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Title page

MULTIPLE AFFINITY STATES OF cGMP-SPECIFIC PDE (PDE5) FOR SILDENAFIL INHIBITION DEFINED BY cGMP-DEPENDENT AND cGMP-INDEPENDENT MECHANISMS

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2. Running title page

a) The Running title: **Super high affinity sildenafil inhibition of PDE5**

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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/PKG signaling pathway. PDE5 has two highly homologous regulatory domains, GAF-A and GAF-B. Previously we showed that PDE5 could be converted from a low activity (non-activated) state to a high activity state upon cGMP binding to the GAF-A domain with higher sensitivities towards sildenafil (Rybalkin et al., 2003). Here we investigated if 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 non-activated recombinant PDE5 for sildenafil, revealing much higher sensitivity to sildenafil inhibition. The apparent affinity for sildenafil increased from the nM range to the pM 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 PDE5, but also through cGMP independent modulation of PDE5 in the non-activated state, possibly through protein/protein interaction. Furthermore, data suggest that non-activated PDE5 with "super" high affinities for sildenafil inhibition may be responsible for therapeutic effects of chronic treatments with low doses of PDE5 inhibitors.

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The cGMP/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) 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) and vardenafil (Levitra), also have been shown to selectively inhibit PDE5 activity and are available for clinical use (Rotella, 2002). In 2005 sildenafil (Revatio) has been approved as treatment of pulmonary arterial hypertension (Ghofrani et al., 2006). At present PDE5 inhibitors are under clinical investigations for the treatments of a number pathological conditions, including lower urinary tract symptoms, cardiac and gastrointestinal disorders, endothelial dysfunction, Raynaud's disease (Sandner et al., 2007). Many of these pathologies are characterized by vascular deficiencies, and PDE5 inhibitors are likely candidates for modulation of NO/PKG controlled smooth muscle tone. Recent reports that sildenafil could improve cardiac functions provided the basis for initiation of the NIH trial of sildenafil for treating heart failure (Takimoto et al., 2005; Takimoto et al., 2009).

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 phoshodiesterases, Anabaena adenylyl cyclases and a bacterial transcription factor FhlA (Aravind and Ponting, 1997). At present more than 7000 GAF domain-

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containing proteins have been found, and these proteins may contain a single, tandem and even multi-domain 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 E. coli FhlA or haem for the DosS GAF domain of Mycobacterium tuberculosis. Although GAF-A and GAF-B domain share a significant sequence homology, they appear to have different binding functions. For example, the GAF-A but not GAF-B domain of PDE5 binds cGMP, while in PDE2 the GAF-B domain is the only cGMP-binding domain. However, in spite of 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, that 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 (CyanB1), 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 (Kanacher 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

Previously, we have demonstrated that cGMP binding to the GAF-A domain is necessary and sufficient to achieve full activation of PDE5, and PDE5 in non-activated and cGMP-activated states differ significantly in their catalytic and inhibitory properties (Rybalkin et al., 2003). Here we investigated if 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.

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MATERIALS and METHODS

Reagents - Pepstatin, leupeptin and Nonidet P-40 were from Roche Molecular Biochemicals. SuperSignal West Pico Chemiluminescent Substrate, AminoLink kit, Coomassie Plus protein assay were from Pierce. Horseradish peroxidase-conjugated goat anti-rabbit IgGs and goat anti-mouse IgGs were from Bio-Rad. Protein G-agarose beads were from Calbiochem. Talon Metal Affinity Resin was from Clontech. Qiagen plasmid maxi kit was from Qiagen. BenchMark pre-stained protein standard, Lipofectamine 2000, pcDNA3 vector, Dulbecco's modified Eagle's medium, SilverXpress kit were from Invitrogen. [^{3}H] cGMP were purchased from PerkinElmer Life And Analytical Sciences. Mono Q anion exchange column HR 5/5, Ficoll-Paque Plus were from Amersham Pharmacia Biotech. HEK 293 cells were obtained from the American Type Tissue Culture Collection. 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.

