA* **105**:13650–13655.
- Zhang KY, Card GL, Suzuki Y, Artis DR, Fong D, Gillette S, Hsieh D, Neiman J, West BL, Zhang C, et al. (2004) A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. *Mol Cell* **15**:279–286.

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