Cell culture and Transfection

HEK 293 cells were grown in DMEM 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 DTT, 10 mg/ml aprotinin, 5 mg/ml pepstatin, 20 mg/ml leupeptin, 1 mM benzamidine, 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),

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GAF B (334-525 aa) and GAF (A + B) (125-539 aa) were expressed in Escherichia coli with polyhistidine tags and purified on a Talon Metal Affinity Resin. GAF proteins were analyzed by Western Blotting and also silver stained using using SilverXpress kit. Only freshly purified proteins or proteins frozen at -80^0C in a 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 antibody (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 GST-fusion protein from the C-terminal part of mouse PDE5 (775-865 aa) and were affinity purified using a AminoLink kit.

Immunoprecipitation and Western Blot Analysis

Protein samples were incubated with different PDE5 antibodies in the immunoprecipitation buffer, containing 0.1% NP-40, 0.1 mM NaCI in the homogenization buffer. The immunopellets were washed three times in the immunoprecipitation buffer and then boiled for 5 minutes 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 (ECL) 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 Ficoll-Paque

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Plus. Platelets were resuspended in incubation buffer, containing 137 mM NaCl, 2.7 mM KCl, 0.77 mM NaH2PO4, 5 mM glucose and 2.0 mM MgCl2, 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 immunoprecipiattion buffer. The immunopellets were preeincubated 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 –10 min at 30°C using different concentrations of cGMP (0.025 – 1.0 □M) as substrate. The total reaction volume (125 □l) contained 40 mM MOPS pH 7.5, 2.0 mM EGTA, 15 mM Mg acetate, 0.2 mg/ml bovine serum albumin and 50 000 c.p.m. [3H] cGMP. IC_{50} values were calculated using GraphPad Prism 2.0 C (GraphPad Software).

Protein concentrations were measured by the Coomassie Plus protein assay.

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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 conformation states for PDE5 has been proposed. For example, it has been suggested that PDE5 could exist in at least two different conformational states: a non-activated 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 towards sildenafil (Rybalkin et al., 2003). The IC_{50} for sildenafil inhibition changed from 2.1 nM to 0.63 nM for non-activated to cGMP-activated PDE5 correspondingly, when PDE5 activity was assayed at 0.1 μM cGMP.

We also measured sildenafil inhibition for PDE5 in these two states at lower substrate concentration – 0.025 μM (Fig.1A). This cGMP concentration is supposed to be closer to the physiological condition present in un-stimulated corpus cavernosum, estimated to be 18 nM (Gopal et al., 2001). We found that at 0.025 μM cGMP, the sildenafil inhibition curves were better fit not by 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 a day and still show differential responses to sildenafil inhibition for non-activated 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 suggested that concentrated samples of PDE5

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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 due to 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. Typically in order to achieve linearity PDE assays require serial dilution of samples to achieve hydrolysis no higher than 10-20% 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, that those seen in the very high dilution states normally required for PDE activity analysis.

First we used individually expressed GAF domain protein to investigate if any protein/protein interactions between GAF domains were possible by performing co-immunoprecipitation 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 co-immunoprecipitation experiments, including the GAF-A domain with and without the N-terminal end (1-320 aa and 125-320 aa), the GAF-B (aa 334-525) 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 μM) domain protein and GAF-B domain (2 μM) protein for an hour on ice could result in forming a protein complex, detected by co-immunoprecipitation, when either mAb/P4A9 or mAb/P3B2 were used for immunoprecipitation (Fig. 2A). GAF-B specific mAb was used to identify GAF-B in the immunopellet by Western blotting.

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Decreasing the concentration of the GAF-B protein (0.2 μM) (the molar ratio 1/0.1 for GAF-A/GAF-B) in the incubation mixture significantly reduced the amount of GAF-B co-immunoprecipitated with the GAF-A domain, while increasing the concentration of GAF-B (20 μM) (molar ratio 1/10) produced the same results as with 2 μM 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. Addition of cGMP (10-100 μM) to the incubation mixture did not affect the binding pattern for co-immunoprecipitation by mAb/P4A9 (Fig. 2B). Moreover, mAb/P3B2, which was also used for these co-immunoprecipation experiments, was found to completely block cGMP binding to the GAF-A domain even at high concentrations of cGMP (100 μM) thus preserving the GAF-A domain into a non-binding 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 if 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 co-immunoprecipitated, 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 -80^0C and used only once after thawing. Purified GAF domain proteins (especially the GAF-B protein) stored at 4^0C 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

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Since we found that *in vitro* GAF-A and GAF-B domains demonstrated inter-domain binding capabilities, we investigated if 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 setting 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 non-activated and cGMP-activated states, assayed at 0.025 μM cGMP.

We found that the most dramatic changes in the sildenafil sensitivity occurred for non-activated PDE5 (Fig. 3A). The whole pattern for sildenafil inhibition moved sharply to the left for this form of enzyme when it was pre-incubated with either the GAF-A or GAF-AB protein. Since the GAF-A and the GAF-AB protein showed very similar responses, it is likely that in these experiments the GAF-A domain was largely responsible for this shift in 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 pM range of concentrations- for sildenafil inhibition (IC_{50}s - 2.9 nM and 1.7 pM).

We also performed co-immunoprecipitation experiments to test if under our experimental conditions any interactions between individual GAF-A domain protein and the full-length PDE5 could be found. PDE5 in the non-activated state was preincubated with the GAF-A domain protein and then immunoprecipitated using a C-terminal polyclonal PDE5 Ab. 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 co-immunoprecipitation, suggesting that changes in the inhibitory properties of PDE5 in the non-activated

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state after addition of the GAF-A domain (or GAF-AB) protein could be the results of protein-protein interactions.

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 suggest that binding competition between the GAF-A and GAF-B domain proteins prevented 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 non-activated 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 non-activated 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 very high concentration. The reported IC_{50} for sildenafil inhibition of platelet PDE5 appears 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, while 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 non-activated state when assayed at 1.0 μM 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). Two-fold increase in cGMP-induced activation of PDE5 has been detected in PKG-depleted platelet lysates. Recently importance of compartmentalization of PDE5 in human platelets has been shown and suggested that distinct pools of cGMP could be selectively regulated by cGMP/PKG activated PDE5 (Wilson et al.,

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2008).

Here we tested if PDE5 from human platelets could be a source of non-activated PDE5 and investigated if its sildenafil inhibitory sites could be modulated by cGMP/GAF-A dependent and independent mechanisms. In order 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 μM cGMP, followed by two washes to remove excess of cGMP. Under these experimental conditions we could detect up to 3-fold activation of platelet PDE5 activity by cGMP (Fig. 5B). Phospho-specific PDE5 Ab 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 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 non-activated (IC_{50} - 5.0 nM) and cGMP-activated PDE5 (IC_{50} - 1.6 nM), when assayed at 0.1 μM cGMP (Fig. 5C).

Since we found that treatments of mouse PDE5 with mAb/P3B2 could preserve the enzyme in the non-activated 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 non-activated PDE5, did not change, however, the appearance of high affinity inhibitory sites for sildenafil was detected. Similar biphasic inhibitory curves and similar conversion of non-activated PDE5 into a state with high affinities for sildenafil inhibition were also obtained when recombinant PDE5 in the non-activated state was treated with mAb/P3B2 under the same conditions (Fig. 6B), also suggesting that cGMP binding sites in the non-bound state are involved in the formation of non-activated conformation of PDE5 with low affinity for sildenafil. mAb/P3B2 may block that region from any interaction thus preserving PDE5 in the non-activated state but with high affinity for sildenafil. These effects appear specific, since other PDE5 mAb

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(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 non-activated 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.

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DISCUSSION

This study demonstrates that a super high affinity site for sildenafil inhibition can be present on PDE5. Our data suggest that non-activated PDE5 is able to adopt two different states, characterized by nM and pM affinities for sildenafil inhibition (Fig. 7). In our experiments the equilibrium between these two states was shifted by addition 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 non-activated PDE5 and GAF domain proteins may emulate the normal physiological situations in tissues as lung or corpus cavernosum, where high concentrations of PDE5 exist, likely 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 H^3-sildenafil

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and unlabeled sildenafil (Blount et al., 2004; Corbin et al., 2003). These studies showed two kinetic components (slow and fast), and for sildenafil two calculated K_d have been determined- 14.7 nM 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.

Determination of conformation states of PDE5 would be more straightforward if crystal structures of PDE5 in the liganded and non-liganded 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 (IBMX, sildenafil) (Huai et al., 2004; Wang et al., 2006; Zhang et al., 2004). 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 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, since 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, PDE10 and the cyanobacterial tandem GAF domains from the cyaB2 adenylyl cyclase have been reported (Handa et al., 2008; Martinez et al., 2005; Martinez et al., 2008; Martinez et al., 2002). In all GAF domain crystal structures ligands, such as cGMP for PDE2 and PDE6 or cAMP for CyaB2, were deeply buried 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

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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 have been crystallized with appropriate antibodies to provide a stabilization effect for the crystallization. Since we found that mAb/P3B2 could bind to the GAF-A domain, block cGMP binding and preserve PDE5 in a non-activated 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.

Since approval of sildenafil as an oral drug for the treatment of erectile dysfunction in 1998, a lot of 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 hrs. Therefore the optimal time of therapeutic use has been recommended to be between 30 min and no longer than 4 hrs. This drug is removed from the plasma mostly through liver metabolism, and by 24 hrs 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 hrs and 12 hrs correspondently after an oral dose of sildenafil (Gingell et al., 2004). A high rate of response (74%) has been reported to sildenafil treatment at 12 hrs after dosing in the open-label uncontrolled study of sildenafil responders (Moncada et al., 2004). The effects of sildenadil lasting up to 24 hrs have also been shown in men beginning sildenafil therapy for erectile dysfunction (Steidle et al., 2007). Similar prolonged effects of tadalafil and vardenafil have also been reported.

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To explain possible mechanisms of such effects it has been proposed that sildenafil could be retained and accumulated in penile VSMC beyond the time of clearance from plasma due to 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 duration of sildenafil action when the concentration of sildenafil falls below therapeutic levels outside of the cell. However, it is not clear why PDE5 shall retain sildenafil for 12-24 hours 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). Also, sildenafil as a moderately lipophilic compound is expected to achieve equilibrium between its intracellar and extracellular/plasma concentrations relatively fast.

Our data provide another possible explanation for sildenafil effectiveness at the time of its declining plasma concentrations into the subnanomolar levels. We show that non-activated PDE5 could be effectively inhibited by sildenafil in the pM 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 modulation of inhibitor sensitivities of non-activated PDE5 are not known, understanding of these mechanisms appears 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 chronic low doses of PDE5 inhibitors.

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Footnotes

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LEGENDS FOR FIGURES

Figure 1. The difference in sildenafil sensitivity between non-activated 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 μM cGMP. (B) The potency of sildenafil to inhibit PDE5 diminishes upon storage of concentrated samples at 4^0C and after one day no differences between non-activated and cGMP-activated PDE5 has been detected. Inhibitory curves were fitted to either a one-site or a two-site competition equations using GraphPad Prism 2.0C (GraphPad Software).

Figure 2. Direct interaction of the GAF domain proteins analyzed by co-immunoprecipitation.

(A) Co-immunoprecipitation of purified GAF-B with GAF-A domain protein. 2 μM GAF-A was preincubated with 2 μM GAF-B (+) at molar ratio 1:1 or with 20 μM GAF-B (++) at molar ratio 10:1 on ice for 1 hour in the homogenization buffer. GAF A specific mAbs (mAb/P3B2 or mAb/P4A9) were used for co-immunoprecipitation with similar results. Immunoprecipitation buffer contained 0.1M NaCI, 1 mg/ml BSA and 0.1% NP-40. After performing SDS-PAGE, GAF-B domain protein was detected by Western blotting with the GAF-B domain specific mAb.

(B) Co-immunoprecipitation of purified GAF-B with GAF-A domain proteins was performed as described above in the presence of 100 μM cGMP, showing cGMP binding to the GAF-A domain did not change co-immunoprecipitation pattern.

(C) Co-immunoprecipitation of purified GAF-B with GAF-AB domain protein. 2 μM GAF-AB (125-539 aa) domain protein was pre-incubated with 2 μM of GAF-B on ice for 1 hr and analyzed using the same

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conditions as above. Light and heavy chain IgGs are visible on the blot since mouse monoclonal antibodies were used for immunoprecipitation and Western blotting.

Figure 3. Interactions between the GAF-A domain containing proteins and full-length PDE5 in the non-activated state lead to significant increases in affinity for sildenafil inhibition.

(A) Recombinant full-length PDE5 was preincubated with 1 □M of purified GAF-A (125-320 aa), GAF-B (334-525 aa) or GAF-AB (125-539 aa) domain proteins for 1 hour 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 non-activated state were fitted to either a one-site or a two-site competition equations using GraphPad Prism 2.0C (GraphPad Software). Similar results were obtained when the concentrations of the GAF domain proteins used in the preincubation step were 10 □M.

(B) Co-immunoprecipitation of PDE5 and GAF-A domain protein after their preincubation under the same condition as above (Fig. 4A) 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 μM) and GAF-B (5 μM) 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 Fig.3A.

Figure 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 □M of purified GAF-A (125-320 aa), GAF-B (334-525 aa) or GAF-AB (125-539 aa) domain proteins for 1 hour on ice. In order to convert non-activated PDE5 to the cGMP-activated state 50 □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

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sildenafil. Using 10 □M of the same GAF domain proteins produced similar results.

PDE5 activities of the control samples for cGMP-activated PDE5 was 0.14 pmol/min/ug protein and 0.013pmol pmol/min/mg protein for non-activated PDE5, measured at 0.025 □M cGMP.

(B) Data from Fig.3A and Fig.4A shown at the same graph to illustrate different responses in sildenafil sensitivity for PDE5 in the non-actiavted and cGMP-activated states. All inhibitor curves were obtained in the same experiment and presented as a representative experiment. At least three more experiments produced similar results. Inhibitory curves were fitted to either a one-site or a two-site competition equations using GraphPad Prism 2.0C (GraphPad Software).

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Figure 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 1uM 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 μM cGMP for 30 min on ice, followed by two washes with the homogenization buffer 1 ml each using quick low speed centrifugation. PDE5 activity was assayed at 0.1 μM cGMP.

(C) Non-activated and cGMP-actiavted PDE5 were obtained as described above and analyzed in the presence of different concentrations of sildenafil at 0.1 μM cGMP.

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Figure 6. Blocking cGMP binding site of PDE5 in the non-activated state with mAb/P3B2 reveals high affinity sites for sildenafil inhibition.

(A, 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 μM cGMP in the presence of different concentrations of sildenafil. Inhibitory curves were fitted to either a one-site or a two-site competition equations using GraphPad Prism 2.0C (GraphPad Software). Data shown are representative of at least three experiments.

Figure 7. Multiple states of non-activated 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 non-activated PDE5. cGMP binding would transform both states into the cGMP activated state with high catalytic activity and high affinity for sildenafil.

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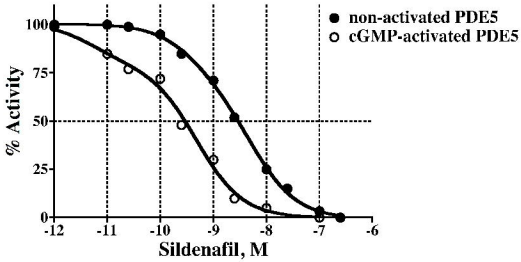
A


non-activated PDE5
cGMP-activated PDE5
% Activity
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
Sildenafil, M

B

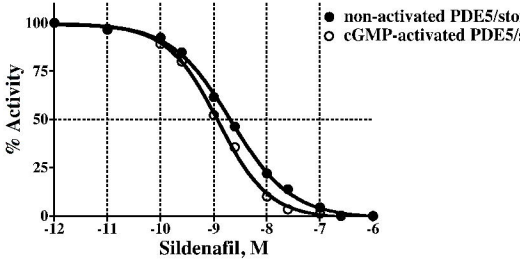

non-activated PDE5/stored
cGMP-activated PDE5/stored
% Activity
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
Sildenafil, M

Figure 1

A

GAF-A		+	+	+
GAF-B	+		+	++

WB: GAF-B mAb

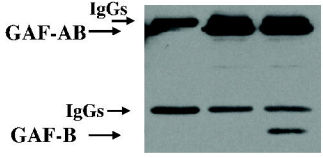
B

GAF-A	+	+
GAF-B	+	+
cGMP		+

WB: GAF-B mAb

C

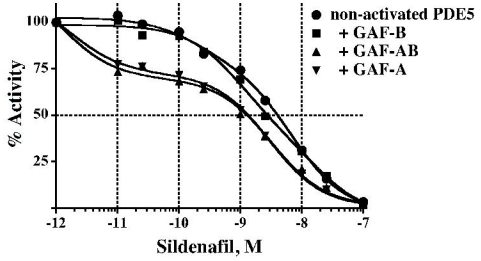
GAF-AB		+	+
GAF-B	+		+



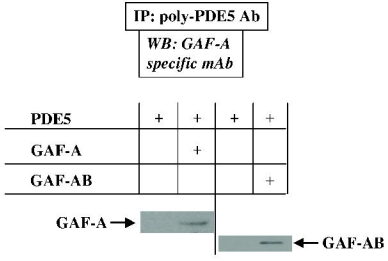
WB: GAF-B mAb

Figure 2

A


non-activated PDE5
+ GAF-B
+ GAF-AB
+ GAF-A
% Activity
100
75
50
25
-12
-11
-10
-9
-8
-7
Sildenafil, M

B


IP: poly-PDE5 Ab
WB: GAF-A specific mAb
PDE5
GAF-A
GAF-AB
+
GAF-A
GAF-AB

C

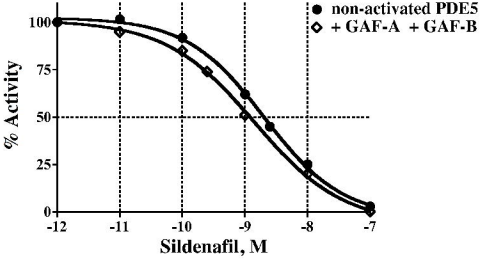
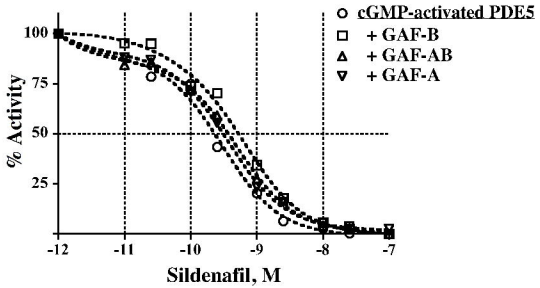

non-activated PDE5
+ GAF-A + GAF-B
% Activity
100
75
50
25
0
-12
-11
-10
-9
-8
-7
Sildenafil, M

Figure 3

A


cGMP-activated PDE5
+ GAF-B
+ GAF-AB
+ GAF-A
% Activity
100
75
50
25
-12
-11
-10
-9
-8
-7
Sildenafil, M

B

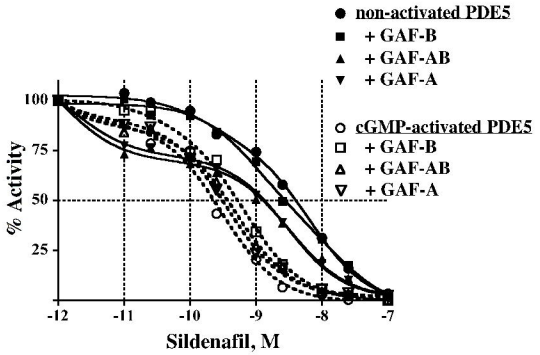
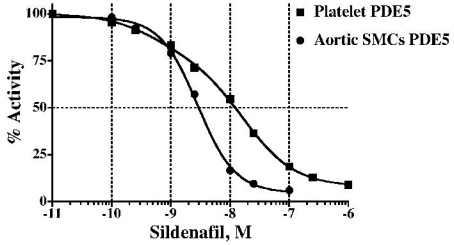
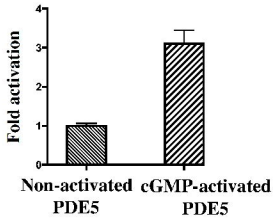

non-activated PDE5
+ GAF-B
+ GAF-AB
+ GAF-A
cGMP-activated PDE5
+ GAF-B
+ GAF-AB
+ GAF-A
% Activity
100
75
50
25
-12
-11
-10
-9
-8
-7
Sildenafil, M

Figure 4

A


Platelet PDE5
Aortic SMCs PDE5
% Activity
100
75
50
25
0
-11
-10
-9
-8
-7
-6
Sildenafil, M

B


Fold activation
4
3
2
1
0
Non-activated PDE5
cGMP-activated PDE5

C

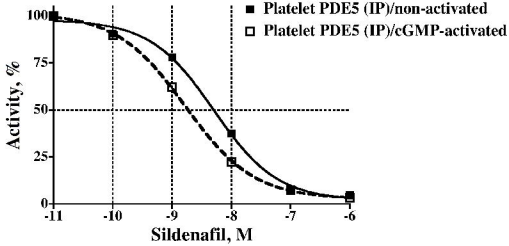
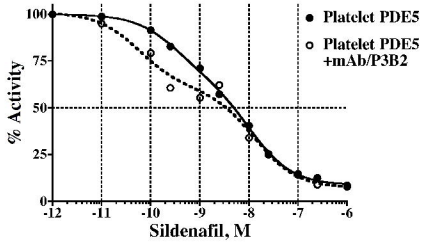

Platelet PDE5 (IP)/non-activated
Platelet PDE5 (IP)/cGMP-activated
Activity, %
100
75
50
25
0
-11
-10
-9
-8
-7
-6
Sildenafil, M

Figure 5

A


100
75
50
25
0
% Activity
-12
-11
-10
-9
-8
-7
-6
Sildenafil, M
Platelet PDE5
Platelet PDE5
+mAb/P3B2

B

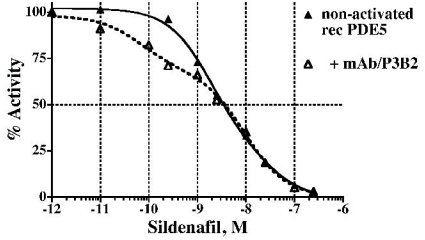

100
75
50
25
0
% Activity
-12
-11
-10
-9
-8
-7
-6
Sildenafil, M
non-activated
rec PDE5
+ mAb/P3B2

Figure 6

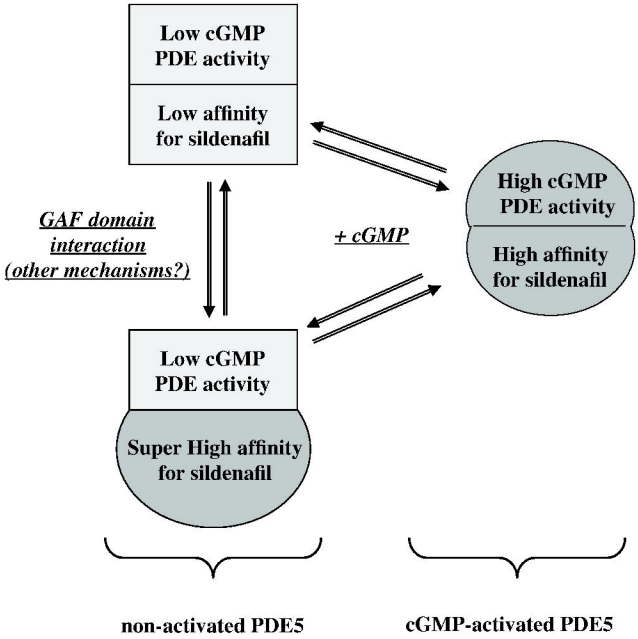

Low cGMP PDE activity
Low affinity for sildenafil
High cGMP PDE activity
High affinity for sildenafil
GAF domain interaction (other mechanisms?)
+ cGMP
Low cGMP PDE activity
Super High affinity for sildenafil
non-activated PDE5
cGMP-activated PDE5

Figure 7